Characterisation of *Escherichia coli* of the bovine intestinal tract

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ABSTRACT

Enterohaemorrhagic *E. coli* (EHEC) are important gastrointestinal pathogens of humans. *E. coli* serotype O157:H7 is the EHEC most commonly associated with human illness. *E. coli* O157:H7 is carried asymptomatically by cattle which form an important reservoir for the bacterium. *E. coli* O157:H7 has been found to colonise at the terminal rectum of cattle in preference to other sites in the bovine gastrointestinal tract. The first objective of this work was to characterise the roles of bacterial secreted components responsible for key functions in the modulation of host defences against EHEC. Data presented here reaffirms the role of flagellin in the elicitation of a proinflammatory response in a cultured human epithelial cell line; however, the response of a bovine epithelial cell line to bacterial secreted products was not affected by the presence or absence of flagellin. A role in the modulation of the host response for the StcE protease was also investigated: although its role in interaction with the bovine host was not established, bovine secretory antibodies to StcE were detected in rectal mucosal scrapings from an *E. coli* O157:H7-challenged calf, suggesting that StcE is expressed and recognised *in vivo*. The second key objective was to isolate *E. coli* from the bovine intestinal tract in order to define the colonisation patterns of *E. coli* within the bovine intestinal tract and relate this to bacterial genotype and to provide bovine *E. coli* isolates to test for inhibitory activity against *E. coli* O157:H7 which may yield bacteria with potential as probiotic agents with a view to reducing the prevalence of EHEC in cattle. Genotypic analysis of bovine resident *E. coli* confirmed that these strains carry a variety of virulence factor-encoding genes; however, certain dominant genotypes were identified and the genomic structure of representative isolates was predicted by genomic microarray. EHEC-related genotypes were found to be positively associated with colonisation at the rectum, whereas non-EHEC genotypes were found to colonise multiple intestinal sites without showing any apparent site-specificity. The third and final objective of this analysis was to carry out genotypic analysis of Scottish EHEC strains in order to predict whether increased incidence of EHEC infection in Scotland may be related to the presence of EHEC strains carrying altered complement of virulence factor-encoding genes. The analysis of EHEC isolated in Scotland revealed that these strains exhibit a genomic profile which is largely typical of EHEC isolated elsewhere, although there were certain differences in the carriage of a certain genomic elements. The results presented here support the proposal that bacteriophages are the key mediators of genetic variability among *E. coli* isolates.

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DECLARATION

The work reported in this thesis was carried out under the supervision of Professor David G. E. Smith at the Moredun Research Institute. All results present, unless otherwise stated, are the sole work of the author, as is the composition of this thesis.

Signed Date

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ABBREVIATIONS

2D/2DGE	Two dimensional (Two dimensional gel electrophoresis)
A/E	Adhering/effacing
aat	Anti-aggregation protein transporter
AB_5	Denotes a toxin with one A subunit and 5 B subunits
***Alu*I**	Restriction endonuclease I encoded by *Arthrobacter luteus*
AP-1	Activator protein-1
APEC	Avian pathogenic *E. coli*
APS	Ammonium persulphate
Arp2/3	Actin-related proteins 2 and 3
BD	Bloody diarrhoea
bfp	bundle forming pilus
BLAST-N, BLAST-P, BLAST-X	Basic local alignment tool (N-nucleic acid query, P-protein, X-translated nucleotide query)
bp	Base pairs
BSA	Bovine serum albumen
C1-INH	C1-esterase inhibitor
Caco-2	Epithelial colorectal carcinoma cell line-2
CCL5	Chemotactic cytokine ligand 5
CDT	Cytolethal distending toxin
CesT	Chaperone for *E. coli* secretion of Tir
***Cfo*I**	Restriction endonuclease I encoded by *Clostridium formicoaceticum*
cfu	Colony-forming units
CGH	Comparative genomic hybridisation
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Che	Chemotaxis
chuA	*E. coli* haeme utilisation gene
Cif	Cell cycle inhibitory factor
CNF	cell necrotising factor
Col	Colicin
CP	Cryptic prophage
CSPD	Chloro-5-substituted adamantyl-1,2-dioxetane phosphate
DAEC	Diffusely-adherent *E. coli*
DC	distal colon
DEH	DIG-easy hyb
DIG	Digoxigenin
DMEM	Dulbecco's minimal essential medium
DMSZ	*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*
DNA	Deoxyribonucleic acid
dNTPs: dATP, dCTP, dTTP and dGTP.	Deoxynucleotide triphosphates: deoxyadenosine, deoxycytidine, deoxythymidine and deoxyguanosine
draA	Gene encoding receptor for Dr blood group antigen receptor haemagglutinin
DTT	Dithiothreitol
eae	*E. coli* attaching and effacing (intimin)
EAggEC	Enteroaggregative *E. coli*
EBL	Embryonic bovine lung
ECACC	European collection of cell cultures

ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
efa-1	EHEC factor for adherence
EHEC	Enterohaemorrhagic *E. coli*
Ehx	Enterohaemolysin
EIA/RIA	Enzyme immunoassay/Radioimmunoassay
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ERK	Extracellular signal-regulated kinase
Esc	*E. coli* secretion complex
Esp (e.g. EspA, EspB *et cetera*)	*E. coli* secreted protein (A, B *et cetera*)
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FAE	follicle associated epithelium
FBS	foetal bovine serum
Fc	Fragment crystallisable
fimA	Gene encoding type I fimbrial subunit A
flhDC	Flagellar regulatory operon
FliC/*fliC*	Flagellin protein/gene encoding flagellin
g	standard coefficient of gravity (approximated to $9.81 ms^{-1}$)
gafD	G-fimbrial lectin-encoding gene
GALT	Gut-associated lymphoid tissue
Gb3	globotriaosylceramide
Gb4	globotetraaosylceramide
GIT	gastrointestinal tract
gluc+/gluc-	glucuronidase positive/negative
H & E	haematoxylin and eosin
H-antigen (e.g. H7)	flagellar antigen (from German *Hauch* meaning "breath" or "film")
HC	Haemorrhagic colitis
HCl	Hydrochloric acid
HeLa	"Henrietta Lacks" cervical epithelial cell line
HEp-2	Human epithelial cell line 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
***Hinc*II**	Restriction endonuclease cII encoded by *Haemophilus influenzae* Rc (ATCC 49699)
HPS	Heath Protection Scotland
H-RFLP	H-antigen RFLP
HRP	Horseradish peroxidise
HUS	Haemolytic uraemic syndrome
ICE	interleukin-1-β converting enzyme
IEC	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IHF	integration host factor
IL	Ileum
IL-	(followed by a number) Interleukin
IMS	Immunomagnetic separation
IPAF	ICE protease activating factor
ipaH	Gene encoding invasion plasmid antigen H
IPG	Immobilised pH gradient
IRAK	IL-1 receptor-associated kinase
iutA	Gene encoding iron utilisation protein A (aerobactin receptor)
IVOC	*In vitro* organ culture

IκBα	Inhibitory κ Bα
JNK	c-Jun N-terminal kinase
K-antigen (e.g. K12)	Capsular antigen (from German: *Kapsel*)
kDa	Kilodalton
kfiC	K5 polysaccharide synthesis gene C
KOH	potassium hydroxide
LB	Luria Bertani
LEE	Locus of enterocyte-effacement
LF	Lymphoid follicle
LM	Lamina muscularis
LP	Lamina propria
LPF	Long polar fimbriae
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-associated laser desorption and ionisation time of flight
MALT	Mucosa-associated lymphoid tissue
Map	Mitochondria-associated protein
MAPK	Mitogen-activated protein kinase
Mcc	Microcin
M-cell	Membraneous cell
MCI	Microbial and Cellular Interactions (Group at Moredun Research Institute)
MEK	Mitogen activated protein kinase kinase
MIP	Macrophage Inflammatory Proteins
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
MPRL	Microbial Pathogenicity Research Laboratory
MUG	4-methylumbelliferyl-D-glucuronide
MyD88	Myeloid differentiation primary response gene (88)
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NAIP-5	NLR family, apoptosis inhibitory protein
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
Nck	non-catalytic region of tyrosine kinase adaptor protein
NCTC	National Collection of Type Cultures
ND	Not determined
NEB	New England Biolabs
neuB	N-acetylneuraminic acid (Neu5Ac) synthase
NF-κB	Nuclear factor κB
Nle (A, B, C...)	Non-LEE encoded effector (A, B, C…)
NLR	NOD-like recepto
NMEC	Neonatal meningitis *E. coli*
NOD	Nucleotide Oligomerisation Domain
NT	Non-typeable
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
O-antigen (e.g. O157)	Somatic antigen (from German *Ohne hauch*: “without breath” or “without film”)
OD (e.g. OD600)	Optical density (e.g. optical density at 600nm wavelength)
Or (e.g. Or7)	O-RFLP pattern (e.g. O-RFLP pattern 7)
ORF	Open reading frame
O-RFLP	O-antigen RFLP
p38	38kDa MAPK

PAMP	Pathogen-associated molecular pattern
papC	gene encoding P-fimbrial usher protein
PAR	Protease-activated receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PerC	Plasmid encoded regulatory protein C
PFGE	Pulsed field gel electrophoresis
PI3K	Phosphoinositide-3-kinase
pO157	large plasmid encoded by *E. coli* O157:H7
PRR	Pattern recognition receptor
PT	Phage type
PTS	phosphoenolpyruvate:sugar phosphotransferase system
Rac1	Ras-related C3 botulinum toxin substrate 1
RAJ	Recto-anal junction
RAPD	random amplification of polymorphic DNA
RDNC	reacted but did not conform to any reference pattern
REML	Restricted maximum likelihood
RFLP	Restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
***Rsa*I**	Restriction endonuclease I encoded by *Rhodopseudomonas sphaeroides*
rStcE	Recombinant StcE
RT	Rectal tropic
RT-PCR	Reverse transcriptase PCR
RTX	Repeats in toxin
SAC	Scottish Agricultural College
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SERL	Scottish *E. coli* Reference Laboratory
SF/nSF	Sorbitol-fermenting/non-sorbitol-fermenting
***sfa* (D/E)**	S-fimbrial antigen genes D/E
sfp	Sorbitol-fermenting O157 P-fimbriae
SMAC (CT-SMAC)	Sorbitol MacConkey medium (cefixime/tellurite-SMAC)
SN	Supernatant
SSC	Saline sodium citrate
SSH	Suppression subtractive hybridisation
StcE	Secreted protease of C1-inhibitor of EHEC
STEC	Shiga-like toxin-producing *E. coli*
Stx	Shiga-like toxin
T3SS	Type III secretion system
TAK-1	TGF-activated kinase-1
Taq polymerase	DNA polymerase from *Thermus aquaticus*
***Taq*I**	Restriction endonuclease I encoded by *Thermus aquaticus*
TBS (TBS-T)	Tris-buffered saline (TBS plus Tween 20)
TBX	Tyryptone/bile salts/X-glucuronide medium
TccP	Tir-cytoskeleton coupling protein
TEMED	*N,N,N',N'*-tetramethylethyldiamine
Tir	Translocated intimin receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumour necrosis factor-α

TOLLIP	Toll-interacting protein
TR	Terminal rectum
TRAF-6	TNF receptor associated factor-6
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSPE4.C2	Anonymous DNA fragment from subtractive library cleaved with the *Tsp*509I restriction endonuclease
***Tsp*509I**	Restriction endonuclease 509I encoded by *Thermus* sp.
TTP	thrombotic thrombocytopaenic purpura
UPEC	uropathogenic *E. coli*
UV	Ultra violet
V	Volts
v/v	volume per volume
VLA	Veterinary Laboratories Agency
VTEC	vero-cytotoxigenic *E. coli*
w/v	weight per volume
wrbA	tryptophan (W) repressor-binding protein
WSU	Washington State University
X (10X, 5X, 2X *et cetera*)	Multiplication of concentrated solution (e.g. a 10X solution is diluted 1:9 to form a 1X working concentration)
Δ(delta)	Before a gene name to denote that gene is deleted (e.g. Δ*stx*)

Chapter 1: General Introduction

1.1 *Escherichia coli*

E. coli is a Gram-negative, facultative anaerobic bacterium of the genus *Escherichia*, type genus of the family Enterobacteriaceae within the class of Gamma Proteobacteria (Scheutz and Strockbine, 2001). *E. coli* and related organisms, including species of the genera *Salmonella*, *Shigella*, *Yersinia*, *Citrobacter* and *Klebsiella* spp. are able to cause an extensive range of diseases in humans and animals; however, *E. coli* form part of the gastrointestinal microflora of many animals where they normally exist without causing disease. The host gastrointestinal microflora is an important part of the healthy gut and so can be described as a component of host innate defences. *E. coli* provide vitamin K_2, which is required for post-translational modification of proteins involved in blood coagulation and bone metabolism and have been recognised as a protective agent against infection by pathogenic bacteria (Dam, 1935;Bentley and Meganathan, 1983;Price, 1988;Mann, 1999;Hudault *et al.*, 2001). However, members of the normal gut microbiota, including *E. coli*, may act as opportunistic pathogens, especially in the immunocompromised host. Different strains of *E. coli* present different complements of molecules, termed virulence factors, which may determine the pathogenic functions of the bacterium, including the host molecules to which it may adhere and the host molecules which the bacterium may affect using toxins and enzymes. As different hosts and different tissues present an altered complement of molecules accessible to bacterial virulence factors, pathogenic *E. coli* often exhibit host and tissue specificity, in that a bacterium may exhibit differing levels of colonisation and pathogenicity in different hosts and at different locations within the host. Differential behaviour in different hosts is an important characteristic of enterohaemorrhagic *E. coli* (EHEC) which is the organism that is the focus of this research. EHEC serotype O157:H7 is the most common EHEC serotype worldwide (Gyles, 2007) and has been found to be carried in the ruminant gastrointestinal tract (GIT) without overt signs of clinical disease (Borczyk *et al.*, 1987) but infection of humans, particularly colonisation of the human ileum (Phillips *et al.*, 2000), may lead to severe disease (Karmali *et al.*, 1983b), as discussed further in Section 1.3.1.

1.2 Molecular classification of *E. coli*

1.2.1 Phenotypic Classification of *E. coli*

Initial classification of bacteria has traditionally relied upon the biochemical activities of the organism. Screening for the presence of a particular bacterium is regularly performed using selective media, often indicator media. Such methods rely on specific fermentative growth characteristics and resistance to antimicrobial compounds. Further profiling of bacterial metabolism can be used to predict bacterial species or subspecies. *E. coli* can be identified on the basis that it produces acidic metabolites and gas on fermentation of D-glucose and that it can utilise acetate but not citrate, malonate or potassium cyanide as a sole carbon sources. The majority of *E. coli* strains produce lysine decarboxylase and indole but do not produce acetyl-methy carbinol (Voges Proskauer negative). *E. coli* strains generally ferment lactose, D-mannitol, melibiose, mucate and D-sorbitol but not D-adonitol, D-arabitol or cellobiose (Scheutz and Strockbine, 2001). These biochemical properties have been exploited for the development of kits which may be used to predict bacterial species, such as the analytical profile index (API, Biomerieux) and the Biolog system (Biolog Inc.). Similarly to other Gram-negative bacteria, *E. coli* exhibit decreased sensitivity to hydrophobic antibiotics compared with Gram-positive organisms. This is due to the decreased permeability of the double membrane and the presence of multidrug efflux pumps (Nikaido, 1996).

E. coli O157:H7 can be distinguished from the majority of *E. coli* subtypes as it is generally non-sorbitol-fermenting (nSF) and is glucuronidase negative (gluc-) (March and Ratnam, 1986;Krishnan *et al.*, 1987;Ratnam *et al.*, 1988). Tryptone-bile salts-X-glucuronide (TBX) and 4-methylumbelliferyl-D-glucuronide (MUG) agar culture media have been used to discriminate *E. coli* O157:H7 from other *E. coli* on the basis of glucuronidase activity (Thompson *et al.*, 1990). Sorbitol MacConkey (SMAC) agar may be used to distinguish sorbitol-fermenting bacterial colonies from non sorbitol-fermenting colonies. On this growth medium sorbitol-fermenting colonies are stained pink, due to the effects of acids, such as lactate, produced on fermentation of sorbitol on phenol red: a pH indicator included in the medium (Rappaport and Henig, 1952). Non-sorbitol-fermenting colonies (such as the majority of *E. coli* O157:H7 strains) remain brown. SMAC contains inhibitory compounds such as bile

salts, which inhibits most Gram-positive organisms. The selective power of this medium for *E. coli* O157:H7 is enhanced by the addition of antimicrobial chemicals cefixime and potassium tellurite (CT-SMAC) which inhibit the majority of organisms other than *E. coli* O157:H7 (Chapman *et al.*, 1991;Zadik *et al.*, 1993). Hence, CT-SMAC has been widely adopted for the detection of *E. coli* O157:H7 in food and environmental samples (de Boer and Heuvelink, 2000). However, not all EHEC strains are non-sorbitol fermenting or glucuronidase negative. The presence of gluc-/nSF/CT-resistant organisms in a patient or biological sample does not confirm that the organisms recovered are disease-causing EHEC. Nevertheless, the prevalence of gluc-/nSF/CT-resistant *E. coli* O157:H7 strains in human infection means that such media remain useful for the prediction of the presence of the most common EHEC subtype in a given biological sample.

Differential immune recognition of bacterial subtypes has been exploited to identify serotypes of bacteria within the species. For *E. coli*, a serotyping scheme has been established, based on the serologically distinct types of O, H and K antigens presented by the bacterium (Kauffmann, 1947). One hundred and eighty six types of somatic antigen, or O-antigen, have been described to date (Guo *et al.*, 2008). O-antigen types are defined by the composition of the sugar residues in the repeating unit of outer membrane lipopolysaccharide (Reeves *et al.*, 1996). The genes underlying O-antigen biosynthesis have been identified as the *rfb*/*gnd* locus and polymorphisms in this region have been exploited for the development of DNA-based methods of O-antigen determination (Wang and Reeves, 1998;Coimbra *et al.*, 2000). Acidic capsular polysaccharide forms the K-antigen, of which there are 80 types currently described (Whitfield and Roberts, 1999).

Variation in the immune recognition of flagellar (H) antigen is based on changes in the primary sequence of flagellin (Kuwajima, 1988). Flagellin is the protein which forms the repeating unit which is polymerised to form the major subunit of the bacterial flagellar filament (Kuwajima *et al.*, 1986;Iino *et al.*, 1988;Fields *et al.*, 1997) as described in more detail in Section 1.4.4. Fifty-six H-types have been described to date, although two have been removed (H13 and H22) as the isolates exhibiting these serotypes were reclassified as *Citrobacter freundii* and H50 has been withdrawn as it is identical to H10 (Scheutz and Strockbine, 2001).

As certain serotypes are commonly associated with particular pathotypes, serotyping has provided advances in the diagnosis, epidemiological analysis, detection and control of bacterial disease. For example: the O157 antigen has provided a target for the isolation and detection of *E. coli* O157 using immunomagnetic separation and latex agglutination assays (Wright *et al.*, 1994). Both the O157 LPS and H7 flagellin antigen have been evaluated as a target for vaccine development (Ahmed *et al.*, 2006;McNeilly *et al.*, 2008). These strategies are valid for the targeting of *E. coli* O157:H7; however, many serotypes of *E. coli* have now been described as EHECs and targeting this serotype alone leaves many EHEC subtypes unaccounted for. In total, 404 different (non-O157) O:H serotypes have been implicated in cases of EHEC infection (a complete list of those identified as of 05/08/2008 is available at: http://www.microbionet.com.au/frames/feature/vtec/brief02.html). A great many *E. coli* isolates, including EHEC, are untypeable by serological methods, including those which do not possess one or more of the antigen types. The O-antigen may be lost under laboratory culture conditions, such as in the case of *E. coli* K12 where a mutation in the rhamnose transferase gene of this bacterium prevents it from producing the O-polysaccharide (Liu and Reeves, 1994). Furthermore, serotyping is an expensive process to implement and is only performed in a few reference laboratories.

The electrophoretic mobilities of bacterial metabolic enzymes have been used to develop a bacterial typing scheme termed multi-locus enzyme electrophoresis (MLEE), whereby bacterial proteins are separated by gel electrophoresis and enzyme activity is detected using indicator compounds (Selander *et al.*, 1986). This technique has been used to infer phylogenetic relationships among *E. coli* and has allowed the designation of several phylogenetic groups within the species (Whittam *et al.*, 1983;Herzer *et al.*, 1990).

Patterns of susceptibility to particular bacteriophages have been exploited to discriminate *E. coli* O157:H7 subtypes (Ahmed *et al.*, 1987;Khakhria *et al.*, 1990). Phage-typing has been widely adopted for epidemiological analysis of *E. coli* O157:H7 (Milford *et al.*, 1990;Izumiya *et al.*, 1998;Lynn *et al.*, 2005). Analysis of phage types of *E. coli* in the British Isles has revealed that by 1997 a recently emerging phage type (PT21/28) had replaced a phage type (PT2) prevalent in the late 1980s and that PT21/28 and PT2 were more frequently associated with HUS than other phage types (Milford *et al.*, 1990;Lynn *et al.*, 2005). This analysis also revealed that phage types differ in their geographical distribution, with phage type 21/28 being

particularly prevalent in Scotland (Lynn *et al.*, 2005). The prevalence of PT21/28 in human infection in Scotland is reflected by the prevalence of strains exhibiting this phage type among colonised cattle in Scotland (Halliday *et al.*, 2006).

Phenotypic typing schemes are a useful tool in the identification and discrimination of bacterial subtypes and generally correlate well with underlying genotypic characteristics, exemplified by the correlation between multi-locus enzyme electrophoresis and phylogenetic group as determined by rRNA-typing (Whittam *et al.*, 1983;Herzer *et al.*, 1990). However, such techniques give only a general indication of the underlying genetic nature of a bacterial type. One consequence of widespread horizontal gene transfer among bacteria is that bacteria that share a phenotypic characteristic, such as serotype, may exhibit extensive genomic variation. One example was this is illustrated in a study by Wu *et al.* (2008) in which *E. coli* isolates of serotype O157 were found to exhibit extensive genomic variation from the majority of *E. coli* O157:H7 isolates. Hence, determination of serotype does not directly predict the genotype of *E. coli* strains.

The resolution of phenotypic typing schemes may be improved by the adoption of proteomic techniques (reviewed in Han and Lee, 2006). Molecular identification of proteins using mass spectrometry is a key technology in this area. MALDI-TOF has recently been applied for the rapid molecular typing of whole bacterial cells and may aid swift identification of bacterial isolates and discrimination between bacterial subtypes (Holland *et al.*, 1996;Krishnamurthy and Ross, 1996;Fenselau and Demirev, 2001;Mellmann *et al.*, 2008).

1.2.2 Genotypic classification of *E. coli*

Recent advances in genomic analysis have allowed DNA-based typing procedures to be developed for the classification of bacterial isolates. The polymerase chain reaction (PCR) and sequencing technology has allowed simplification of the genotyping process over existing phenotypic methods. Bacterial taxonomy has been standardised by the use of 16S ribosomal RNA-encoding gene sequences, allowing species and subspecies groupings to be assigned (reviewed in Staley, 2006). A protocol for multi-locus sequence typing (MLST) which groups organisms according to nucleotide polymorphisms in selected genes, has been developed for

E. coli, based on the sequence types of 7 housekeeping genes (Wirth *et al.*, 2006). The resolution of the standard MLST method is comparable to that of MLEE (Section 1.2.1).

PCR may also be used to screen the genomes of *E. coli* isolates for genes including those encoding virulence factors. This process aids the prediction of pathotype (Section 1.3). PCR has also been utilised for random amplification-based procedures such as "random amplification of polymorphic DNA" (RAPD) whereby arbitrary oligonucleotides are utilised to generate an amplification profile for each strain analysed, allowing comparison of related bacteria (Welsh and McClelland, 1990). Random amplification using octamer oligunucleotides was utilised to identify two lineages of *E. coli* O157:H7, one of which is more prevalent in human infection and the other more prevalent in bovine colonisation (Kim *et al.*, 1999). Subsequently, genomic markers for these lineages were determined by comparative genomic hybridisation (CGH) using microarray (Zhang *et al.*, 2007). The sensitivity of PCR means that it may be performed on extracts from biological samples to detect and identify organisms present without prior culture. Upgrades to PCR, such as the development of real-time PCR, allow the quantification of certain sequences in a biological sample.

Restriction endonucleases, which cleave DNA at specific sequences, are useful tools in the molecular profiling and identification of different genotypes. Sequence polymorphisms at restriction sites are detected by restriction fragment length polymorphism (RFLP). This may be coupled with PCR, to allow typing of individual genes or genomic regions (PCR-RFLP) or performed on a genomic scale using pulsed-field gel electrophoresis (PFGE). RFLP-based methods have been developed for the typing of numerous virulence factors and to predict both O and H serotypes (Fields *et al.*, 1997;Coimbra *et al.*, 2000). PFGE has been widely adopted for the epidemiological tracking of *E. coli* O157:H7 subtypes (Gerner-Smidt *et al.*, 2006).

As many of the high resolution genotyping techniques are complex, difficult to interpret or expensive, there is often scope for simplification. This requires understanding of the basis of a polymorphism observed for the more complex techniques. For example, sequencing of 16S rRNA loci may be simplified using PCR-RFLP (ribotyping) (Desjardins *et al.*, 1995). *E. coli* phylogenetic groupings established by MLEE, MLST and ribosomal RNA sequencing may be predicted rapidly and reproducibly using a simple triplex PCR (Clermont *et al.*, 2000).

Similarly to phenotypic classification schemes, genotyping technologies give an indirect view of the genome of an organism, as they rely on variation within a small subset of genomic loci. To obtain a global view of bacterial genomes, whole genome microarray allows the comparison of an isolated strain's genome against that of one or more strains for which genome sequence has been resolved. This technique can be highly descriptive and gives a direct reflection of the genes encoded by the organism; however, it can only identify diversity by presence or absence of sequences printed onto the array chip and can not be easily used to identify acquisitions of sequences that have not yet been described. Whole genome sequencing is the highest resolution and most descriptive bacterial genotyping scheme available currently. The cost of this technology has decreased recently and the speed, detail and accuracy of computerised analysis have improved, making this technique more accessible. Nevertheless, higher-throughput low-cost techniques, such as PCR-based methodology, are still heavily relied upon for preliminary characterisation of isolated bacteria, in that many isolates may be characterised simultaneously. A limitation of genomic and genotypic analysis is that the expression, biological function and interaction of gene products with other molecules (the defining features of bacterial phenotype) are not yet simple to predict from the data generated. It is envisaged that improved bioinformatic analysis coupled with the accessibility of genome sequencing technology may yield improved predictions which may be testing by analysis of gene expression and biological function of the organism *in vitro* and *in vivo*. To date (01/08/2008) nineteen *E. coli* genome sequences are currently available, including 2 isolates of *E. coli* O157:H7 (Perna *et al.*, 2001;Hayashi *et al.*, 2001b). Many more are currently in preparation and the results of a large scale sequencing project of *E. coli* O157:H7 genomes at the J. Craig Venter Institute (http://msc.jcvi.org/o157h7/index.shtml) are expected. These data, coupled with sequences of single genes submitted to the NCBI provide an extensive and constantly improving reference database for comparative genomic analysis of *E. coli* isolates.

1.3 Pathogenic *E. coli* of animals and man

Pathogenic *E. coli* include certain types which may establish enteric infections and those which may establish extraintestinal infections. *E. coli* may be grouped according to their pathotype referring to the organism's virulence mechanisms, its host specificity and the resultant disease symptoms. Currently described pathotypes of *E. coli* known to affect humans are summarised in Table 1.1.

The virulence mechanisms of each *E. coli* pathotype are determined by the carriage of key virulence factors. These include factors promoting colonisation of the host, cellular adherence/invasion, evasion of host defences and disruption of host cell signalling pathways. These include surface receptors, secreted enzymes and toxins. Several pathotypes of *E. coli* which cause enteric disease in humans have been described. The first recognised were enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) (Bray, 1945). ETEC encodes one or more enterotoxins which may include heat labile (LT) or heat stable (ST) which act to activate adenylate and guanylate cyclases respectively, triggering events which lead to fluid accumulation in the gut lumen (Gill and Richardson, 1980). Enteroinvasive *E. coli* (EIEC) shares virulence mechanisms with *Shigella* spp. in that it is able to invade and multiply within intestinal epithelial cells of the large intestine (Lan and Reeves, 2002). Other diarrhoeagenic *E. coli* pathotypes include enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC). These two pathotypes may be distinguished by their patterns of adherence to cultured epithelial cells of the HEp-2 line (Nataro and Kaper, 1998). Their pathogenic mechanisms are poorly understood, although putative virulence factors have been related to these pathotypes (Table 1.1).

EPEC and Enterohaemorrhagic *E. coli* (EHEC) belong to a related group of organisms which adhere to and efface the membrane of enterocytes. This activity depends upon the functions of a type III secretion system (T3SS) and an outer membrane protein termed intimin (discussed in detail in Sections 1.3.1 and 1.4.1). Attaching and effacing (A/E) bacteria include pathogens of many different host species, including *Citrobacter rodentium*, which has been employed as a mouse model for prediction of the mechanisms of A/E bacterium-host interactions (reviewed in Mundy, 2005).

Extraintestinal pathogenic *E. coli* (ExPEC) establish infections at a variety of locations. Most common among ExPEC infections are those affecting the urinary tract, although *E. coli* have been noted to cause neonatal meningitis and pneumonia (reviewed in Russo and Johnson, 2000). Human ExPEC are phylogenetically distinct from non-pathogenic ("true commensal") strains and those which cause enteric infections and are frequently found among the normal faecal and environmental bacterial flora. ExPEC often express adhesins such as P and S fimbriae, iron acquisition systems (e.g. presentation of the aerobactin receptor) and toxins including cytolethal distending toxin (CDT), cell necrotising factor (CNF) and α-haemolysin (Johnson *et al.*, 2002;Usein *et al.*, 2003).

E. coli infections in animals reflect the diversity of the human *E. coli* pathotypes. *E. coli* has been observed to have an aetiological role in various diarrhoeal and extraintestinal diseases of livestock. ETEC infections are particularly prevalent in neonatal calves, lambs and piglets (Wray *et al.*, 1993). ExPEC is an important agent of mastitis in cows (reviewed in Hogan and Smith, 2003). ExPEC-like organisms (termed avian pathogenic *E. coli*; APEC) are commonly associated with avian colibacilliosis (Johnson *et al.*, 2007). Host and tissue specificity may be related to the presentation of different fimbriae by the bacterium and differential presentation of molecules with affinity for those fimbriae by host cells. For example, *E. coli* isolated from diarrhoeic calves have been observed to express K99 (F5) and F41 fimbriae (ETEC-like strains) or F17 fimbriae (ExPEC-like strains) (Discussed further in Section 1.5.2) (Acres, 1985;Bertin *et al.*, 1996a;Bertin *et al.*, 1996b;Contrepois *et al.*, 1998;Güler *et al.*, 2008).

Pathotype	Common O-serotypes	Disease characteristics	Virulence factors and mechanisms
Enteropathogenic *E. coli* (EPEC)	O26, O55, O86, O88, O103, O111, O119, O125ac, O126, O127, O128ab, O142, O145, O157, O158.	Watery diarrhoea	Initial localised adherence via bundle-forming pili (BFP) followed by intimate adherence
Enterotoxigenic *E. coli* (ETEC)	O6, O7, O8, O9, O11, O15, O17, O20, O21, O25, O27, O29, O48, O55, O56, O63, O64, O65, O71, O73, O77, O78, O85, O86, O88, O105, O114, O115, O119, O126, O128ac, O133, O138, O139, O141, O147, O148, O149, O153, O159, O166, O167.	Watery diarrhoea.	Heat labile/Heat stable enterotoxin
Enterohaemorrhagic *E. coli* (EHEC)	O26, O103, O111, O121, O145, O157. *	Watery or bloody diarrhoea, haemorrhagic colitis progression to systemic diseases.	T3SS, Shiga-like toxins, enterohaemolysin, plasmid encoded protease (EspP) (See section 1.3)
Enteroaggregative *E. coli* (EAggEC)	O3, O15, O44, O86, O111, O125.	Persistent mucoid diarrhoea.	Aggregative adherence, dependent on plasmid-encoded genes such as AAF-fimbriae. Also heat stable enterotoxin (EASTI) and plasmid-encoded enterotoxin (PET)
Enteroinvasive *E. coli* (EIEC)	O28ac, O29, O112ac, O115, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167, O173.	Watery or bloody diarrhoea.	Cellular invasion similar to *Shigella* spp. dependent on proteins encoded on pInv plasmid, including a T3SS.
Diffusely adherent *E. coli* (DAEC)	O126:H27	Watery diarrhoea	Diffuse adherence F1845 fimbriae, AIDA-I adhesin
Extraintestinal pathogenic *E. coli* (ExPEC)	UPEC: O1, O2, O4, O6, O7, O18ac, O75, O16, O15 NMEC: O7, O18ac, O1, O6, O83	UTIs, pneumonia, wound infections, intra-abdominal infections, osteomyelitis, meningitis.	P-fimbriae, S-fimbriae, K1 capsule, α-haemolysin, CDT, CNF.

Table 1.1: Common pathotypes of disease-causing *E. coli* of humans: Compiled from Nataro & Kaper (1998) and Scheutz & Strockbine (2001). UPEC: uropathogenic *E. coli*. NMEC: neonatal meningitis *E. coli.* * The most common O-serotypes of EHEC associated with human disease are listed here although many more have been described as EHEC (Section 1.2.1).

1.3.1 Enterohaemorrhagic *E. coli*

As mentioned in Section 1.3, EHEC is the pathotype which is central to this research. EHEC shares evolutionary origins with EPEC and both share common adherence mechanisms, which depend on the locus of enterocyte effacement (LEE; Section 1.4.1). Possibly the most important difference between EHEC and EPEC is the acquisition of genes encoding verotoxin (also referred to as verocytotoxin and shiga-like toxin; Stx) by EHEC through the action of integrative bacteriophages which aid the spread of these virulence factor-encoding genes (Konowalchuk *et al.*, 1977;Scotland *et al.*, 1983). Symptoms of EHEC infection include abdominal pain accompanied by watery diarrhoea and can progress to haemorrhagic colitis (HC) which is manifested as grossly bloody diarrhoea (BD) (Riley *et al.*, 1983). HC sufferers may develop more severe sequelae, including haemolytic uraemic syndrome (HUS) which is particularly prevalent in younger children (Griffin and Tauxe, 1991). HUS symptoms include microangiopathic haemolytic anaemia, thrombocytopaenia and acute renal dysfunction (often renal failure). Stx induces these symptoms which are the result of the action of the toxin on the endothelial cells of blood vessels especially in the kidney (Karmali *et al.*, 1983a). Thrombotic thrombocytopaenic purpura (TTP) is another consequence of EHEC infection, generally occurring in adults. It can occur without an initial diarrhoeic phase of infection and can result in neurological disorders (Pickering *et al.*, 1994).

There is currently no effective targeted treatment in instances of human EHEC infection. Treatment is largely supportive including fluid management and dialysis when required. There have been reports of the efficacy of antibiotics in the prevention of EHEC disease progression when administered early. This remains controversial since adequate case-control studies have not been carried out and it has been suggested that the use of antibiotics could exacerbate EHEC-induced disease (reviewed in Nataro & Kaper 1998). The potential severity of human EHEC infection coupled with the lack of adequate treatment methods means that effective control and prevention strategies would be of significant benefit to human health.

1.3.2 Epidemiology of EHEC infection

Although O157:H7 remains the most commonly detected serotype among cases of human EHEC infection other EHEC serotypes are prevalent in certain geographical areas. *E. coli* O157:H- (sorbitol-fermenting) isolates were recently identified as a threat to human health (Karch *et al.*, 1990). Outbreaks related to sorbitol-fermenting *E. coli* O157:H- strains were confined to continental Europe until outbreaks occurred recently in Scotland (Karch and Bielaszewska, 2001;Taylor *et al.*, 2003). Non-O157 EHEC, particularly O26, O103, O111, O121, O145 and ONT (O-untypeable) are significant in human infection and are prevalent in southern Europe, South America and Australasia (Lopez *et al.*, 1989;Ojeda *et al.*, 1995;Goldwater and Bettelheim, 2002;Tozzi *et al.*, 2003;Gyles, 2007).

The range of outcomes of human EHEC infection suggests that there is a significant host-factor in the progression of EHEC-induced disease. The young and elderly are at particular risk for the progression of the disease to more severe sequelae yet in healthy adults infection may be restricted to watery diarrhoea or may even be sub-clinical (Wilson *et al.*, 1996;Wilson *et al.*, 1998;Rios *et al.*, 1999;Silvestro *et al.*, 2004). Asymptomatic carriage of *E. coli* O157:H7 and other VTEC has been detected in numerous domestic animal hosts, most frequently in ruminants but also in pigs, cats, dogs and chickens (Doyle and Schoeni, 1987;Griffin and Tauxe, 1991;Beutin *et al.*, 1993;Nakazawa and Akiba, 1999). Since cattle are the principal hosts of *E. coli* O157:H7 many outbreaks and sporadic cases of EHEC infection have been linked with bovine faecal contamination of items consumed by humans, including undercooked beef, unpasteurised milk, vegetables, fruit juices or drinking water (Riley *et al.*, 1983;Borczyk *et al.*, 1987;Morgan *et al.*, 1988;Besser *et al.*, 1993;Swinbanks, 1996;Yarze and Chase, 2000;Licence *et al.*, 2001). Outbreaks have also been traced to caprine and ovine hosts (Bielaszewska *et al.*, 1997;Ogden *et al.*, 2002). Direct contact with cattle and the farming environment is a risk factor for EHEC infection, surpassing foodborne incidences of illness in the UK (Locking *et al.*, 2001;O'Brien *et al.*, 2001). *E. coli* O157:H7 has been isolated from wild animals, including deer and rabbits, suggesting that interaction between farm animals and wild animals could be important in the spread of EHEC between farms (Renter *et al.*, 2001;Pritchard *et al.*, 2001). Despite the variety of hosts of *E. coli* O157:H7, the bovine host remains the most significant in terms of the threat to human health (Locking *et al.*, 2001;O'Brien *et al.*, 2001;Kassenborg *et al.*,

2004). Epidemiological studies have estimated that prevalence of *E. coli* O157:H7 in cattle is just under 2% in Japan, Australia and the USA (Faith *et al.*, 1996;Miyao *et al.*, 1998;Cobbold and Desmarchelier, 2000). Estimates of 4.7% in England and Wales and 8.6% in Scotland have been reported (Synge and Paiba, 2000;Paiba *et al.*, 2002). The frequency of human *E. coli* O157:H7 infection reflects these data as incidence of disease in Scotland has been consistently above the UK average, as shown in Figure 1.1.

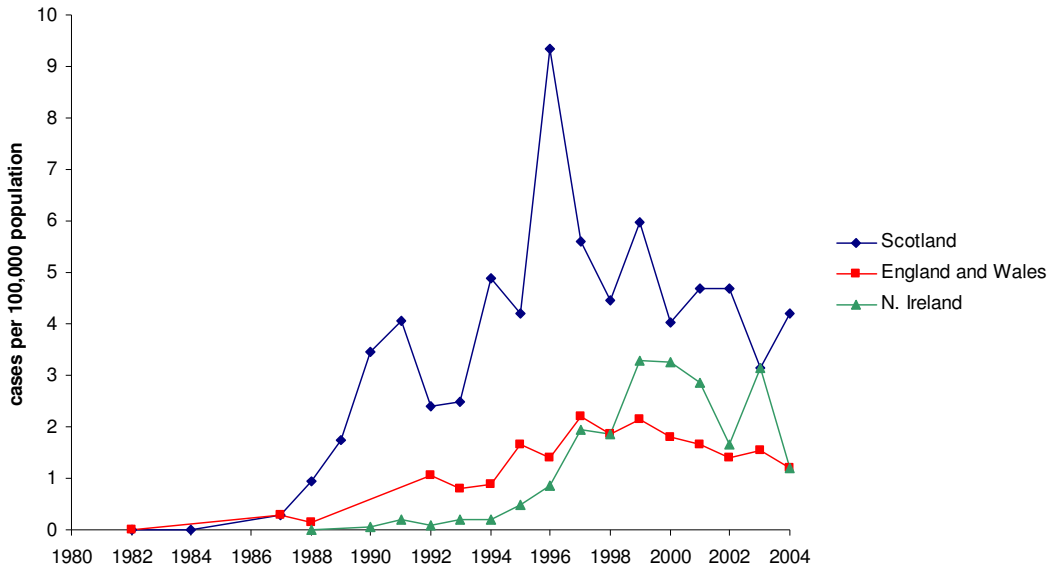


Figure 1.1: Prevalence of *E. coli* O157 in Scotland compared to the rest of the UK: Cases of EHEC infection in Scotland have been consistently higher than the UK average (redrawn from Lynn *et al.*, 2005).

1.3.3 Enterohaemorrhagic *E. coli* in cattle

As highlighted in Section 1.3.2, cattle provide a reservoir for *E. coli* O157:H7, which is important for its transmission to humans (reviewed in Caprioli *et al.*, 2005). *E. coli* O157:H7 is essentially non-pathogenic in cattle although it has been associated with diarrhoea in very young calves (Kang *et al.*, 2004). Non-O157 serotypes also identified as human EHEC, including O5, O26 and O118, have been noted to induce diarrhoea in weaned cattle exhibiting extensive A/E lesion formation in the bovine large intestine although progression towards haemorrhagic symptoms, as observed in human EHEC infection, has only been noted in very young calves (Wieler *et al.*, 1996;Pearson *et al.*, 1999;Stevens *et al.*, 2002;Sandhu and Gyles, 2002). The lack of clinical signs and asymptomatic tolerance of *E. coli* O157:H7 in ruminants is likely to be an important factor in the prevalence of this serotype in human disease. Unlike the strains associated with diarrhoea in cattle (mentioned above) *E. coli* O157:H7 does not persist throughout the large intestine in weaned cattle and has been found to colonise in the largest numbers at the recto-anal junction (RAJ) (Naylor *et al.*, 2003;Low *et al.*, 2005;Lim *et al.*, 2007). Several efforts have been made to define the gastrointestinal localisation of EHEC in the ruminant host. Early studies concluded that *E. coli* O157:H7 is able to colonise the bovine GIT at multiple sites with a general predominance at large intestinal sites (Cray, Jr. and Moon, 1995;Brown *et al.*, 1997;Dean-Nystrom *et al.*, 1998a;Laven *et al.*, 2003). However, Grauke *et al.* (2002) noted that colonisation of these intestinal sites was only observed up to 16 days post infection, although faecal shedding was observed up to 34 days later. Naylor *et al.* (2003) hypothesised that lengthened shedding may be due to rectal colonisation and were able to show that *E. coli* O157:H7 shows a preference for the terminal 5cm of the rectum, proximal to the recto-anal junction (RAJ). Preference for this site has since been reported in naturally colonised cattle, confirming that this is not merely a feature of experimental challenge (Low *et al.*, 2005;Lim *et al.*, 2007).

It has been proposed that persistent rectal colonisation is the major feature of "super-shedders" (Cobbold *et al.*, 2007). These comprise a small proportion of cattle which are responsible for a large proportion of the transmission of *E. coli* O157:H7 among herds (Matthews *et al.*, 2006a;Matthews *et al.*, 2006b;Chase-Topping *et al.*, 2007). Rectal *E. coli* O157:H7 is proposed to coat the faeces on egestion, allowing shedding and transmission of

the organism to be maintained. The site of colonisation at the terminal rectum is an area densely populated by lymphoid follicles (LFs). Bacterial interaction with LF-dense mucosal tissue is discussed in detail in Section 1.5.2.

Due to the preference shown for the bovine terminal rectum by *E. coli* O157:H7 this region could represent a target for the development strategies for the control of EHEC in cattle. As the area is fairly accessible, topical applications of antimicrobial agents to the rectum have been investigated as a method of control (Naylor *et al.*, 2007) although the impracticality of such a strategy is likely to prohibit widespread adoption of such methods.

As the bovine gastrointestinal tract provides a refuge for the propagation of EHEC, this poses a significant risk to human health due to the potential for human contact either via food or via contact with cattle or environments contaminated with cattle faeces. Intervention at this point of contact would theoretically reduce the incidence of human EHEC infection.

1.3.4 Options for control of EHEC infection

As mentioned previously, there is no effective treatment for EHEC in humans once it has established infection. Therefore, prevention of infection by targeting environmental and animal sources of the organism is likely to have positive benefits in terms of reduction of the transmission of EHEC to humans. As the bovine host represents an important vector for EHEC, the reduction of EHEC prevalence in cattle may in turn help to reduce incidence of human infection. Simplistic interventions include improvements in drinking water, feed and pen hygiene (Garber *et al.*, 1999;Lejeune *et al.*, 2001;Smith *et al.*, 2001;Dodd *et al.*, 2003). Prevention of contact between wildlife and cattle is another possible control point, as many animals may carry EHEC, facilitating its transmission (Daniels *et al.*, 2003). Modulation of gastrointestinal acidity by switching feed from grain to hay prior to slaughter has been suggested as a means of reduction of the carriage of *E. coli* O157:H7 but this is a subject of contention and has not been reproduced on a larger scale (Diez-Gonzalez *et al.*, 1998;Hovde *et al.*, 1999).

Control methods employing antimicrobial substances has also been proposed (Elder *et al.*, 2008) although the use of antibiotics poses problems such as disruption of the normal gastrointestinal microbiota of the animal and promoting the proliferation of pathogenic antibiotic-resistant bacteria. Numerous vaccination strategies have been devised, although none have yet led to an effective product (Konadu *et al.*, 1999;Dean-Nystrom *et al.*, 2002;Potter *et al.*, 2004;Ahmed *et al.*, 2006;McNeilly *et al.*, 2008).

Biological control is another option for targeted control of EHEC in cattle. Exclusion of *E. coli* O157:H7 using bacteriophages has been validated in murine models but not yet implemented in ruminants (Sheng *et al.*, 2006a). Probiotic bacteria have been tested for this purpose, most significantly *Lactobacillus acidophilus* which is widely available in USA for addition to feed which is proposed to reduce the numbers of bacterial pathogens present in the ruminant gut (Elam *et al.*, 2003). Probiotic *E. coli* strains have also been found to have efficacy in reduction of EHEC prevalence in farm animals (Zhao *et al.*, 1998;Tkalcic *et al.*, 2003;Zhao *et al.*, 2003;Schamberger *et al.*, 2004;Schamberger and Diez-Gonzalez, 2004). The most promising strains for this application are those able to elaborate bacteriocins, including colicins (large protein bacteriocins) and microcins (small peptide bacteriocins) (Sable *et al.*, 2000;Callaway *et al.*, 2003). These are molecules produced by *E. coli* which have antibacterial activity, increasing the competitive fitness of the bacteriocin-producing strain. Direct application of bacteriocins to food products has also been considered as a strategy for post-harvest control of EHEC in foodstuffs (Nandiwada *et al.*, 2004;Abercrombie *et al.*, 2006).

1.4 Molecular basis of EHEC colonisation and pathogenesis

EHEC-pathogenicity is complex and multifactorial. EHEC possess elaborate mechanisms which aid the subversion of host defences and the progression of EHEC pathogenesis.

1.4.1 Type III Secretion System

The genomes of EHEC and closely related EPEC carry a pathogenicity island known as the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995). This locus encodes the genes necessary for the bacterium to induce attaching/effacing (A/E) lesions associated with EHEC and EPEC colonisation, including a functional type III secretion system (T3SS) (Jerse *et al.*, 1990;Jarvis *et al.*, 1995). The LEE of EPEC O127:H6 is wholly sufficient for the induction of A/E lesions, whereas the LEE of EHEC O157:H7 requires additional factors encoded elsewhere on the genome (McDaniel and Kaper, 1997;Elliott *et al.*, 1999). The LEE carried by EPEC and EHEC is approximately 35.5kbp and the genes encoded are organised into 5 operons: LEE1-LEE5 (Figure 1.2) (McDaniel *et al.*, 1995). Operons 1-3 encode the proteins required to form the secretion apparatus, the *E. coli* secretory complex or *esc* genes. The LEE4 operon encodes EspA (*E. coli* secreted protein A) which forms the needle structure of the T3SS, EspB and EspD which form a pore in the host cell membrane (Sekiya *et al.*, 2001;Ogino *et al.*, 2006). Through this complex a number of proteins are injected into the cell. These include LEE5-encoded Tir (translocated intimin receptor) which becomes embedded in the host cell membrane. Tir interacts with intimin (also LEE5-encoded) which is a bacterial outer membrane protein and this interaction facilitates intimate adherence between bacterium and host (Kenny *et al.*, 1997;Deibel *et al.*, 1998). Intimin is proposed to have a second, Tir-independent, role in adherence to host enterocytes in that initial bacterial adherence to host cells is proposed to be aided by interaction between intimin and host protein nucleolin (Sinclair and O'Brien, 2002).

Tir protein is central to the divergent mechanisms of EHEC- and EPEC-induced A/E lesion formation (Ismaili *et al.*, 1995;DeVinney *et al.*, 2001). Intimate adherence of EHEC or EPEC to the enterocyte, facilitated by Intimin-Tir binding, triggers the process of actin polymerisation to form the pedestal structure of the A/E lesion (Cantarelli *et al.*,

2000;Goosney *et al.*, 2001). EPEC-encoded Tir is phosphorylated on a tyrosine residue and recruits Nck adaptor protein leading to activation of neuronal Wiskott Aldrich syndrome protein (N-WASP). N-WASP then acts to activate the Arp2/3 complex leading to actin nucleation (Kalman *et al.*, 1999;Higgs and Pollard, 2001;Rohatgi *et al.*, 2001;Campellone *et al.*, 2004a). EHEC-encoded Tir requires non-LEE encoded effector $EspF_U$ (or TccP) which is functionally similar to host adaptor protein Nck and mediates signalling between EHEC and N-WASP (Garmendia *et al.*, 2004;Campellone *et al.*, 2004b). Intimate adherence between bacterium and host cell leads to microcolony formation. The subsequent condensation of filamentous actin leads to effacement of microvilli and formation of the pedestal structure of the A/E lesion.

In addition to the aforementioned effectors A/E lesion-inducing bacteria (EHEC, EPEC and *Citrobacter rodentium*) inject a number of effector proteins into the host cell, many of which interact in some way with the cell cytoskeleton. Mitochondria-associated protein (Map) and EspF are proposed to aid disruption of transepithelial resistance and mitochondrial membrane potential (McNamara and Donnenberg, 1998;McNamara *et al.*, 2001a). Map, along with EspH and EspB, also have roles in the modulation of the cytoskeleton to induce elaboration of filopodia (Kenny *et al.*, 2002;Tu *et al.*, 2003). EspF has been observed to contribute significantly to the induction of cell death (Crane *et al.*, 2001; Viswanathan *et al.*, 2004) and EspG induces destruction of microtubule network (Matsuzawa *et al.*, 2004). Certain effector proteins are encoded outwith the LEE. Several T3SS effector proteins have not been assigned a function, including LEE-encoded EspZ and Non-LEE-encoded NleA(EspI), NleB, NleC, NleD, NleE, NleF and NleH (Deng *et al.*, 2004;Gruenheid *et al.*, 2004;Kanack *et al.*, 2005). NleA is proposed to inhibit protein export by the host cell (Kim *et al.*, 2007). NleD has been proposed to aid colonisation of experimentally infected calves, although the mechanism by which it achieves this is as yet unknown (Dziva *et al.*, 2004). Certain non-O157 strains of EHEC and EPEC have been observed to inject a cell cycle inhibitory protein (Cif) into the host cell (Marches *et al.*, 2003). Bioinformatic analysis suggests that the extent of the function of the T3SS is greater than previously imagined. Mining of the *E. coli* O157:H7 genome revealed a number of putative T3SS effectors of which 39 were confirmed as injected proteins using translocation assays (Tobe *et al.*, 2006); however, functions are not yet assigned to the majority of these injected proteins. Thus, type III secreted proteins perform a range of

function, some of which remain to be elucidated, which may aid the hijacking of host cell function for the benefit of the bacterium.

Complex regulatory control ensures the expression of the T3SS at the optimal stage in bacterium-host interaction. Regulation of the expression of LEE-encoded operons is defined by the action of Ler which is encoded on the LEE1 operon. Ler acts to upregulate both LEE-encoded and non-LEE-encoded virulence factors (Mellies *et al.*, 1999;Friedberg *et al.*, 1999;Sperandio *et al.*, 2000;Sanchez-SanMartin *et al.*, 2001). Quorum sensing has been implicated as a key signal which triggers transcription of the LEE-encoded genes (Sperandio *et al.*, 1999).

As mentioned earlier, LEE-encoded intimin protein is an important determinant of adherence to human enterocytes both in combination with the T3SS, in the form of intimin-Tir binding and independently of Tir. Intimin-Tir binding has also been shown to be important for the colonisation of ruminants and A/E lesions have been observed in EHEC-infected bovine and ovine intestinal tissues, even though the ruminant host generally remains healthy throughout EHEC infection (Dean-Nystrom *et al.*, 1998b;Cornick *et al.*, 2002;Woodward *et al.*, 2003;Naylor *et al.*, 2005).

The intimin gene exhibits significant diversity between A/E *E. coli* subtypes and currently 14 serologically distinct intimin types have been described denoted by Greek letters α (alpha) to ξ (xi) (Agin and Wolf, 1997;Adu-Bobie *et al.*, 1998;Oswald *et al.*, 2000;Tarr and Whittam, 2002;Zhang *et al.*, 2002;Jores *et al.*, 2003). The substitution of intimin types has been noted to alter the tissue tropism of *E. coli*, as determined using *in vitro* organ culture (IVOC) (Phillips and Frankel, 2000;Hartland *et al.*, 2000;Fitzhenry *et al.*, 2002). This feature could relate to a Tir-independent receptor binding such as the affinity of intimin γ of *E. coli* O157:H7 for nucleolin (Sinclair and O'Brien, 2002). Thus, intimin subtypes may be related to tissue and host specificity.

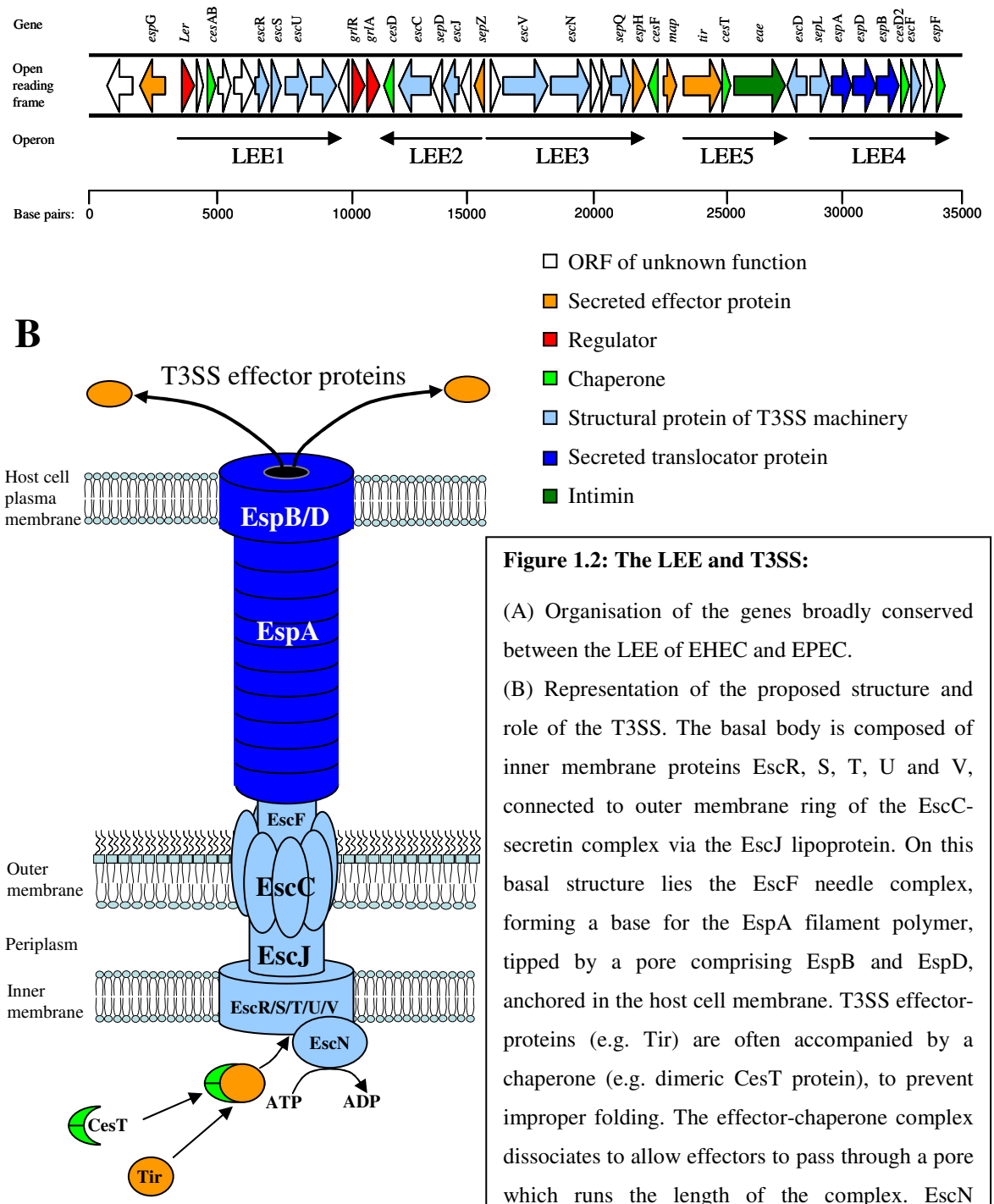


Figure 1.2: The LEE and T3SS:

(A) Organisation of the genes broadly conserved between the LEE of EHEC and EPEC.

(B) Representation of the proposed structure and role of the T3SS. The basal body is composed of inner membrane proteins EscR, S, T, U and V, connected to outer membrane ring of the EscC-secretin complex via the EscJ lipoprotein. On this basal structure lies the EscF needle complex, forming a base for the EspA filament polymer, tipped by a pore comprising EspB and EspD, anchored in the host cell membrane. T3SS effector-proteins (e.g. Tir) are often accompanied by a chaperone (e.g. dimeric CesT protein), to prevent improper folding. The effector-chaperone complex dissociates to allow effectors to pass through a pore which runs the length of the complex. EscN provides energy for this process through hydrolysis of ATP (redrawn from Garmendia *et al.* 2005).

1.4.2 Shiga-like/Vero-toxins

As denoted in Section 1.3, Shiga-like toxins have a major role in the severity of EHEC induced disease. Stx is responsible for the majority of the damage to the intestinal mucosal vasculature during haemorrhagic colitis and damage to the renal, circulatory and central nervous system in cases of HUS and TTP (O'Brien and Holmes, 1987). The importance of Stx in EHEC-infection has been highlighted by the discovery that the neutralisation of Stx activity reduced the effects of infection in animal models (Matise *et al.*, 2001;Paton *et al.*, 2001a;Sheoran *et al.*, 2003). Stx is an example of an AB_5 toxin, comprising a catalytic A-subunit (32kDa) and a pentameric B-subunit (five 7.7kDa monomers) (Donohue-Rolfe *et al.*, 1984;Stein *et al.*, 1992;Fraser *et al.*, 2004). The pentameric B subunit binds to the globotriaosylceramide (Gb_3) receptor (also known as CD77) (Lindberg *et al.*, 1987;Lingwood, 1996). The toxin is then internalised by endocytosis, in clathrin coated pits. Depending on the cell type, internalised toxin may have two fates: (i) the toxin is transported to the cytoplasm via the endoplasmic reticulum and Golgi apparatus followed by the cleavage of the A1 peptide of the A-subunit by furin, releasing a 28kDa catalytically active protein which acts to cleave 28S rRNA (Endo *et al.*, 1988;Lea *et al.*, 1999); resultant inhibition of protein synthesis causes apoptosis (Sandvig *et al.*, 2002) (ii) in cell types which present Gb_3 receptors but resist the action of verotoxin internalised toxin vesicles are fused with lysosomes leading to degradation of the toxin (Falguieres *et al.*, 2001;Hoey *et al.*, 2003).

The host inflammatory response to EHEC (examined further in Section 1.5.3) may enhance the effects of Stx as TNFα and IL-1β levels increase presentation of Gb3 receptors on human vascular endothelial cells (Louise and Obrig, 1991;van de Kar *et al.*, 1992;Kaye *et al.*, 1993;Louise *et al.*, 1995). Polymorphonuclear leukocytes (PMLs) localised to the site of EHEC infection are proposed to facilitate the carriage of Stx to target organs as Stx has been observed to bind to the surface of these cells and this may allow the toxin to be transported systemically. Stx is proposed to have greater affinity for Gb3 receptors presented by epithelial cells; hence, the toxin may be delivered by PMLs to regions where the greatest Stx-mediated damage is observed (Hurley *et al.*, 2001;te Loo *et al.*, 2001a;te Loo *et al.*, 2001b).

It has not yet been determined whether Stx is toxic to the bovine host. Cattle remain largely unaffected by colonisation by Stx-producing *E. coli* strains, despite the detection of significant levels of Stx in bovine faecal matter (Ball *et al.*, 1994;Hyatt *et al.*, 2001). Immune recognition of Stx by the bovine host is likely to occur, as anti-Stx antibodies have been detected in bovine serum, colostrum and mucosal material (Pirro *et al.*, 1995;Johnson *et al.*, 1996). The insensitivity of the bovine host to Stx may be due to the lack of Gb3 receptors on bovine vascular endothelial cells (Pruimboom-Brees *et al.*, 2000). The differential consequences of EHEC infection of the bovine host could depend on the method of processing the toxin by Gb3-positive basal crypt cells, which have been shown to target the protein to lysosomes (Hoey *et al.*, 2003). Stx may also modulate the immune response of cattle and there is evidence that the toxin reduces the proliferation and activation of peripheral blood mononuclear cells (PBMCs) and inhibits proinflammatory cytokine secretion by intraepithithelial lymphocytes (Menge *et al.*, 1999;Menge *et al.*, 2004). In this way, Stx could contribute to immune tolerance of EHEC in the bovine host, may be important in defining the different outcomes of EHEC colonisation of bovine and human hosts, especially as immune/inflammatory damage contributes to the severity of the disease in humans and the likelihood of progression to HUS (Westerholt *et al.*, 2003).

Two distinct subgroups of Stx are produced by EHEC (Scotland *et al.*, 1983;Strockbine *et al.*, 1986). Stx1 is indistinguishable from shiga toxin produced by *Shigella dysenteriae*, whereas Stx2 exhibits 56% amino acid identity to Stx1. Isolates expressing Stx2 have been linked to increased likelihoods of causing HUS (Gewirtz *et al.*, 2001a;Zhou *et al.*, 2003;Khan *et al.*, 2004). Up to 11 variants of Stx2 have been identified. These include "conventional" $Stx2_{EDL933}$ (also termed $Stx2_{vha}$) Stx2c (also termed $Stx2_{vhb}$) Stx2d, Stx2e, Stx2f and Stx2g each are antigenically non-cross reactive (Perera *et al.*, 1988;Schmitt *et al.*, 1991;Schmidt *et al.*, 2000;Brett *et al.*, 2003). $Stx2_{EDL933}$/$Stx2_{vha}$ will be termed simply "Stx2" or "conventional Stx2" in this study as it is the prototype Stx2 allele encoded by reference strains of *E. coli* O157:H7. Intestinal mucous has been observed to increase the activity of Stx2d, due to the action of elastase, cleaving $Stx2_d$ to form a toxic mature protein (Melton-Celsa *et al.*, 1996). $Stx2_e$-producing *E. coli* causes oedema disease in swine and differs from other Stx subtypes in that it binds to globotetraosylceramide (Gb_4) receptor (DeGrandis *et al.*, 1989). Thus, verotoxin subtype may aid determination of host specific pathogenesis among shiga-toxin producing *E. coli*. Levels of toxicity of Stx vary between Stx subtypes. For example: toxicity of activated Stx2d is greater than that of

Stx2/Stx2c and the activity of Stx1 and Stx2e are the least verotoxic (O'Brien and LaVeck, 1983;Samuel *et al.*, 1990;Paros *et al.*, 1993;Lindgren *et al.*, 1994). Hence, the Stx subtype could be important in the levels of cellular damage caused by pathogenic STEC and the increased toxicity of Stx2 could explain the increased severity of infections caused by Stx2 producing EHEC strains.

The action of Stx has also been proposed to aid gastrointestinal colonisation, perhaps by increasing presentation of nucleolin which is proposed to act as an initial receptor for intimin, enhancing bacterial adherence to the enterocyte (Robinson *et al.*, 2006). This was determined using epithelial cell line and a mouse model of infection and verification of this function in relevant host species has not been performed. Nevertheless, this discovery suggests a link between Stx and intimin which further highlights the complex interplay between EHEC virulence factors during intestinal colonisation.

1.4.3 Plasmid-encoded factors

The majority of human EHEC isolates generally carry a large (~90kbp) plasmid, which contributes to virulence (Levine *et al.*, 1987;Beutin *et al.*, 1994;Schmidt *et al.*, 1994). In the majority of *E. coli* O157 isolates the plasmid carries genes encoding several putative virulence factors including *ehxA*, *espP* and *tox*B which are described below. It has been noted that the plasmid confers enhanced adherence to cultured cells when compared with plasmid-cured *E. coli* O157:H7 derivatives and increases adherence of transformed *E. coli* K12 to the rabbit intestinal epithelium (Toth *et al.*, 1990;Dytoc *et al.*, 1993) suggesting that the plasmid carries genes contributing to adherence mechanisms.

EspP is a member of the SPATE (serine protease autotransporters of the Enterobacteriaceae) family of proteases secreted by the type V secretion system (reviewed in Henderson *et al.*, 2004). EspP is highly expressed under laboratory culture conditions and is also recognised during the course of human infection, as anti-EspP antibodies have been detected in the sera of EHEC-infected patients (Brunder *et al.*, 1997). EspP cleaves pepsin A and human coagulation factor V and thus could act to exacerbate the haemorrhagic effects of EHEC infection (Brunder *et al.*, 1997). EspP has also been noted

as an enhancer of bacterial adherence to bovine enterocytes (Dziva *et al.*, 2007); hence, this protein could aid colonisation of the bovine host by *E. coli* O157:H7.

Plasmid-encoded enterohaemolysin (EhxA) belongs to the RTX (repeats in the structural toxin) toxin family, which are expressed by several Gram-negative pathogens of humans and animals, including *Mannheimia haemolytica*, *Bordetella pertussis* and *Actinobacillus* spp. (Bauer and Welch, 1996;Frey and Kuhnert, 2002). EhxA is highly conserved among EHEC strains but its role in pathogenesis remains undefined (Schmidt *et al.*, 1996;Boerlin *et al.*, 1999). EHEC strains which express EhxA exhibit weak calcium dependent haemolysis of sheep erythrocytes, which is dependent on Ca^{2+} ions (Beutin *et al.*, 1989;Bauer and Welch, 1996). Low levels of haemolysis have been attributed to the lack of a fully functional export system for this protein and complementation of *E. coli* O157:H7 with the further components of this system vastly increases EhxA-dependent haemolytic activity (Schmidt *et al.*, 1995a). Expression of EhxA is increased under anaerobic conditions and *in vivo* expression is also suggested, as antibodies to Ehx have been detected in the sera of HUS patients (Schmidt *et al.*, 1995a;Chart *et al.*, 1998); however, whether this protein influences virulence functions of the bacterium *in vivo* remains unclear.

StcE (secreted protease of C1 inhibitor from EHEC), also referred to as TagA for its similarity to the ToxR-regulated lipoprotein of *Vibrio* spp., was identified as an antigen in the sera of HUS patients (Paton and Paton, 2002). Like EspP, it is a plasmid-encoded protease which cleaves a factor which is important to host immunity by catalysing the cleavage of C1-esterase inhibitor (C1-INH) which is an inhibitor of the complement cascade (Lathem *et al.*, 2002). The cleavage of C1-INH by StcE increases the inhibitory activity of C1-INH, hence decreasing complement-mediated cell lysis (Lathem *et al.*, 2004). Secretion of StcE depends upon the *etp* gene cluster which encodes a functional type II secretion system, also encoded on the plasmid (Schmidt *et al.*, 1997;Lathem *et al.*, 2002). The precise role of this protein in EHEC infection is not yet confirmed, although prevention of the death of host cells could aid the survival of EHEC-infected cells and hence aid persistence of the bacterium as EHEC relies on adherence to enterocytes for survival and proliferation within the host. StcE is also proposed to have mucinase activity and may affect surface proteins of the enterocyte, which could increase bacterial adherence (Grys *et al.*, 2005;Grys *et al.*, 2006).

Also encoded on the plasmid is ToxB, a homologue of the Tox proteins of *C. difficile* (Burland *et al.*, 1998). Homologues of ToxB are also encoded by sorbitol-fermenting *E. coli* O157:H-, non-O157 EHEC and EPEC termed Efa-1 or LifA (Nicholls *et al.*, 2000;Klapproth *et al.*, 2000;Janka *et al.*, 2002). ToxB and Efa-1 have been suggested to increase adherence of EHEC to cultured cells and aid colonisation of cattle by mediating increased T3SS expression (Nicholls *et al.*, 2000;Stevens *et al.*, 2002). However, mutation of the *tox*B gene did not significantly affect the length of faecal shedding of *E. coli* O157:H7 in cattle (Stevens *et al.*, 2004).

Although a similar large plasmid is carried by many EHEC subtypes, considerable strain-dependent variation exists in its genetic composition. The plasmid encoded by sorbitol-fermenting *E. coli* O157:H- (pSFO157) is roughly 30kbp larger than that carried by *E. coli* O157:H7. It lacks *kat*P (catalase peroxidise), *espP* and *tox*B genes and carries a larger proportion of genes related to conjugal transfer, along with a novel fimbrial locus termed *sfp*, which has homology to P-fimbriae encoded by ExPEC strains (Burland *et al.*, 1998;Brunder *et al.*, 2001;Brunder *et al.*, 2006). Plasmid-encoded genes for a novel auto-agglutinating adhesin have been identified in LEE-negative STEC strains including *E. coli* O113:H21 (Paton *et al.*, 2001b). The virulence factor encoding genes *espP* and *ehxA* also exhibit strain-specific polymorphisms (Brunder *et al.*, 1999;Cookson *et al.*, 2007). Certain subtypes of EspP are inactive against pepsin A and human coagulation factor V (Brockmeyer *et al.*, 2007), although the roles of the proteolytically inactive forms of EspP as adhesins have not been investigated. The significance of *ehxA* variants remain to be determined, although all subtypes identified by Cookson *et al.* (2007) were found to have comparable haemolytic activities.

1.4.4 Flagella

The flagella are filamentous appendages which the bacterium may rotate to facilitate motility (Berg and Anderson, 1973). The flagellar filament is made up of 11 protofilaments, which are essentially chains of polymerised flagellin (FliC protein) monomers (Samatey *et al.*, 2001). The FliC monomers are connected via the interactions of concave and convex domains (Samatey *et al.*, 2001;Smith *et al.*, 2003;Yonekura *et al.*,

2003). The filament is attached to a hook complex, which is anchored to the basal body, forming the flagellar motor (reviewed in MacNab, 2003). Flagella and T3SS share structural and functional similarities and polymerisation of flagellin is proposed to occur by a similar mechanism to the translocation of T3SS effector proteins (Galan *et al.*, 1992).

Motility coupled with regulation of motility using two-component regulatory systems (e.g. Che proteins) allows chemotaxis of the organism, facilitating the acquisition of nutrients, evasion of toxic environments and translocation to preferred sites in the host (Adler and Tso, 1974). However, the flagellum provides a target for host innate immunity and is recognised by host pattern recognition receptors (PRRs) including Toll like receptor (TLR) 5 (discussed further in Section 1.5.1). The conserved amino and carboxy termini of flagellin are recognised by TLR5 (Eaves-Pyles *et al.*, 2001;Donnelly and Steiner, 2002;Smith *et al.*, 2003;Murthy *et al.*, 2004). Flagellin has also been found to activate alternative PRR-signalling pathways, including TLR2 and glycolipids such as gangliosides (McNamara *et al.*, 2001b;Adamo *et al.*, 2004;Ogushi *et al.*, 2004). Adaptive immune responses in the form of antibodies are generally observed against the variable central domain and his variability has given rise to the serological diversity exemplified by the H-serotyping scheme, described in Section 1.2.1 (Kuwajima, 1988;Newton *et al.*, 1991;He *et al.*, 1994;Yoshioka *et al.*, 1995).

Although a role for the flagellum in adherence of EPEC to host cells has been suggested several lines of evidence suggest that H7-flagellin is unlikely to have a direct role as an EHEC adhesin, in that antibodies against H7-flagellin do not inhibit adherence and purified H7 flagellin does not adhere directly to cells (Giron *et al.*, 2002). EHEC also shuts down flagellar expression in microcolonies and upregulates expression of the T3SS, at a time when adherent mechanisms are required to function at their maximal levels. This switching mechanism is mediated by integration host factor (IHF) which activates the expression of Ler and prevents expression of the *flhDC* regulon, shutting down expression of flagellar components (Yona-Nadler *et al.*, 2003). However, recent evidence suggests that H7 flagellin is important in binding to intestinal mucins (Erdem *et al.*, 2007) and therefore may have an initial role in localising the bacterium to the mucosal surface.

1.4.5 Additional EHEC virulence and colonisation factors

The resolution of the complete genome sequences of two *E. coli* O157:H7 isolates has allowed the prediction of further potential virulence factors (Perna *et al.*, 2001;Hayashi *et al.*, 2001b). These are encoded on genomic regions present in *E. coli* O157:H7 genome which are absent from the *E. coli* K12 MG1655 genome, termed "O-islands" (Perna *et al.*, 2001). Candidate factors with predicted roles in the infective process include fimbrial adhesins such as two long polar fimbriae operons (LPF1 and LPF2) encoded on O-island 154 (Torres *et al.*, 2002;Torres *et al.*, 2004). Long polar fimbriae have been suggested to aid initial adherence of the bacterium to host enterocytes (Jordan *et al.*, 2004). Curli fimbriae are also expressed by *E. coli* O157:H7 and may have a role in adherence (Torres and Kaper, 2003). Outer membrane proteins OmpA and Iha (IrgA homologue adhesin) have also been put forward as adhesins in EHEC and UPEC strains. Both of these adhesins are also believed to have functions in bacterial iron-uptake mechanisms (Tarr *et al.*, 2000;Torres and Kaper, 2003;Leveille *et al.*, 2006). EHEC also produces a pilus which is also common among human commensal *E. coli* isolates, termed "*E. coli* common pilus" and has been suggested to aid adherence to epithelial cells (Rendon *et al.*, 2007). A role in adherence was established using HEp-2 cells as an epithelial model (derived from a larynx carcinoma) and its role in adherence to the intestinal epithelium or in interactions with non-human hosts remains to be established.

The level of horizontal gene transfer among *E. coli* especially facilitated by bacteriophages has lead to the differential carriage of potential virulence factors by EHEC. Sorbitol-fermenting *E. coli* O157:H- and other non-O157 STEC serotypes have been observed to produce cytolethal distending toxin, generally associated with ExPEC strains, encoded by genes *cdtB* (encoding the catalytic subunit), *cdt*A and *cdt*C (which facilitate the delivery of the catalytic subunit to the host cell) (Janka *et al.*, 2003;Bielaszewska *et al.*, 2005). The production of cytolethal distending toxin by EHEC strains could contribute to the induction of endothelial cell death in cases of human EHEC infection although this is yet to be ascertained. Certain EHEC strains, including EHEC serotypes O113 and O157, have been observed to produce subtilase toxin. Subtilase toxin shares similarity with toxins produced by *Bacillus* spp. and *Yersina pestis*. Similarly to Stx, subtilase is an AB_5 toxin and has been proposed to be a major inducer of HUS in response to EHEC strains that lack conventional *stx* genes (Schmidt *et al.*, 1999;Paton *et al.*, 2004).

In summary, EHEC interactions with the host are complex and mediated by many factors. The multiple adherence mechanisms proposed show that there may be functional redundancy, possibly providing backup adherence mechanisms. The wide range of adhesive and pathogenic mechanisms could be important for the interaction of *E. coli* with different hosts or different tissue types.

1.5 Bacteria and the Gut

1.5.1 *E. coli* and the gastrointestinal microflora

Bacteria populate the mammalian gut in substantial numbers. In the human GIT, colonisation ranges from 10^2-10^5 cfu/ml of gut contents in the upper regions of the GIT to 10^{11}-10^{12}cfu/ml in the colon (Evaldson *et al.*, 1982). The gut microbiota comprises a large variety of bacterial genera and there are estimated to be more than 500 different species of bacteria commonly found in the human gut. Obligate anaerobes predominate, especially species of the genera *Clostridium* and *Bacteroides* (Eckburg *et al.*, 2005). Facultative anaerobes including *E. coli* are thought to comprise only a small proportion of the microbial flora (~0.1%) but are consistently present (Suau *et al.*, 1999;Hayashi *et al.*, 2002;Wang *et al.*, 2003). The presence of the gastrointestinal microflora has significant benefits for both bacterium and host and can be considered as an example of symbiosis. The host provides a supply of nutrients and maintains an optimal temperature for bacterial growth, while the microflora benefits the host by protection from cellular injury, regulation of lipid storage and stimulation of the development of intestinal tissues (Stappenbeck *et al.*, 2002;Rakoff-Nahoum *et al.*, 2004;Backhed *et al.*, 2004). The constant basal level of pattern recognition receptor (PRR)-stimulation (discussed in more detail in Section 1.5.3) by the gastrointestinal microbiota is important in these functions (Rakoff-Nahoum *et al.*, 2004). The normal gut microflora is also important for the prevention of colonisation of pathogenic bacteria, evidenced by the fact that clearing of the gastrointestinal microflora using antibiotics can lead to the proliferation of pathogens such as *Clostridium difficile* (Zwiener *et al.*, 1989). Thus, the gut microflora serves as a source of opportunistic bacterial pathogens including *E. coli* such as ExPEC (described in Section 1.3). In addition, dysregulated responses of the human host to the intestinal microflora are proposed to be central to the progression of inflammatory gut diseases (reviewed in Tlaskova-Hogenova *et al.*, 2004) .

The benefits of non-pathogenic strains of *E. coli* in promotion of human health have been recognised. Certain strains of *E. coli* have proven probiotic effects, such as the Mutaflor isolate of *E. coli* (Nissle 1917) which has been shown to reduce gastrointestinal colonisation by pathogenic bacteria and have efficacy for the treatment of inflammatory

disorders, such as IBD and Crohn's disease, by modulation of epithelial signal transduction pathways (Lodinova-Zadnikova and Sonnenborn, 1997;Lodinova-Zadnikova *et al.*, 2003;Kamada *et al.*, 2008). Probiotic *E. coli* strains have shown promise as agents which may be applied to reduce the carriage of *E. coli* O157:H7 in cattle; however, further investigation is required in order to develop an applicable probiotic strategy for commercial use in the cattle farming industry (examined in more detail in Chapter 5).

1.5.2 Gastrointestinal physiology and Bacterial interactions with the host

Bacteria are found in large numbers in the faeces but the most successful gastrointestinal colonists include those which are able to adhere to the gastrointestinal mucosal surface allowing persistence and propagation of the organism within the host. In order to adhere to host enterocytes, certain bacteria have evolved diverse adherence mechanisms which are often based on the binding bacterial surface molecules with receptor molecules presented by the host cell. *E. coli* strains elaborate a variety of fimbriae, which enhance adherence to particular cellular receptors. Many of the fimbrial subtypes have affinity for particular sugar residues contained in host glycoproteins or glycolipids. Alteration of the presentation of host cell surface molecules differs both between host species and regionally within a host, often due to host or tissue specific regulation of glycosyltransferases (reviewed in Varki, 1996). As different tissue types present a different complement of receptor molecules, the expression of different adhesin molecules by a bacterium aids definition of its tissue and host specificity.

The most commonly encoded *E. coli* adhesins are type I fimbriae, which have affinity for mannosides, laminin, fibronectin and plasminogen (Krogfelt *et al.*, 1990;Sokurenko *et al.*, 1998;Pouttu *et al.*, 1999;Pouttu *et al.*, 2000). The affinity of type I fimbriae for such complexes is important in colonisation during *E. coli*-induced meningitis (Pouttu *et al.*, 1999), although the ubiquitous nature of these fimbriae are among *E. coli* suggests that they are a general adherence molecule of both pathogenic and commensal organisms. P-, Prs- and S- fimbriae are commonly expressed by ExPEC and also *E. coli* strains which persist in the human gastrointestinal tract (Nowrouzian *et al.*, 2001). These fimbriae target galactose-based surface carbohydrates (Hull *et al.*, 1981;Korhonen *et al.*, 1984;Lund *et al.*, 1988). K88 (F4) and K99 (F5) fimbriae, which are prevalent among pathogenic ETEC

strains of pigs and calves respectively, also recognise carbohydrate-based cellular receptors (Smit *et al.*, 1984;Erickson *et al.*, 1994). The ligand recognised by K99 fimbriae: N-glycosylneuraminic acid (NeuGc) is not presented by human enterocytes; hence, K99 presenting ETEC strains have not been identified in cases of human infection (Smit *et al.*, 1984;Mouricout *et al.*, 1990). Production of F17 fimbriae has been observed among strains which cause diarrhoea and septicaemia in cattle (Bertin *et al.*, 1996b) and also among bovine commensal *E. coli* (Güler *et al.*, 2008). F17 fimbriae have affinity for receptors containing *N*-acetyl glucosamine residues (Saarela *et al.*, 1996;Bertin *et al.*, 1996a). In this way, pathogenic and commensal *E. coli* elaborate a variety of fimbrial adhesins many of which are dependent on polysaccharide components of the host cell membrane; hence, differential presentation of these polysaccharides may contribute to definition of *E. coli* host and tissue specificity.

E. coli O157:H7 has been proposed to exhibit tissue specificity in colonisation of the GIT of the bovine and human hosts. The bacterium localises preferentially to areas of mucosal tissue which are densely populated by lymphoid follicles especially at the ileal Peyer's patches during human infection and at the recto-anal junction in cattle (Phillips and Frankel, 2000;Phillips *et al.*, 2000;Naylor *et al.*, 2003).

The mammalian GIT is sporadically populated with gatherings of mucosa-associated lymphoid tissue (MALT, also referred to as GALT: Gut associated lymphoid tissue). MALT is important for both innate and acquired immunity in that MALT is characterised by large populations of lymphocytes. They consist of a central core of B-lymphocytes embedded in a network of dendritic cells. Dendritic cells and "membraneous" (M-) cells perform antigen sampling at these sites (Rescigno and Borrow, 2001). Smaller populations of T-lymphocytes are also integral to the immune function of this site. MALT may be present as individual lymphoid follicles (LFs) or aggregates of multiple lymphoid nodules. The epithelium which overlays the lymphoid follicle is thinner than non-lymphoid regions, lacks prominent villi and is heavily infiltrated by lymphocytes. The distribution of lymphoid tissue differs between animal species, although certain features are ubiquitous among mammals, such as the ileal Peyer's patch which is most LF-dense close to the terminal ileum. The bovine, ovine and caprine gut share similar GALT-distribution patterns. In addition to terminal-ileal Peyer's patches and interspersed jejunal Peyer's Patches, sites at the terminal rectum and proximal colon are particularly densely populated

with lymphoid follicles (reviewed in Liebler-Tenorio and Pabst, 2006). Targeting of the ileal Peyer's patches has been noted for other bacterial enteric pathogens such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Shigella* spp. (Grutzkau *et al.*, 1990;Perdomo *et al.*, 1994;Jones *et al.*, 1994;Jensen *et al.*, 1998;Vazquez-Torres and Fang, 2000;Chong *et al.*, 2007). The prevalence of phagocytic cells at these regions means that bacteria frequently associate with such regions. This represents a feature of the antagonism between host-mediated destruction of invading pathogens by binding and phagocytosis and bacterial subversion of this process.

The genome sequence of *E. coli* O157:H7 has revealed 14 putative fimbrial loci encoded by *E. coli* O157:H7 (Perna *et al.*, 2001). Some of those with a proposed role in cellular adherence are summarised in Section 1.4.5. Several adhesins have been implicated in the preference shown by the bacterium for the follicle associated epithelium (FAE). Intimin gamma was found to induce FAE-tropism in EPEC, when *eae* γ was cloned and expressed in EPEC (Δ*eae*) (Fitzhenry *et al.*, 2002). As mentioned earlier, intimin is proposed to bind to cellular receptors, such as nucleolin (Robinson *et al.*, 2006), which enhances adherence of the bacterium to the mucosal surface. It is possible that host cells present at lymphoid follicle dense regions present receptors for intimin (or other bacterial molecules) at greater levels than enterocytes at regions outwith these sites. *E. coli* O157:H7 has been noted to elaborate long polar fimbriae (Torres *et al.*, 2002;Torres *et al.*, 2004). These may also aid colonisation of the FAE as has been noted for *Salmonella enterica* serovar Typhimurium (Baumler *et al.*, 1996; Jordan *et al.*, 2004;Fitzhenry *et al.*, 2006).

E. coli O157:H7 has been observed to form A/E lesions in the bovine GIT (Naylor *et al* 2005) but has not been reported to persist in the bovine ileum or target bovine ileal Peyer's patches. The lack of persistence in the ileum could be a key factor in the absence of further clinical manifestations of EHEC colonisation. The reasons why *E. coli* O157:H7 does not colonise at the ileal Peyer's patches in the bovine ileum are unclear, but could potentially rely on differential receptor presentation at the sites. Alternatively, immune tolerance of *E. coli* O157:H7 at the terminal rectum and not at the ileum could be the basis for the observed absence of the bacterium at the ileum.

1.5.3 Host responses to bacterial colonisation

Host innate and adaptive immune systems are vital in defining the results of bacterial colonisation of the mammalian GIT. Immune recognition of colonising bacteria is delicately balanced in that non-recognition of a potentially pathogenic bacterium leads to a permissive host state, allowing pathogen-mediated damage to host tissues. Conversely, an excessive host immune response to an invading microorganism may succeed in elimination of the bacterial pathogen but may also exacerbate the damage caused to the host system by inflammatory-mediated tissue injury. Hence, for an organism to remain in the host GIT without causing damage or eliciting a response which leads to its elimination, an equilibrium is maintained between bacterium and host defences.

Pattern recognition receptors (PRRs) are vital in pathogen recognition by the host and initiation of cellular responses. PRRs include the Toll like receptors (TLRs) normally presented on the surface of mammalian cells and Nod proteins, which are intracellular PRRs. PRRs recognise pathogen-associated molecular patterns (PAMPs) which are molecular motifs that are broadly conserved among pathogens. To date, eleven distinct TLR types have been recognised in mammals (Roach *et al.*, 2005). Certain TLRs have been related to recognition of molecules produced by bacteria. Of these, TLR2, 4 and 5 are perhaps the most important. TLR4 recognises lipid A of bacterial lipopolysaccharides (LPS) via LPS binding protein, CD14 and MD-2 (Poltorak *et al.*, 1998;Shimazu *et al.*, 1999;Bernheiden *et al.*, 2001). TLR2 forms heterodimers with either TLR1 or TLR6 and these complexes recognise components of the bacterial membrane such as lipoproteins, diacyl and triacyl lipopeptides and atypical LPS (Medzhitov *et al.*, 1997;Werts *et al.*, 2001;Takeuchi *et al.*, 2001;Takeuchi *et al.*, 2002;Alexopoulou *et al.*, 2002). TLR5 recognises flagellin proteins, which make up the major flagellar subunit. Recognition is through a conserved domain which interacts directly with TLR5 (Hayashi *et al.*, 2001a). Thus, multiple sensing systems are possessed by the mammalian host, required for recognition and responses to invading microorganisms. The mechanism by which the mammalian cell responds to activation of TLRs is a highly complex network joining together many facets of cellular signal-transduction centered around transcription factors NF-κB, AP-1 and the mitogen-activated protein kinases (MAPKs). A summary of the TLR-dependent response to bacterial products is provided in Figure 1.3.

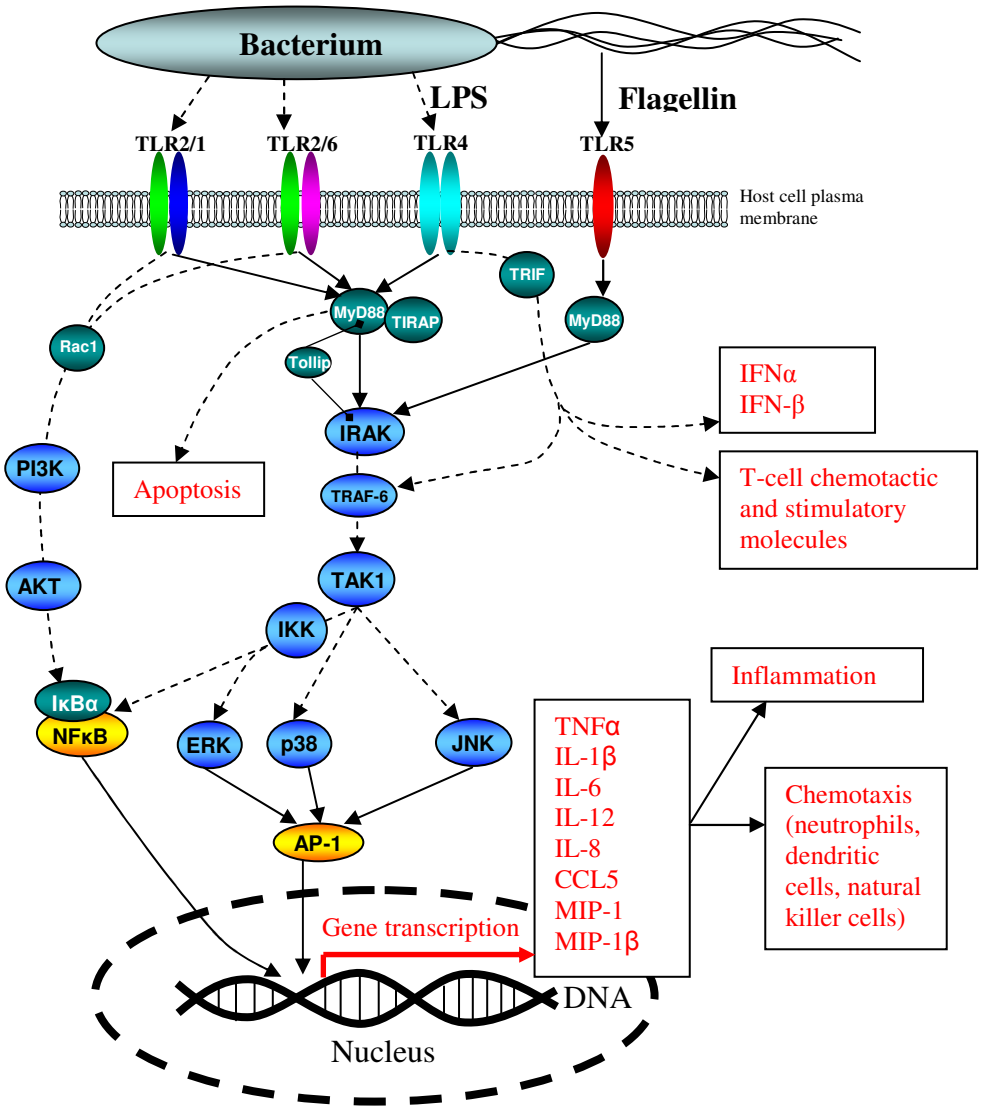


Figure 1.3: Bacterial interactions with Toll-like receptors: Direct effects are denoted by solid arrows while indirect transduction via intermediary factors is denoted by dashed arrows. Bacterial products such as LPS, flagellin and other ligands (set out in Section 1.5.2) interact with their respective TLR. The activation of each of the TLRs depicted causes the activation of MyD88 and subsequent signalling through IRAK, TRAF6 and TAK1. TAK1 forms a branch-point activating the IKK complex and MAPKs p38 and JNK. IKK activation leads to activation of ERK1/2 which (along with JNK and p38) activates AP-1. IKK also causes degradation of IκBα allowing NF-κB to enter the nucleus. NF-κB and AP-1 act as transcription factors facilitating production of proinflammatory cytokines. Two MyD88-bypass mechanisms are depicted: (i) TLR4-dependent signalling via TRIF which activates both TRAF-6 and a mechanism leading to production of interferons (IFN) and T-cell interacting molecules (ii) signalling via Rac1, PI3K and AKT, leading eventually to NFκB activation. Activation of TLR2 or TLR4 also leads MyD88-dependent caspase activation and caspase-dependent apoptosis.

Nucleotide binding and oligomerisation domain (NOD) -like receptors (NLRs) are intracellular PRRs. NLRs include Nod1 which recognises a peptidoglycan fragment more common in Gram-negative bacteria, whereas Nod2 recognises bacterial peptidoglycan from Gram-positive and Gram-negative bacteria (Girardin *et al.*, 2003a;Girardin *et al.*, 2003b). NLRs including neuronal apoptosis inhibitory protein (NAIP)-5 and ICE protease activating factor (IPAF) are activated by bacterial flagellin (Molofsky *et al.*, 2006;Franchi *et al.*, 2006). NLRs activate both NFκB and proapoptotic pathways (reviewed in Delbridge and O'Riordan, 2007).

The distribution and localised presentation of PRRs by the cell has implications for the recognition of pathogenic bacteria and the tolerance of non-pathogenic bacteria. IECs *in vivo* have been shown to present TLR5 at the basolateral surface and not the apical surface (Gewirtz *et al.*, 2001a). This ensures that a proinflammatory response to flagellin is initiated only when bacteria have penetrated the epithelium or flagellin is transported across the epithelium (Gewirtz *et al.*, 2001b). Both TLR2 and TLR4 are underexpressed by IECs and therefore the responses to bacterial LPS lipoproteins and lipopeptides are reduced (Abreu *et al.*, 2001;Naik *et al.*, 2001;Melmed *et al.*, 2003). As Nod proteins are intracellular, they respond only to ligands which are introduced to the cell, especially (but not exclusively) those produced by intracellular bacteria (Philpott *et al.*, 2002;Girardin *et al.*, 2003c). Hence, the epithelial proinflammatory responses generally occur following epithelial damage, cellular invasion, tight junction disruption or translocation of bacterial products across the epithelial layer. Responses to normal bacterial flora of the gut lumen are therefore limited in the healthy gut. Dysregulation of PRR signalling can be detrimental to the host. Inflammatory disorders of the human gastrointestinal tract are thought to be related PRR activation by the normal bacterial flora of the gut (Farrell and LaMont, 2002;Inohara *et al.*, 2003). Conversely, transient activation of PRR signalling is proposed to be required for development of the normal gut (Rakoff-Nahoum *et al.*, 2004). It must be noted that the above observations pertain to the distribution of PRRs expressed by human IECs. It remains to be determined if bovine IECs show similar patterns of PRR presentation.

The result of PRR activation is the production of proinflammatory immune effectors including cytokines, chemokines and antimicrobial peptides. These molecules act to activate responsive components of the immune system including phagocytic and antigen-

presenting cells (e.g. neutrophils, macrophages and dendritic cells). The action of these components of innate immunity is important for the priming of adaptive responses such as humoral immunity, in the form of specific antibodies produced by B-lymphocytes and cell-mediated immunity dependent on the action of T-lymphocytes (reviewed in Lanzavecchia and Sallusto, 2007). In the case of non-invasive bacteria such as EHEC, B-cell-produced antibodies are arguably more significant in the host response to colonisation. Antibodies are composed of immunoglobulin proteins (Ig). The most important antibodies with respect to microbial infections are IgG (serum antibodies), IgA (mucosal secretory antibodies) and IgM (B-cell surface-presented antibodies, important in early stages of infection). Antibodies may act to inhibit the functions of invading microorganisms by binding to presented epitopes. Antibodies coating the surface of a microorganism may interfere with its binding to host cells, preventing invasion or close adherence and also increase the likelihood of macrophage mediated phagocytosis, as macrophages carry receptors for the conserved domain (Fc) of antibodies. Antibodies also induce complement-mediated killing of microorganisms and infected cells, as the complement C1 complex binds to the Fc component of antibodies bound to the surface of bacterial cells. Complement consists of a cascade of proteins which facilitate the killing of cells via the formation of a membrane attack complex (reviewed in Janeway *et al*., 2001).

As IgA is most predominant at the mucosal surface, it is important in the recognition of microorganisms which are present at these sites. Secretory IgA is produced in response to the resident gut microflora which may be important for the prevention of invasion by normally non-pathogenic microorganisms and also may aid biofilm formation (Johansen *et al.*, 1999;Bollinger *et al.*, 2003;Macpherson and Uhr, 2004;Bollinger *et al.*, 2006). Secretory IgA also aids sampling of gut bacteria by M-cells (Corthesy, 2007). The secretory IgA response may also be also important in resistance to invasion by enteric pathogens and the action of their toxins (Uren *et al.*, 2005;Wijburg *et al.*, 2006). Serum IgG functions by binding antigens which are transferred into the bloodstream although IgG may be present at the mucosal surface especially in cases where tissue damage has occurred.

As set out in earlier sections, the human and bovine host produce both serum (IgG) and mucosal (IgA) antibodies against multiple components of *E. coli* O157:H7. Identification of bacterial components recognised by host antibodies has recently been enhanced by the adoption of high-throughput proteomic techniques such as PELS (Proteomics-based

Library Screening) (John *et al.*, 2005;Kudva *et al.*, 2006). There have been several efforts to produce a vaccine effective against *E. coli* O157:H7 (as denoted in Section 1.3.4). The detection of specific antibodies against key components of EHEC by the host indicates that those components are expressed by the bacterium *in vivo* and recognised by the host; hence, detection of specific antibodies produced by the host in response to EHEC colonisation may give rise to candidate targets for vaccine development.

1.6 Aims

The above summarises the current knowledge regarding the danger to human health posed by EHEC, its asymptomatic existence in the bovine gut and the intricate mechanisms of its interactions with the host. In order to explore these issues further the work performed in this study aimed to:

1) Characterise the responses of bovine and human epithelial cell lines to exported products of *E. coli* O157:H7 and further characterise the action of the StcE protease (Chapter 3).

2) Define the genotypes of resident *E. coli* of the bovine gastrointestinal tract and relate these genotypes to colonisation patterns (Chapter 4).

3) Assess the inhibitory activity of bovine *E. coli* isolates against *E. coli* O157:H7 in order to predict whether these strains may have applicability as probiotic agents (Chapter 5).

4) Evaluate the genotypic variation among Scottish EHEC isolates and assess the potential acquisition of additional virulence factors by outbreak-related *E. coli* O157 isolates (Chapter 6).

Chapter 2: Materials and Methods

2.1 Source of materials

All chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. All PCR primers were obtained from MWG-Biotech. Purified recombinant StcE (rStcE) in phosphate-buffered saline (PBS) was provided by Rodney Welch (University of Wisconsin, Madison).

2.2 Bacterial strains

Bacterial strains used as reference isolates for this study are listed in Table 2.1. Bovine *E. coli* strains isolated during this study or provided by Stuart Naylor (Scottish Agricultural College) or Neil Paton (Moredun Research Institute) are described in detail in Chapter 4.

Reference number	Strain/serotype (original designation)	Supplier	Applications
MCI 0010	*E. coli* O157:H7 (WallaWalla3)	M. Reynolds, Emory University, Atlanta	Antimicrobial susceptibility testing (Chapter 5)
MCI 0011	*E. coli* O80	M.J. Woodward VLA Weybridge	O-RFLP reference (Chapter 4)
MCI 0022	*E. coli* O43:H28	M. Stevens, IAH Compton	O-RFLP reference (Chapter 4)
MCI 0024	*E. coli* O157:H7 (NCTC12900)	A. Best, VLA	Wild type parent strain of MCI 0025 (Chapter 3) Antimicrobial susceptibility testing (Chapter 5)
MCI 0025	*E. coli* O157 Δ*fliC* (DM4)	A. Best, VLA	*fliC* mutant strain of MCI0024 (Chapter 3)
MCI 0045	*E. coli* O157:H7 (EDL933)	G. Pósfai, Institute of Biochemistry, Szeged, Hungary.	O-RFLP reference (Chapter 4) H-RFLP reference (Chapter 4) Positive control for: *stx*1, *stx*2, *eae* (γ), *espP*, *escN*, *espA*, *ehxA* (Chapters 4 & 6). Microarray control strain (Chapters 4 & 6) Antimicrobial susceptibility testing (Chapter 5) Subtractive hybridization driver strain (Chapter 6)
MCI 0050	*E. coli* O26 (MPRL4269)	MPRL, Aberdeen	O-RFLP reference (Chapter 4)
MCI 0052	*E. coli* O5:H- (95-4107/291D18)	T. Besser, WSU	O-RFLP reference (Chapter 4)
MCI 0053	*E. coli* O111:H21 (DEC/15A)	T. Besser, WSU	O-RFLP reference (Chapter 4)
MCI 0066	*E. coli* O157:H7 (WallaWalla1)	M. Reynolds, Emory University, Atlanta	Wild-type parent strain of MCI 0010
MCI 0070	*E. coli* O116:H21 (EH42)	E. Hartland, University of Melbourne	O-RFLP reference (Chapter 4)
MCI 0071	*E. coli* O130:H11 (EH43)	E. Hartland, University of Melbourne	O-RFLP reference (Chapter 4)
MCI 0072	*E. coli* O1:H7 (EH69)	E. Hartland, University of Melbourne	O-RFLP reference (Chapter 4)
MCI 0076	*E. coli* O103:H2 (PMK5::Δ*stx*)	M. Stevens, IAH, Compton	O-RFLP reference (Chapter 4) Positive control for: eae (ε) (Chapter 4)

MCI 0078	*E. coli* O157:H7 (Sakai::Δ*stx*)	C.Sasakawa, University of Tokyo	O-RFLP reference (Chapter 4) Microarray control strain (Chapters 4 & 6) Antimicrobial susceptibility testing (Chapter 5)
MCI 0132	*E. coli* O9:K30 (EC46/04)	R. M. La Ragione, VLA	O-RFLP reference (Chapter 4)
MCI 0133	*E. coli* O149:K91 (EC439/04)	R. M. La Ragione, VLA	O-RFLP reference (Chapter 4)
MCI 0134	*E. coli* O147:K89 (EC1203/04)	R. M. La Ragione, VLA	O-RFLP reference (Chapter 4)
MCI 0191	*E. coli* O157:H7 (85-170)	M. Stevens, IAH Compton	Antimicrobial susceptibility testing (Chapter 5)
MCI 0200	*E. coli* O157:H7 (TUV933-0)	D. Miller, Bristol University	O-RFLP reference (Chapter 4) Antimicrobial susceptibility testing (Chapter 5)
MCI 0234	*E. coli* O157 (SERL5)	L. Allison, SERL	Positive control for: *stx*2 (Chapters 4 & 6)
MCI 0236	*E. coli* O157 (SERL7)	L. Allison, SERL	Positive control for: *stx*1, *stx*2 (Chapters 4 & 6)
MCI 0246	*E. coli* O162 (SERL17)	L. Allison, SERL	O-RFLP reference (Chapter 4)
MCI 0247	*E. coli* O113 (SERL18)	L. Allison, SERL	O-RFLP reference (Chapter 4)
MCI 0248	*E. coli* O177 (SERL19)	L. Allison, SERL	O-RFLP reference (Chapter 4)
MCI 0249	*E. coli* O118 (SERL20)	L. Allison, SERL	O-RFLP reference (Chapter 4)
MCI 0254	*E. coli* K12 (MG1655)	M. Hough, Edinburgh University	O-RFLP reference (Chapter 4) Microarray control strain (Chapters 4 & 6)
MCI 0255	*E. coli* K12 (NCTC10418)	M. Hough, Edinburgh University	Antimicrobial susceptibility testing (Chapter 5)
MCI 0278	*E. coli* O127:H6 (E2348/69)	D. Gally, Edinburgh University	O-RFLP reference (Chapter 4) Positive control for: *eae* (α1), *espA*, *escN* (Chapter 4).
MCI 0279	*E. coli* O157:H7 (TT12B)	D. Gally, Edinburgh University	O-RFLP reference (Chapter 4) Antimicrobial susceptibility testing (Chapter 5)
MCI 0291	*E. coli* O44:H18 (EAggEC 042)	A. Scott-Tucker, University of Birmingham	O-RFLP reference (Chapter 4) Positive control for: *aat* (Chapters 4)

MCI 0296	*E. coli* O157:H- (NCTC13125)	R. M. La Ragione, VLA	O-RFLP reference (Chapter 4)
MCI 0478	*Shigella sonnei* (539)	I.R. Henderson, University of Birmingham	Positive control for: *ipaH* (Chapter 4)
MCI 0479	*E. coli* O78:H11 (H10407)	I.R. Henderson, University of Birmingham	O-RFLP reference (Chapter 4)
MCI 0480	*E. coli* O6:K5:H1 (DSM 6601)	Ardeypharm, Germany	O-RFLP reference (Chapter 4) Positive control for: *neuB* (K5 capsule) (Chapter 4)
MCI 0481	*E. coli* O6:K?:H1 CFT073	D. Gally, Edinburgh University	O-RFLP reference (Chapter 4)
MCI 0482	*E. coli* O18:K1:H7 (RS218)	D. Gally, Edinburgh University	Positive control for: *kfiC* (K1 capsule) (Chapter 4)
MCI 0652	*E. coli* O78:K80	F. Dziva, IAH, Compton	O-RFLP reference (Chapter 4)
MCI 0662	*E. coli* O4:K6 (J96)	D. Gally, Edinburgh University	O-RFLP reference (Chapter 4)
MCI 0663	*E. coli* O6:K15:H31 (536)	D. Gally, Edinburgh University	O-RFLP reference (Chapter 4)
MCI 0685	*E. coli* O101 (EC25/06)	R. M. La Ragione, VLA	Positive control for: F5, F41 (Chapter 4)
MCI 0686	*E. coli* O9 (EC92/07)	R. M. La Ragione, VLA	Positive control for: F5, F41 (Chapter 4)
DNA5	*E. coli* O84:H4 (4795/87)	R. M. La Ragione, VLA	*eae* (ζ), *escN* (Chapter 4)
DNA6	*E. coli* O145:H4 (7476197)	R. M. La Ragione, VLA	*eae* (ι), *escN* (Chapter 4)
DNA7	*E. coli* O125:H- (CF/11201)	R. M. La Ragione, VLA	*eae* (η), *escN* (Chapter 4)
DNA8	*E. coli* O118:H5 (6044/95)	R. M. La Ragione, VLA	*eae* (κ), *escN* (Chapter 4)
DNA9	*E. coli* O111:H8 (CL37)	R. M. La Ragione, VLA	*eae* (θ), *escN* (Chapter 4)

Table 2.1: Reference strains used in this study: Bovine gastrointestinal *E. coli* isolates are described in Chapter 4. A full list of EHEC isolates is given in Table 6.1.

2.3 Microbiological techniques

2.3.1 Bacterial culture

2.3.1.1 General culture and maintenance of *E. coli* isolates

All culture media prepared in-house were sterilised by autoclaving at 15 psi for 15 minutes at 121˚C prior to use. All non-presterilised heat-sensitive additive solutions, including antibiotics, were passed through a Millipore 0.22μM filter and added to media after autoclaving, once media had cooled to below 55˚C. Selective antimicrobial substances employed included ampicillin, streptomycin, cefixime and potassium tellurite. Stock ampicillin solution (50mg/ml) was prepared in water, filter-sterilised and diluted in media to give a final concentration of 50μg/ml. Stock streptomycin (10mg/ml) was prepared in water and diluted in media to give a final concentration of 25μg/ml. Cefixime-tellurite solution (Oxoid) was prepared according to the manufacturers instructions and added to media as required, to give final concentrations of cefixime, 2.5μg/ml; and potassium tellurite, 0.05μg/ml.

E. coli was resuscitated on Luria-Bertani (LB: 1% w/v Bacto-tryptone, 0.5% w/v Bacto-yeast extract 1.5% w/v Bacto-agar) medium or nutrient agar (NA; Oxoid, Basingstoke, Hampshire) overnight at 37°C. Subculture was generally performed in LB broth (as LB medium without agar). Minimal essential medium-HEPES modification (MEM-HEPES, M7278 Sigma) was used when bacteria were to be cultured for addition to cultured cell lines. Broth cultures were incubated at 37˚C with 200rpm shaking.

Selective indicator media employed for isolation of *E. coli* and verification of culture purity included Tryptone/bile-salts/X-glucuronide (TBX) medium and Sorbitol MacConkey Agar (SMAC) and were both supplied by Oxoid.

To select transformants carrying cloned plasmids, S-Gal/LB Agar Blend (Sigma C-4478) was employed. One sachet was added to 500ml distilled water before autoclaving. Ampicillin was included at a final concentration of 50μg/ml.

2.3.1.4 Long term storage of *E. coli* isolates

Glycerol stocks of *E. coli* isolates were prepared by mixing 500µl of *E. coli* overnight culture with 500µl sterile LB containing 30% v/v glycerol in a 1.5ml sterile centrifuge tube or Cryovial. Alternatively, 5 colonies of a pure overnight culture on LB or NA medium were transferred to a Protect vial (Prolabs, Neston, Wirral, UK) using a sterile disposable plastic loop. The vial was vortexed briefly and the liquid removed and discarded. Glycerol stocks and Protect vials were stored at -70°C until required.

2.3.2 Isolation and enumeration of *E. coli* from cattle

Isolation of *E. coli* associated with gastrointestinal mucosal surfaces followed a protocol adapted from Naylor *et al.* (2003). Sections of the gastrointestinal tract (roughly 20cm in length) were removed from the animal post mortem. Tract portions were opened longitudinally and visible gastrointestinal contents were carefully shaken off (or gently rinsed off with sterile PBS where this was not possible). The mucosal layer was parted from underlying connective tissue and rinsed gently but thoroughly with sterile PBS. Three $1cm^2$ pieces of tissue were cut from the mucosal layer and added to 5ml aliquots of sterile PBS. Tubes containing tissue sections in PBS were held on ice for no more than one hour before further processing. For bacterial enumeration tissue sections in aliquots of PBS were vortexed for one minute and serial tenfold dilution was performed using sterile PBS. Spread plates on TBX medium (Oxoid) were prepared in triplicate using 100µl of the undiluted sample and diluted samples. The resulting plates were incubated at 37°C overnight. Blue (glucuronidase positive) and white (glucuronidase negative) colonies were counted. The vast majority of *E. coli* isolates (96-97%) produce an active glucuronidase enzyme, although certain serotypes (including strains of serotype O157:H7) are glucuronidase negative (Ratnam *et al.*, 1988). β-glucuronidase activity has been found to be restricted to the genera *Escherichia*, *Shigella* and *Salmonella* among the Enterobacteriaecae (Kilian and Bulow, 1979); hence, detection of glucuronidase activity is an important (though not absolute) indicator that a bacterial isolate is likely to belong to *Escherichia* or related genera. The dilution yielding plates with the highest number of colonies (where that number was less than 1000) were used to calculate the average

number of *E. coli* per 100µl of the undiluted sample. At least 18 colonies were picked per site for each animal (where 18 colonies were available) and transferred to 10ml aliquots of LB. The ratio of blue:white colonies picked was made to reflect the ratio of blue:white colonies obtained at each site. The colonies in LB were incubated overnight at 37°C with 200rpm shaking. These cultures were used to prepare glycerol stocks (Section 2.3.4.1) and genomic DNA (Section 2.6.1).

To visualise mucosal tissue morphology at the sites sampled, two additional pieces of tissue were taken from each site, adjacent to the sections taken for microbiological examination. One was preserved in a 10ml aliquot of formal saline and the other was placed in a plastic tissue cassette with one drop of OCT (TissueTek) and transferred to liquid nitrogen. Snap-frozen OCT-embedded tissue was subsequently stored at -70˚C. Further processing of formal-saline-fixed tissue was performed by the Pathology Unit, Moredun Research Institute. Tissue sections were stabilised in plastic cassettes, embedded in paraffin wax and used to prepare haematoxylin and eosin-stained sections according to standard methodology. Slides were visualised using an Olympus BX50 microscope and an Olympus DP70 camera. Cell^{F} Imaging Software (Olympus) was used to convert images to the TIFF file format.

Sampling of *E. coli* from live cattle was performed by insertion of a swab into the anus of the animal and gently rubbing this against the recto-anal mucosa for approximately 5 seconds. Swabs were used to prepare streak plates on TBX medium using standard methodology.

E. coli isolates provided by Stuart Naylor were isolated from necropsy tissue sections by the method described above, except that "colony sweeps" were provided, whereby colonies from TBX-agar plates were gathered using a sterile spreader and transferred directly to glycerol stocks. Individual isolates were derived from these mixtures in the course of this study, by performing streak plates on TBX-agar. Strains provided by Neil Paton were isolated from live cattle by rectal mucosal swab (described above) or by faecal "free-catch" whereby faecal matter was caught in a clean glove on emergence from the animal. Swabs or faecal matter were used to prepare streak plates on CT-SMAC and individual sorbitol-fermenting (pink) colonies were selected and used to prepare glycerol stocks or Protect vials as set out in Section 2.3.1.4.

2.3.3 Cell lines and culture conditions

2.3.3.1 T84

The T84 cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, England); originally derived from a lung metastasis of colon carcinoma in a 72 year old male (Murakami and Masui, 1980). T84 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM, Sigma: D6546) and Nutrient Mixture Ham's F12 (Sigma N4888) supplemented with 10% (v/v) gamma irradiated heat-inactivated foetal bovine serum (FBS, BioSera) and 2mM L-glutamine. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3.3.2 Caco-2

The Caco-2 cell line was provided by ECACC and was originally derived from a primary colonic tumour (Fogh *et al.*, 1977). Caco-2 cells were maintained in DMEM supplemented L-glutamine (2 mM) and 10% FBS. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3.3.3 EBL

The epithelial bovine lung (EBL) cell line was kindly provided by John March (MRI) and was originally obtained from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* (DMSZ). The cell line was originally isolated from lung of a 7-month-old bovine fetus (Rutter and Luther, 1984). EBL cells were maintained in Eagles Minimal Essential Medium (EMEM, Sigma: M2279) supplemented with 10% FBS and 2mM L-glutamine. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C.

2.3.4 Preparation of bacterial conditioned medium (supernatants)

Single colonies of LB-grown bacteria were picked and used to inoculate 10ml aliquots of MEM-HEPES or MEM-HEPES including streptomycin. These were incubated overnight at 37˚C 200rpm shaking. Cultures were subsequently diluted 1:10 in fresh MEM-HEPES (±

streptomycin 25µg/ml) (prewarmed to 37˚C) and incubated at 37°C with 200rpm shaking. OD_{600} of mid-log and late-log phase cultures was determined to be approximately 0.35 and 0.6 respectively; estimated by the construction of growth curves. Upon reaching this absorbance, cultures were centrifuged at 5000g for 10 minutes at 4˚C and the supernatant harvested. Supernatant samples were filter-sterilised using Millipore disposable 0.22µm low protein binding filters. 7ml aliquots were stored at -70˚C until required.

E. coli O157 (*fliC*-) MCI 0025 was grown in MEM-HEPES containing 25µg/ml streptomycin. When bacterial supernatants from streptomycin sensitive strains were used, streptomycin was added to the supernatants after filtering, to ensure that all challenge materials contained an equal amount of streptomycin. Streptomycin was also included in the control media (MEM-HEPES) used in subsequent sections to determine basal responses.

2.3.5 Stimulation of cultured cell lines cells using bacterial supernatants

Cell monolayers (T84 or EBL) were grown in 24-well tissue culture plates until the first day after they became fully confluent. Prior to challenge all material to be added to the cells was prewarmed to 37˚C. Residual cell medium was removed from the cell culture plate and each well was washed twice in serum-free cell culture medium. Cell culture medium was replaced with bacterial conditioned medium or sterile MEM-HEPES (25µg/ml streptomycin) diluted 1:1 in sterile serum-free cell culture medium. Untreated wells incubated with sterile serum-free cell culture medium alone were included to assess plate-plate variation and edge-effects. Challenges were carried out for 24 hours, after which time, the medium was drawn off and stored at -70°C until required.

2.3.6 Challenge of cultured cell lines cells using live bacteria

EBL-cell monolayers were grown in 12-well tissue culture plates until the first day after they became fully confluent. Medium was removed and replaced with serum-free medium 24 hours prior to challenge. Bacteria were grown and subcultured in MEM-HEPES (± streptomycin 25µg/ml) as detailed in Section 2.3.4. Subcultured bacteria were grown to mid-log phase (OD_{600}=0.35). Prior to challenge all material to be added to the cells was prewarmed to 37˚C.

Residual cell medium was removed from the cell culture plate and each well was washed twice in serum-free cell culture medium (prewarmed to 37°C). For challenge with a MOI of 100 bacteria per cell the volume in the wells was adjusted to 800µl using serum-free cell culture medium. 200µl bacterial culture was added to the cells and the plates were incubated at 37˚C until the specified timepoint.

For live-challenge timecourse experiments, medium containing bacteria was removed at the specified timepoints. Bacteria and cell debris were removed by passing the sample through a 0.22µM filter and the samples stored at -20˚C until analysed by ELISA (Section 2.5.2).

A method based on the gentamicin protection assay (Tang *et al.*, 1993) was also employed, whereby live challenges were carried out as above. After 1 hour or 3 hours, cell medium was removed and the cells washed twice with serum-free culture medium. 1ml serum-free cell culture medium containing 100µg/ml gentamicin was added to each well. Cells were incubated until the total time after the addition of the bacteria had progressed to 24 hours. After this time, culture medium was drawn off, centrifuged and the supernatant transferred to a fresh tube. Samples were stored at -20˚C until required for analysis by ELISA (Section 2.5.2).

2.3.7 Treatment of cultured cell lines with the StcE protease

Three-day post-confluent Caco-2 cells were prepared and maintained in 6-well tissue culture plates according to Section 2.3.3.2. Medium was removed and replaced with medium lacking foetal bovine serum 24 hours prior to treatment. Cells were treated with 25µg/ml rStcE in serum-free Caco-2 cell medium for 4h at 37°C in a humidified atmosphere containing 5% CO_2. Cells treated with a similar volume of PBS were used as a negative control. Cell monolayers were treated with 150µl 2D lysis buffer per well (8M Urea, 4% (w/v) CHAPS, 40mM Tris, including Protease Inhibitor Cocktail (1 tablet to 10ml buffer) Boehringer Manheim) and samples were analysed by 2-DGE as described in Section 2.4.3.

2.4 Proteomic techniques

2.4.1 SDS-PAGE

Two glass plates were clamped together vertically and a resolving gel comprising acrylamide:*N,N*'-methylenebisacrylamide (37.5:1, 10% w/v), Tris (375mM, pH8.8). SDS (0.1% w/v), ammonium persulphate (APS, 0.025% w/v) and *N,N,N',N'*-tetramethylethyldiamine (TEMED, 0.005% v/v), was poured between them, leaving approximately 3cm between the surface of the gel and the top of the glass plates. SDS (200µl, 0.1% w/v) was layered on top of this to produce a smooth level surface and exclude air. When polymerisation of the separating gel was complete the layer of SDS was removed and a stacking gel composed of acrylamide:*N,N*'-methylenebisacrylamide (37.5:1, 3% v/v), Tris (125mM, pH6.8), SDS (0.1% w/v), APS (0.1%w/v) and TEMED (0.14% v/v) was poured above the resolving gel. A plastic comb was inserted into the stacking gel to form wells for the loading of samples. Following polymerisation of the stacking gel, the comb was removed and the assembled gel placed in a Biorad Mini-Protean III™ electrophoresis tank. Both reservoirs were then filled with 1X running buffer (25mM Tris, 192mM glycine, 0.1% SDS). Once the separating and stacking gels were prepared, protein samples were mixed 3:1 with 4X Laemmli sample buffer (250mM Tris, pH 6.8, 40% v/v glycerol, 8% w/v SDS, 520mM dithiothreitol (DTT), 0.01% w/v bromophenol blue) or prepared in 1X Laemmli sample buffer, boiled for 10 minutes, then chilled on ice. Samples and molecular weight markers (PageRuler: Fermentas) were loaded into the wells of the gel using gel loading tips. A current was passed across the gel at a constant voltage of 135V at room temperature until the dye front had reached the bottom of the resolving gel.

2.4.2 Non-denaturing Polyacrylamide gel electrophoresis (Acidic conditions)

Acidic native gel electrophoresis was performed essentially as SDS-PAGE, with the following alterations to buffers and conditions. Resolving gel was composed of acrylamide:*N,N'*-methylene-bisacrylamide (37.5:1, 10% v/v), Acetate-KOH (34mM, pH4.3), glycerol (11.5% v/v), APS (0.12% w/v) and TEMED 0.15%. 300µl of n-butanol was layered on top of this to produce a smooth level and exclude air. Once the resolving gel was fully polymerised, the n-butanol was removed and stacking gel (3% v/v acrylamide:*N,N'*-methylene-bisacrylamide

(37.5:1), 62mM Acetate-KOH (pH6.8), 0.1% v/v APS, 0.1% v/v TEMED) was added and a plastic comb inserted. Running buffer (348mM β-alanine, 20.5% acetic acid, pH4.3) was added to both reservoirs of the electrophoresis tank. Samples were mixed 1:8 with dissolving buffer (37% v/v glycerol, 64mM Acetate-KOH, pH6.8, including a trace amount of Methyl green) and kept on ice. Protein ladder was prepared by combining equal amounts of α-Lactalbumin, carbonic anhydrase, chicken egg albumin and bovine serum albumin and combining this mixture with an equal volume of dissolving buffer. Samples and protein ladder were loaded into the wells and a current of 135V was passed across the gel with reverse polarity for approximately 1.5hrs at 4°C.

2.4.3 2D Gel Electrophoresis (2-DGE)

The method used for 2-DGE was based on that recommended by Amersham Bioscience. Commercially-available precast gels were used for both the first and second dimensions which were carried out using an IPGphor isoelectric focusing unit (first dimension) and a flat-bed Multiphor (second dimension). Commercially-available 18 cm pH3-10 non-linear immobilised pH gradient (IPG) strips were employed (Amersham Biosciences). The second dimension was performed using 12.5% Ettan DALT Gels.

Samples (prepared according to Section 2.3.7) were treated with the 2-D Clean-Up kit (Amersham Biosciences) according to the manufacturer's instructions. Briefly, 100µl aliquots of protein sample were mixed with 300µl precipitant solution, vortexed thoroughly and incubated on ice for 15 minutes. 300µl co-precipitant was added and the mixtures were vortexed briefly. Tubes were centrifuged at 12,000g in a microcentrifuge for 5 minutes. Supernatant was completely removed, aided by a brief pulse-centrifuge to recover residual liquid for removal. 40µl of co-precipitant was carefully layered on the pellet and the tubes left on ice for 5 minutes. Tubes were centrifuged again for 5 minutes at 12,000g. Liquid was carefully removed without disturbing the pellet. 25µl distilled water was added, dispersing the pellet. 1ml chilled wash buffer (-20˚C) and 5µl wash additive were added to each pellet and tubes were vortexed for 10 seconds. Tubes were incubated at -20˚C for 30 minutes with intermittent vortexing. Tubes were centrifuged at maximum speed in a microcentrifuge (17,000g) for 5 minutes. Wash buffer was discarded and pellets allowed to air dry briefly, before resuspension in 100µl 2D rehydration solution (8M urea, 2% (w/v) CHAPS, 0.2%

(w/v) DTT, 1% (v/v) IPG buffer and 0.002% (w/v) bromophenol blue). The resultant mixture was vortex for 30 seconds and subsequently centrifuged to reduce foam.

Duplicate 1µl and 5µl aliquots were transferred to 1.5ml centrifuge tubes and analysed using the 2D Quant Kit (Amersham) according to the manufacturer's instructions. Briefly, a set of BSA standards was prepared, from 0-25µg. 500µl precipitant was added to each sample and standard. Tubes were vortexed and left at room temperature for 2-3 minutes. 500µl co-precipitant was added to each tube and the tubes were vortexed briefly then centrifuged at 10,000g for 5 minutes. All visible supernatant was removed from each tube and 100µl copper solution was added, along with 400µl distilled water. 1ml colour reagent was added to each tube and mixed quickly by vortexing. Absorbance at 480 nm was determined, using water as a reference. A standard curve was plotted and the protein concentration of the original sample was predicted using this curve.

The volume of each original sample was adjusted using rehydration solution to give approximately 250µg protein in 400µl rehydration solution, which was used to rehydrate IPG strips. IPG strips were rehydrated for a minimum of 12 hours in a DryStrip reswelling tray (Amersham). Strips were subsequently transferred to universal IPG Strip holders and then placed on an IPGphor.

Isoelectric focusing was carried out using a gradually increasing voltage, 30V for the first hour, 150V for the second hour; then the voltage was doubled every hour until the 6^{th} hour (2400V). 8000V were applied thereafter, until approximately 80kVh had been applied to strips. At this point strips were stored at -80°C until electrophoresis in the second dimension could be carried out. Strips were removed from -80°C storage and allowed to warm up to room temperature. A two step equilibration process was performed, using equilibration buffer (an aqueous solution containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue). Strips were incubated at room temperature for 20 minutes on a shaking platform with in equilibration buffer containing dithiothreitol (1% w/v). This equilibration buffer was discarded and replaced with fresh equilibration buffer containing iodoacetamide (4% w/v) and incubated for a second 20 minute period.

First dimension strips were then removed from the equilibration buffer and carefully blotted to remove excess traces of buffer before being placed onto the polyacrylamide for electrophoresis. Electrophoresis was carried out at 600V, 20 mA, 40 W for 30 minutes. The first dimension strips were removed from the gel and discarded. Electrophoresis was then continued at 600 V, 40 mA, 40 W until the tracking dye had migrated to the anodic buffer strip. Gels were stained with colloidal coomassie according to Section 2.4.3

2.4.4 Polyacrylamide Gel Staining

In order to visualise all protein bands/spots after PAGE, gels were fixed in 50% v/v methanol, 10% v/v acetic acid in distilled water for at least 30 minutes. Staining was carried out using Genomic Solutions Colloidal Coomassie solution in 20% v/v methanol overnight. Gels were destained in 25% methanol for at least 4 hours. For visualisation of glycosylated proteins after SDS-PAGE, gels were stained using the GelCode Glycoprotein Staining Kit (Pierce Biotechnology) according to the manufacturer's instructions. All incubations were carried out at room temperature with gentle agitation on a rocking platform. Briefly, gels were fixed in 100ml of 50% (v/v) methanol for 30 minutes, washed twice for 10 minutes in 3% (v/v) acetic acid and subsequently transferred to 25ml Oxidising solution for 15 minutes. Gels were then washed 100ml 3% acetic acid three times for 5 minutes each time and subsequently transferred to 25ml GelCode Glycoprotein Staining Reagent for 15 minutes, followed by 25ml reducing solution for 5 minutes. Gels were washed for at least one hour in 3% acetic acid, then for at least one hour in distilled water.

2.4.5 MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was carried out at the Moredun Proteomics Facility according to standard methodology. Briefly, protein bands from the SDS-page gels were treated with 100mM ammonium bicarbonate/50% acetonitrile three times. They were then dehydrated in 100% acetonitrile for 10mins and dried for 20mins under a vacuum, followed by incubation with 10mM DTT in 100mM ammonium bicarbonate at 56ºC for 1hr. The supernatant was then removed and the bands incubated in a solution of 55mM iodoacetimide in 100mM ammonium bicarbonate, in the dark for 30mins. The supernatant was removed and

bands were treated with 100mM ammonium bicarbonate/50% acetonitrile, dehydrated and dried as previous steps. The bands were subjected to trypsinolysis and analysed by matrix assisted laser desorption ionisation-time of flight-mass spectrometry (MALDI-TOF-MS). Resultant peptide profiles were compared predicted patterns using the MASCOT search engine (Matrix Science Inc) to search the NCBI non-redundant and Swissprot databases.

2.5 Immunochemical Techniques

2.5.1 Western Blotting

Immediately following SDS-PAGE, proteins in gels were transferred onto Hybond-C membranes (Millipore) by electrophoretic blotting. The gel was pressed against a piece of nitrocellulose membrane which had been pre-soaked in transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol) and supported between two Whatmann 3MM papers and two sponge pads. This assembly was placed in a BioRad Mini Trans-Blot™ tank which was filled with transfer buffer (25mM Tris, 192mM Glycine, 20% v/v Methanol). Transfer was carried out by applying a current of 240mA for 1h 45min. Tanks were cooled by the inclusion of ice packs containing transfer buffer, frozen at -20˚C prior to use.

Membranes were blocked for at least 2 hours in Tris-buffered saline/Tween-20 (TBS-T: 20mM Tris/HCl, 150mM NaCl, pH7.2, 0.03%v/v Tween-20) including skimmed milk powder (Marvel™, 5% w/v). Primary antibody was diluted to the appropriate concentration (see below) in TBS-T/Marvel™ (0.5% w/v) and membranes were incubated in this solution overnight with constant agitation at 4^{o}C. The membranes were then washed with TBS-T for approximately 1.5 hours, changing the buffer every 15 min. Horseradish peroxidise (HRP) - conjugated secondary antibodies were diluted to the appropriate concentration in TBS-T/Marvel™ (0.5%w/v) and incubated for 2 hours with the membranes, which were then washed. Membranes were then developed using the enhanced chemiluminesence (ECL) system (Amersham). X-ray film (Kodak) was exposed to the membranes, which were developed using an X-ray developer.

Two different primary/secondary antibody combinations were employed during this study; Firstly: the goat anti-human-C1-esterase inhibitor whole antiserum (Sigma, C8159) used at a working dilution of 1/400, followed by HRP-conjugated rabbit anti-goat IgG secondary antibody (Sigma, A5420) at a working dilution of 1/5000.

Secondly, whole bovine mucosal scrapings from calf experimentally infected with *E. coli* O157:H7 (MCI 0010) were provided by Dr Arvind Mahajan. The mucosa was scraped using a

glass slide and the scrapings (approximately 50mg) were suspended in 5 ml of ice cold phosphate buffered saline (PBS, pH 7.2) plus protease inhibitor cocktail (Complete-Mini, Roche, Mannheim, Germany), vortexed and clarified by centrifugation at 16,000g and 4 °C for 15 min. This solution was used as a primary antibody at a working dilution 1/10 in TBS-T. Blots probed with this mixture were probed with HRP-conjugated anti-bovine IgA (Serotec) at a working dilution of 1/1000.

2.5.2 Enzyme-linked Immunosorbent Assay (ELISA)

The Human IL-8 ELISA Duoset (R&D Systems) was employed according to the manufacturer's instructions. 96-well EIA/RIA stripwell plates (Costar) were coated overnight in the Capture Antibody (4.0 µg/ml in PBS, 100µl/well). Liquid from the wells was removed by aspiration and each well was washed 3 times with 400µl PBS, containing 0.05% v/v Tween-20. Residual washing buffer wash removed by blotting on clean tissue paper. The plates were then blocked using PBS containing 1% w/v BSA (300µl/well) for a minimum of 1 hour; then aspiration and washing was carried out as before. Samples of cell culture supernatant (generated as detailed in Section 2.3.5 and 2.3.6) were added in 100µl volumes and incubated for 2 hours at room temperature. IL-8 standards were also included in serial 1:1 dilutions in Reagent Diluent (0.1%w/v BSA, 0.05% v/v Tween-20, 20mM Tris Base, 150mM NaCl) from 2000 to 62.5pg/ml. Three wells containing reagent diluent alone were also included. Wells were aspirated and washed as before and the Detection Antibody (20ng/ml in Reagent Diluent) was added (100µl/well). Plates were incubated for 2 hours at room temperature, then plates were aspirated and washed as before. To each well was added 100µl Streptavidin-HRP (diluted 1:200 in Reagent Diluent). The plate was incubated for 20 minutes in the dark. The plate was aspirated and washed as before. 100µl Substrate solution (60ng/ml Tetramethyl benzidine in acetate:citrate buffer pH5.5, 0.04% v/v hydrogen peroxide) was added to each well and the plate again incubated in the dark for 20 minutes. 50µl stop solution (10%w/v sulphuric acid) was then added to each well and the plate read at 450nm using the ELx808IU Ultra Microplate Reader (BIO-TEK Instruments Inc.). A standard curve was constructed and a third order polynomial trendline was fitted to the datapoints using Microsoft ExCel. The equation generated was used to convert absorbance readings to concentration figures.

2.6 Genomic Techniques

2.6.1 Preparation of bacterial DNA

2.6.1.1 DNeasy blood and tissue kit (QIAGEN)

To obtain high purity bacterial genomic DNA required for DNA microarray hybridisation, the DNeasy blood and tissue kit was employed according to the manufacturer's instructions. Briefly, bacterial isolates were cultured overnight in LB. Two 1.5ml aliquots from each strain were harvested by centrifugation at 8,000g for 5 minutes. The supernatant was discarded and pellets were resuspended in 180µl Buffer ATL. 20µl proteinase K was added and the tubes were mixed thoroughly using a vortex mixer. Tubes were incubated at 56˚C for 3h. After this period, tubes were allowed to cool to room temperature. 1µl RNase A (100mg/ml, QIAGEN) was added to each tube, mixed thoroughly by vortexing and incubated for 2 minutes at room temperature. 200µl Buffer AL and 200µl absolute ethanol were added to each sample and these were mixed thoroughly by vortexing. The mixtures were subsequently transferred to a DNeasy Mini Spin column, placed in a 2ml collection tube. Columns were centrifuged at 6,000g (8,000rpm) for 1 minute in a microcentrifuge. Flow-through was discarded and the columns were washed using 500µl Buffer AW1 and secondly using 500µl Buffer AW2, centrifuging at 6000g (8,000rpm) discarding flow-through after each wash. After the second wash, the columns were replaced in the collection tube and centrifuged at 20,000g (14,000rpm) for 2 minutes to remove residual wash buffer from the column. The columns were placed in sterile 1.5ml centrifuge tubes. For each strain (represented by 2 columns containing genomic DNA) 200µl AE buffer was added to the first column, DNA was eluted by centrifugation at 6,000g (8,000rpm) for 2 minutes. The eluted sample from the first column was transferred to the second column and used to elute the second column in the same way. Thus, 2 columns were eluted with one volume of AE buffer, which resulted in an optimal yield of DNA required for DNA microarray (Section 2.6.6). DNA samples were stored at -20˚C until required.

2.6.1.2 Wizard genomic DNA kit (Promega)

For the production of high concentrations of high molecular weight bacterial genomic DNA suitable for Long-template PCR (Section 2.6.4.1) and genomic subtractive hybridisation (Section 2.6.8) the Wizard genomic DNA purification kit was utilised according to the manufacturer's instructions, with some modifications. For each isolate, 1.5ml of a 10ml overnight culture in LB was pelleted by centrifugation at 10,000g for 2 minutes. The supernatant was removed and discarded. The pellet was thoroughly resuspended in 300µl Nuclei Lysis Solution by gentle pipetting. The mixture was incubated at 80°C for 5 minutes to lyse the cells. The mixture was cooled to room temperature and 1µl RNase A was added. The tubes were inverted 2-5 times to mix and left at room temperature for 5-15 minutes. To the lysate, 100µl Protein Precipitation Solution was added and the mixture was immediately vortexed for 20 seconds and transferred to ice and allowed to stand for a minimum of 5 minutes. The mixture was centrifuged at maximum speed (17,000g) in a microcentrifuge for 5 minutes at 4°C. The supernatant was transferred to a clean tube containing 300µl room temperature isopropanol and mixed thoroughly until the DNA formed a visible mass. The DNA was pelleted by centrifugation at 13000g for 2 minutes. The supernatant was removed and replaced with 750µl of room temperature 70% v/v ethanol. The tubes were inverted several times to wash the DNA pellet and the inside of the tube. The tubes were centrifuged at 13,000g for 2 minutes to recover the DNA pellet. The ethanol was then aspirated and discarded and the pellet was allowed to air-dry until all visible ethanol had evaporated. The pellet was rehydrated by the addition of 100µl TE buffer (Sigma, 10mM Tris, 1mM EDTA, pH8.0) and the tube was gently tapped to aid dispersion of the pellet. Rehydration was allowed to continue overnight at room temperature. Purified DNA was subsequently stored at 4°C or -20°C. To increase the yield of DNA produced using this procedure (required for genomic subtractive hybridisation) a scaled-up protocol was adopted. The same procedure was performed as in the small-scale protocol except for the following alterations. 50ml Falcon centrifuge tubes were used throughout. All centrifugation steps were carried out in a Sigma 4K15 centrifuge at 5,000g. 10ml overnight culture was processed. Lysis was carried out using 6ml Nuclei Lysis Solution. Lysate was treated with 10µl RNase A. Protein precipitation was carried out using 2ml Protein Precipitation Solution. DNA was precipitated in 6ml isopropanol and the pellet washed in 10ml 70% v/v ethanol. DNA was rehydrated in 200µl TE buffer.

2.6.1.3 Rapid genomic DNA preparation

Although the Wizard genomic DNA kit provided chromosomal DNA of high purity, it was found not to contain detectable levels of pO157 plasmid DNA when analysed by PCR for the *espP* gene. Therefore, to provide template DNA suitable for screening bacterial isolates for virulence factor-encoding genes and for a rapid method of PCR-screening of transformed clones, a third method was adopted. Briefly, single, well-isolated bacterial colonies were picked from overnight cultures on LB medium, nutrient agar or S-gal-ampicillin medium (Section 2.3.1.1). Each colony was resuspended in 50µl well-mixed Instagene Chelex Matrix (BioRad) by vigorous vortexing for 10 seconds. The mixture was heated to 100°C in a heating block for 10 minutes. The mixture was allowed to cool briefly and vortexed again for 10 seconds. Samples were stored at -20˚C until required. Before each use, samples were centrifuged at 12,000g for 1 minute.

2.6.1.4 Isolation of Plasmid DNA

Plasmid DNA from bacteria transformed with cloning vectors was isolated using the Wizard SV miniprep kit (Promega) according to the manufacturers' instructions. Briefly, 10ml overnight bacterial culture, grown in LB containing 50µg/ml ampicillin (Section 2.3.1.1) was centrifuged at 13,000g for 5 minutes in a microcentrifuge. The supernatant was discarded and the pellet resuspended in 250µl cell resuspension solution. To this was added 250µl of cell lysis solution and the tube was vortexed thoroughly to mix. 10µl alkaline protease solution was added to this mixture and this was inverted several times to mix. 350µl Neutralisation solution was added and the mixture was vortexed vigorously for 10 seconds. The resultant lysate was transferred to a spin column and centrifuged for 1 minute at 13,000g. The flow-through was discarded and 750µl wash buffer was added to the column. This was centrifuged again at 13,000g and the resultant flow-through discarded. This washing step was repeated using 250µl wash buffer; centrifugation was carried out at 13,000g for 2 minutes. The column was transferred to a sterile 1.5ml centrifuge tube and plasmid DNA was eluted by addition of 100µl nuclease-free water to the column and centrifugation for 1 minute at 13,000g. Plasmid DNA samples were stored at -20˚C until required.

2.6.2 Spectrophotometric analysis of DNA

Purified DNA was quantified by measurement of absorbance at 260nm using a Nanodrop ND-1000 spectrophotometer. Absorbance ratios (260/280nm and 260/230nm) were used to determine DNA purity.

2.6.3 PCR-analysis of *E. coli* isolates

PCR primers were obtained from MWG-Biotech (where). Primers used in this study are detailed in Table 2.2. All PCR reactions were carried out using Thermo-Hybaid thermal cyclers. PCR mixes and cycling conditions are detailed in Table 2.3. Results of PCR analysis were visualised by agarose gel electrophoresis (Section 2.6.5).

Target gene		Sequence	Reference
chuA	chuF	GACGAACCAACGGTCAGGAT	Clermont *et al.*
	chuR	TGCCGCCAGTACCAAAGACA	(2000)
yjaA	yjaF	TGAAGTGTCAGGAGACGCTG	Clermont *et al.*
	yjaR	ATGGAGAATGCGTTCCTCAAC	(2000)
TSPE4.C2	tspF	GAGTAATGTCGGGGCATTCA	Clermont *et al.*
	tspR	CGCGCCAACAAAGTATTACG	(2000)
stx (RFLP)	LinF	GAACGAAATAATTTATATGT	Bastian *et al.*
	LinR	TTTGATTGTTACAGTCAT	(1998)
*stx*1	stx1F	ATAAATCGCCATTCGTTGACTAC	Aranda *et al.*
	stx1R	AGAACGCCCACTGAGATCATC	(2004)
*stx*2	stx2F	GGCACTGTCTGAAACTGCTCC	Aranda *et al.*
	stx2R	TCGCCAGTTATCTGACATTCTG	(2004)
ipaH	ipaF	GTTCCTTGACCGCCTTTCCGATACCGTC	Aranda *et al.*
	ipaR	GCCGGTCAGCCACCCTCTGAGAGTAC	(2004)
aat	aatF	CTGGCGAAAGACTGTATCAT	Aranda *et al.*
	aatR	CAATGTATAGAAATCCGCTGTT	(2004)
bfpA	bfpF	AATGGTGCTTGCGCTTGCTGC	Aranda *et al.*
	bfpR	GCCGCTTTATCCAACCTGGTA	(2004)
eae	eaeF	CTGAACGGCGATTACGCGAA	Aranda *et al.*
	eaeR	CCAGACGATACGATCCAG	(2004)
eae (RFLP)	eaeVF	AGYATTACTGAGATTAAG	Ramachandran
	eaeVR	AAATTATTYTACACARAY	*et al.* (2003)
	eaeZetaVR	AGTTTATTTTACGCAAGT	
	eaeIotaVR	TTAAATTATTTTATGCAAAC	
espP	espPF	AAACAGCAGGCACTTGAACG	McNally *et al.*
	espPR	AGACAGTTCCAGCGACAACC	(2001)
espP (RFLP)	PRFLPf	AAACAGCAGGCACTTGAACG	Brunder *et al.*
	PRFLPr	GGAGTCGTCAGTCAGTAGAT	(1999)
escN	escNf	CGCCTTTTACAAGATAGAAC	Kyaw *et al.*
	escNr	CATCAAGAATAGAGCGGAC	(2002)
cdtB	cdtF	AGCACCCGCAGTATCTTTGA	Janka *et al.*
	cdtR	AGCCTCTTTTATCGTCTGGA	(2003)
cnf	cnfF	CTGGACTCGAGGTGGTGG	Blanco *et al.*
	cnfR	CTCCTGTCAACCACAGCC	(1996)

fimA	fimF	CGACGCATCTTCCTCATTCTTCT	Nowrouzian
	fimR	ATTGGTTCCGTTATTCAGGGTTGTT	*et al.* (2001)
sfaD/E	sfaF	CTCCGGAGAACTGGGTGCATCTTAC	Nowrouzian
	sfaR	CGGAGGAGTAATTACAAACCTGGCA	*et al.* (2001)
papC	papF	GACGGCTGTACTGCAGGGTGTGGCG	Nowrouzian
	papR	ATATCCTTTCTGCAGGGATGCAATA	*et al.* (2001)
draA	draF	GCCAACTGACGGACGCAGCAC	Nowrouzian
	draR	CCCCAGCTCCCGACATCGTTTTT	*et al.* (2001)
neuB	neuF	CTACCCCTTTTGACGAAGAC	Nowrouzian
	neuR	ACACACCTGACCCCAATAC	*et al.* (2001)
kfiC	kfiF	GCCACCAACTGTCGCAAAA	Nowrouzian
	kfiR	TGTCGCCCAAACAAAAAGATT	*et al.* (2001)
iutA	iutF	GGCTGGACATCATGGGAACTGG	Nowrouzian
	iutR	CGTCGGGAACGGGTAGAATCG	*et al.* (2001)
ehxA	ehxF	ATGACAGTAAATAAAATAAAGAAC	Cookson *et al.*
	ehxR	TCAGACAGTTGTCGTTAAAGTTG	(2007)
F5 (K99)	F5F	TATTATCTTAGGTGGTATGG	Franck *et al.*
	F5R	GGTATCCTTTAGCAGCAGTATTTC	(1998)
F17	F17F	GCAGAAAATTCAATTTATCCTTGG	Bertin *et al.*
	F17R	CTGATAAGCGATGGTGTAATTAAC	(1996)
F41	F41F	GCATCAGCGGCAGTATCT	Franck *et al.*
	F41R	GTCCCTAGCTCAGTATTATCACCT	(1998)
espA	espAF	TGAGGCATCTAARGMGTC	China *et al.*
	espAaR	GCTGGCTATTATTGACCG	(1999)
	espAgR	ATCACGAATACCAGTTACCA	
	espAbR	TGCCTTTCTTATTCTTGTCA	
H-RFLP	F-FLIC1	ATGGCACAAGTCATTAATACCCAAC	Fields *et al.*
	R-FLIC2	CTAACCCTGCAGCAGAGACA	(1997)
O-RFLP	O482	CACTGCCATACCGACGACGCCGATCTGTTGCTTGG	Coimbra *et al.*
	O412	ATTGGTAGCTGTAAGCCAAGGGCGGTAGCGT	(2000)
mccH47	mccH47F	CACTTTCATCCCTTCGGATTG	Gordon & O'Brien
	mccH47R	AGCTGAAGTCGCTGGCGCACCTCC	(2006)
mccV	mccVF	CACACACAAAACGGGAGCTGTT	Gordon & O'Brien
	mccVR	CTTCCCGCAGCATAGTTCCAT	(2006)
mccJ25	mccJ25F	TCAGCCATAGAAAGATATAGGTGTACCAAT	Gordon & O'Brien
	mccJ25R	TGATTAAGCATTTTCATTTTAATAAAGTGT	(2006)

colB	ColBF	AAGAAAATGACGAGAAGACG	Gordon & O'Brien (2006)
	ColBR	GAAAGACCAAAGGCTATAAGG	
colE2	ColE2F	TTTATGAGCGGTGGCGAT	Gordon & O'Brien (2006)
	ColE2R	TCGGGTTACTGCGTTGCTAA	
colE7	ColE7F	CCCTCCCTGACTTGACAGAGAAAATAATGGCG	Gordon & O'Brien (2006)
	ColE7R	GCCTCTGTGTAATCACTAATACTATTTTTCAG	
Plasmid inserts	M13F	GTAAAACGACGGCCAGT	
	M13R	AACAGCTATGACCATG	

Table 2.2: Sequences of PCR primers used during this study.

Target gene(s)	PCR Mix	Cycling Conditions
chuA, *yjaA*, TSPE4.C2	12.5µl GoTaq Green Mastermix (Promega) 0.5µM each of 6 primers Wizard genomic DNA:1µl Total volume: 25µl	**1 cycle** 94°C (2 min) **30cycles:** 94°C (30s) 55°C (30s) 72°C (30s) **1 cycle** 72°C (7min)
*stx*1, *stx*2, *ipaH*.	0.1µM each of stx2F and stx2R primers 0.12µM each of stx1F and stx1R primers 0.05µM each of ipaF and ipaR primers 5µl: 5X Q solution (QIAGEN) 0.25µl Accuprime DNA polymerase 2.5µl Accuprime buffer Total volume: 25µl	**1 cycle** 95°C (5 min) **40 cycles** 95°C (45 s) 50°C (1 min) 72°C (1 min) **1 cycle** 72°C (7 min)
aat, *bfpA*, *eae*.	0.2µM each of aatF and aatR primers 0.1µM each of eaeF, eaeR, bfpF and bfpR primers 5µl: 5X Q solution (QIAGEN) 0.25µl Accuprime DNA polymerase (Invitrogen) 2.5µl Accuprime buffer (Invitrogen) Total volume: 25µl	**1 cycle** 95°C (5 min) **40 cycles** 95°C (40 s) 58°C (1 min) 72°C (1 min) **1 cycle** 72°C (7 min)
espP	1µM each of primers espPF and espPR 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **30 cycles** 95°C (45s) 59°C (45s) 72°C (90s) **1 cycle** 72°C (10min)
escN	As for espP, except using escNF and escNR primers	**1 cycle** 95°C (15 min) **35 cycles** 95°C (1 min) 54°C (40s) 72°C (1 min) **1 cycle** 72°C (5 min)
cdtB	0.5µM each of primers cdtF and cdtR 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) 5µl: 5X Q solution (QIAGEN) Total volume: 25µl	**1 cycle** 95°C (15 min) **30 cycles** 95°C (30s) 54°C (1 min) 72°C (1 min) **1 cycle** 72°C (5 min)

cnf	As for cdtB, except using cnfF and cnfR primers	**1 cycle** 95°C (15 min) **30 cycles** 95°C (1 min) 55°C (1 min) 72°C (1 min) **1 cycle** 72°C (5 min)
fimA, *sfaD/E*, *papC*, *draA*	0.45µM each of primers fimF, fimR, papF, papR, sfaF, sfaR, draF and draR 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **30 cycles** 95°C (1 min) 55°C (1 min) 72°C (1 min) **1 cycle** 72°C (5 min)
neuB, *kfiC*, *iutA*	0.45µM each of neuF, neuR, iutF and iutR. 0.9µM each of primers kfiF and kfiR. 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 1.5µl $MgCl_2$ (25mM) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	As *fimA*, *sfaD/E*, *papC*, *draA* PCR.
espA	1µM each of primers espAF, espAaR, espAgR and espAbR 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **30 cycles** 95°C (30s) 48°C (30s) 72°C (30s) **1 cycle** 72°C (5 min)
F5	0.4µM each of primers F5F and F5R Total volume: 25µl 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **25 cycles** 95°C (30s) 50°C (45s) 72°C (90s) **1 cycle** 72°C (10 min)
F17	0.45µM each of primers F17F and F17R 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **25 cycles** 95°C (2 min) 50°C (1 min) 72°C (1 min) **1 cycle** 72°C (5 min)
F41	As for F5, except using F41F and F41R primers	As F5
mccJ25	0.2µM each of primers mccJ25F and mccJ25R 12.5µl GoTaq Green Mastermix (Promega) Total volume: 25µl	**1 cycle** 94°C (4 min) **25 cycles** 94°C (30s) 55°C (30s) 72°C (3 min) **1 cycle** 72°C (3 min)
mccV	As mccJ25 except using mccVF and mccVR primers	As *mcc*J25

mccH47, colB	As mccJ25 except using mccH47F, mccH47R, colBF and colBR primers	As *mcc*J25
colE2	As mccJ25 except using colE2F and colE2R primers	As *mcc*J25
colE7	As mccJ25 except using colE7F and colE7R primers	As *mcc*J25
ehxA	As for F5, except using primers ehxF and ehxR	**1 cycle** 95°C (15 min) **25 cycles** 95°C (45s) 52°C (45s) 72°C (2 min) **1 cycle** 72°C (5 min)
stx (RFLP)	0.5µM each of primers LinF and LinR 0.2µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 40µM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **35 cycles** 94°C (45s) 45°C (1 min) 72°C (1 min) **1 cycle** 72°C (10 min)
eae (RFLP)	1µM each of primers eaeVF, eaeVR, eaeZetaVR and eaeIotaVR 0.5µl $MgCl_2$ (25mM) 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **35 cycles** 94°C (1 min) 41°C (1 min) 72°C (1 min) **1 cycle** 72°C (10 min)
espP (RFLP)	0.6µM each of primers PRFLPf and PRFLPr 0.25µl HotStarTaq (QIAGEN) 2.5µl 10X PCR Buffer (QIAGEN) 0.1mM dNTPs (Roche Applied Sciences) Total volume: 25µl	**1 cycle** 95°C (15 min) **30 cycles** 94°C (30s) 56°C (1 min) 72°C (2min 30s) **1 cycle** 72°C (5 min)

Table 2.3: PCR amplification conditions: 2µl Template DNA was included in each reaction prepared according to Section 2.6.1.3, unless stated otherwise. Reactions were made up to their respective total volumes using Sigma Ultrapure Water (molecular biology grade).

2.6.4 PCR-RFLP analysis of *E. coli* isolates

2.6.4.1 Molecular serotyping

Flagellar (H) antigen determination by PCR-RFLP was carried out employing a previously described method (Fields *et al.*, 1997;Ramos Moreno *et al.*, 2006). Genes encoding the FliC major flagellar protein were amplified using the primers F-FLIC1 and R-FLIC2 (Table 2.2). Amplification was carried out in 25µl volumes containing 1µM each primer, 1µl template DNA (prepared as set out in Section 2.6.1.2) 160µM dNTPs, 1.5mM $MgCl_2$, 0.1µl GoTaq Flexi DNA polymerase and 1X Promega PCR buffer (colourless). PCR cycling parameters were as follows: 1 cycle (94°C/4 min) was followed by 35 cycles (94°C/30s, 60°C/1 min, 72°C/2 min) and 1 cycle (72°C/5 min). After amplification, 5µl of the mixture was analysed by agarose gel electrophoresis. 1µl *Rsa*I restriction enzyme (Roche Applied Science) was added to each amplified sample and mixed by pipetting. Restriction digest mixtures were incubated at 37°C for at least 3 hours. After this time, 8µl loading buffer was added and the resultant mixture was analysed by agarose gel elecrophoresis. Observed fragment sizes were compared to expected sizes, generated by *in silico* restriction analysis using the University of Alberta Sequence Manipulation Suite: http://www.ualberta.ca/~stothard/javascript/rest_digest.html.

Somatic (O) antigen determination (O-RFLP) was attempted by the method proposed by Coimbra *et al.* (2000). The *rfb* locus was amplified using the primers O-412 and O-482 (Table 2.2). Amplification was carried out in 25µl volumes containing 0.3µM each primer, 1µl template DNA (prepared as set out in Section 2.6.1.2), 500µM dNTPs, 0.375µl Long Template DNA polymerase and 1X Long template PCR buffer 3. PCR cycling parameters were as follows: 1 cycle (94°C/2 min) was followed by 10 cycles (94°C/10s, 64°C/30s, 68°C/15 min) then 20 cycles (94°C/10s, 64°C/30s, 68°C/15 min with a 20s increment each cycle) and finally 1 cycle (72°C/7 min). After amplication, 5µl 10X NEB restriction buffer 2, was added and the volume adjusted to 49.5µl with ultrapure water. To this 0.5µl (2.5U) *Mbo*II restriction enzyme was added. The mixture was thoroughly mixed and incubated at 37˚C for at least 3 hours. After this time the mixture was heated to 72˚C for 10 minutes to dissociate the enzyme from the DNA. Subsequently, 15µl of the mixture was analysed by agarose gel

electrophoresis. Isolates representing unknown O-types were compared with those of the available reference types (detailed in Table 2.1). Selected isolates with a pattern that did not match the reference types were submitted to the Health Protection Agency, Laboratory of Enteric Pathogens (Colindale Avenue, London) for serological O-antigen determination.

2.6.4.2 PCR-RFLP analysis of candidate virulence factor-encoding genes

PCR amplification was carried out as detailed in Table 2.3. 7µl aliquots of the amplified products of the *stx* (RFLP) PCR (Table 2.3) were mixed with 1.25µl NEBuffer 3, 4µl distilled water and 0.25µl (2.5U) *Hin*cII (New England Biolabs). 7µl aliquots of the amplified products of the *espP* (RFLP) PCR were mixed with 1µl SureCut Buffer A, 1.7µl distilled water and 0.3µl (3.33U) *Alu*I (Roche Applied Science). Similarly 7µl aliquots of the amplified products of the *ehx* PCR were mixed with 1µl SureCut Buffer B, 1.7µl distilled water and 0.3µl (3.33U) *Taq*I (Roche Applied Science). Each of these mixtures were incubated at 37°C for 3 hours and subsequently analysed by agarose gel electrophoresis.

2.6.5 Agarose gel electrophoresis

Samples resulting from DNA isolation, PCR and restriction digest were analysed by agarose gel electrophoresis according to standard methodology. Gel Red (Biotium Inc) was included in gels as per the manufacturers instructions. The percentage of agarose used depended on the size of fragments expected. For applications resulting in fragments below 500bp, 2% agarose gels were employed, whereas when fragments were expected greater than 500bp, 1% agarose gels were employed. Where fragments of a range of sizes were expected, 1.5% agarose gels were utilised.

2.6.6 Genomic microarray

2.6.6.1 Composition of the microarray

Pre-printed microarray slides were provided by Dr Wu Guanghui of the Veterinary Laboratory Agency, Weybridge, Surrey. The array comprised the Array-ready Oligo Set for the *E coli*

genome Version 1.0 (Operon) containing 5978 70-mer probes representing open reading frames (ORFs) of three *E. coli* strains, MG1655, O157:H7 (EDL933) and O157:H7 (Sakai). In addition, 110 Oligos representing genes encoded on plasmids pO157 and pOSAK1 were also included on the array. The composition, printing and validation of the array are detailed in Wu *et al.* (2008) and (Carter *et al.*, 2008). Oligonucleotides were set out on the slide in triplicate on each slide, arranged in 21 x 21 subgrids. Spacing between spots was approximately 0.21 µm and the average diameter of spots was approximately 120 µm.

2.6.6.3 Genomic DNA preparation, Labelling and hybridisation

As a control sample the DNA from three sequenced strains (MG1655, EDL933 and Sakai) was mixed, each strain contributing a third of the total DNA (i.e. 0.66 µg each). Two micrograms of the control or test DNA (from a single strain) was labelled using the protocol of the Institute of Food Research (http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/protocols.html) with some minor adjustments. Briefly, 2 µg of genomic DNA in 23.5 µl of nuclease-free water was mixed with 20 µl of 2.5X random primer–reaction buffer mix from the BioPrime DNA labelling system (18094-011; Invitrogen). The mixture was boiled for 5 min and cooled on ice for 5 min. Then, 5 µl of 10X deoxynucleoside triphosphates (1.2 mM each dATP, dGTP and dTTP and 1.1 mM dCTP; Amersham Biosciences) and 0.5 µl of Cy5 or Cy3 dCTP (1 mM stock; Amersham Biosciences) were added. Finally, 1 µl of Klenow enzyme from the kit was added and the reaction was carried out at 37°C for 1.5 h. Excess Cy3 and Cy5 dCTP were removed from the labeled DNA with a MinElute PCR purification kit (28006; Qiagen). Hybridization was performed with a protocol similar to the one developed by the BµG@S group (http://bugs.sgul.ac.uk/bugsbase/index.php). The slide was submerged in prehybridization solution (3.5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) and 10 mg/ml bovine serum albumin) at 65°C for 20 min. Then, it was rinsed in 400 ml of water for 1 min and followed by 400 ml of propan-2-ol for 1 min before being dried by centrifugation at 1,500 rpm (480g) for 5 min. The labeled DNA was prepared in hybridization solution (4X SSC, 3% SDS), heated at 95°C for 2 min and allowed to cool at room temperature before being added to a slide covered with a LifterSlip. Hybridization was carried out in a Genetix hybridization chamber and incubated at 65°C in a hybridization oven for 16 to 20 h. After the hybridization, the slide was washed twice gently in

wash buffer A (1X SSC, 0.05% SDS) at 65°C for at least 5 min each time. The slide was then washed in 400 ml wash buffer B (0.06X SSC) for 2 min at room temperature.

2.6.6.4 Data acquisition and analysis

The processed slides were scanned using a GenePix 4000B scanner (Axon Instruments, Inc) and data was compiled using GenePix Pro 4.1. A spot was determined to be present if a minimum 65% of its pixels have intensities greater than the background plus 2 standard deviations. The resultant data was normalised per spot by dividing signal (532nm wavelength, Cy3) intensity by control (635nm wavelength, Cy5) intensity. Per-chip normalisation was performed using a set of genes present in a single copy in each of the three control strain genomes. Cut-off values were determined according to Carter *et al.* (2008). Pixel intensity values below 0.25 were marked absent, those above 0.5 were marked present and those between 0.25 and 0.5 were considered to be ambiguously detected.

2.6.7 Virulence gene nanoarray

A miniaturised DNA Microarray described by Anjum *et al.* (2007) was used, comprising 39 oligonucleotide probes complementary to virulence factors, 7 complementary to bacteriocin genes and 15 control genes. The method set out by (Ballmer *et al.*, 2007) was used for multiplex linear DNA amplification and labelling. Briefly, for each assay, 1µg DNA prepared by the DNeasy method was combined with 1 µl 10X ThermoPol amplification buffer (New England Biolabs), 1 µl deoxynucleoside triphosphate mixture (1 mM each dATP, dCTP and dGTP; 0.65 mM dTTP), 0.35 µl biotin-16-dUTP (Roche Applied Science), 1 µl mixture of the 60 oligonucleotide primers described by Anjum *et al.* (2007) at a final concentration of 0.135µM and 0.1 µl Therminator DNA polymerase (New England Biolabs) in a total volume of 10µl.

The linear amplification steps comprised 5 min at 96°C, followed by 45 cycles of 62°C/20s, 72°C/40s and 96°C/60s. Printed ArrayTubes obtained from Clondiag were placed in a thermomixer (Eppendorf) and washed with 500 µl of deionized water for 5 min at 55°C (550 rpm) then with 500 µl hybridization buffer 1 (Clondiag) for 5 min at 30°C and (550 rpm). 10µl

of the labeled DNA was diluted with 90 µl hybridization buffer 1, denatured for 5 min at 95°C, cooled on ice for 2 min and then added to the ArrayTube.

The hybridization was carried out at 55°C and with shaking at 550 rpm for 1 h. After hybridization the ArrayTubes were washed for 5 minutes in each of three wash solutions: the first using 500 µl 2X SSC (0.3M NaCl, 0.03M sodium citrate, pH 7.0) containing 0.2% w/v SDS at 40°C then with 500 µl 2X SSC at 30°C, followed by 500 µl 0.2X SSC at 30°C, with each step performed for at 550 rpm. The arrays were blocked using 100 µl 6X SSPE solution (60 mM sodium phosphate, 1.08 M NaCl, 6 mM EDTA, pH 7.4) containing 0.005% Triton X-100 and milk powder (2% w/v) for 15 min at 30°C (550 rpm). 100µl 200pg/µl Peroxidase-streptavidin conjugate (Sigma) in 6X SSPE–Triton X-100 was added to each tube and the mixture was incubated for 15 min at 30°C and 550 rpm. ArrayTubes were washed using 500µl 2X SSC containing 0.01% (v/v) Triton X-100 at 30°C, 500 µl 2X SSC at 20°C and 500µl 0.2X SSC at 20°C, for 2 min each, with each step repeated once.

100µl True Blue peroxidase substrate (Kirkegaard & Perry Laboratories) was added to the ArrayTubes and signals were detected with the ATR01 ArrayTube reader (Clondiag). Signals were recorded at 25°C for 5 min and analyzed by using the IconoClust, version 2.2, software (Clondiag). Images were examined for smudging and debris leading to false positives and these were removed from the analysis. Values for 1-(mean pixel intensity/background pixel intensity) were calculated and a cut-off applied, whereby values above 0.3 were designated positive, while values below 0.1 were considered negative. Values between 0.1 and 0.3 were considered to be ambiguous.

2.6.8 Subtractive hybridisation

DNA was prepared using the Wizard genomic DNA kit (Section 2.6.1.2). This DNA was used for genomic subtractive hybridisation, using the PCR-select Bacterial Genome Subtraction Kit (Clontech) according to the manufacturer's instructions. In each case, genomic DNA from a genome-sequenced *E. coli* O157:H7 EDL933 (MCI0045) was used as the driver DNA. Genomic DNA from strains MCI 0453, MCI 0489, MCI 0507 were used individually as tester DNA. Briefly, tester and driver DNA samples (2-10µg) were digested to completion in 50µl reaction volume including 1.5µl *Rsa*I restriction enzyme, 5µl 10X *Rsa*I Restriction Buffer. The

reaction was stopped by the addition of 2.5µl 0.2M EDTA (pH 8.0) and 50µl phenol:chloroform:isoamyl alcohol (25:24:1) was added. The mixture was vortexed and centrifuged at 14,000g for 10 minutes at room temperature. The aqueous layer was recovered and added to 50µl chloroform:isoamyl alcohol (24:1) and centrifuged as before. The aqueous layer was recovered to a clean tube. DNA was precipitated by the addition of 0.5 volume of ammonium acetate and 2.5 volumes of 95% ethanol. After vortexing briefly, precipitated DNA was pelleted by centrifugation. The supernatant was removed and the pellet washed by the addition of 200µl 80% ethanol. Pellets were recovered by centrifugation, the ethanol was removed and pellets were air-dried briefly. Digested precipitated DNA was resuspended in nuclease-free water. DNA concentration was determined using a Nanodrop spectrophotometer (Section 2.6.2) and adjusted to 120ng/µl with nuclease free water. A 1µl aliquot of each tester DNA was mixed with 2µl Adaptor 1 and another 1µl aliquot mixed Adaptor 2R. To these mixtures were added 4µl sterile distilled water, 2µl 5X Ligation buffer and 1µl T4 DNA Ligase (400U). After overnight incubation at room temperature, 1µl 0.2M EDTA (pH 8.0) was added and samples were heated to 72˚C to stop the reaction and inactivate the ligase.

At this point, ligation and digestion efficiency was determined by the method prescribed by the manufacturer. Briefly, each adaptor-ligated tester DNA sample was used as template DNA for PCR-amplification using a primer complementary to the *E. coli* 23S rRNA gene and a primer complementary to sequence carried on the adaptor (both supplied with the kit). A reaction whereby the DNA was amplified using two internal 23S rRNA primers was included as a positive control. Ligation efficiency was considered to be adequate if the intensity of the band generated by the adaptor-specific primer/23S rRNA primer amplification was greater than 25% of that generated by amplification using the two internal 23S rRNA primers. If the amplification using the adaptor-specific primer/23S rRNA primer generated larger products than the expected 374bp band, the restriction digest was considered to be incomplete.

Two rounds of hybridisation of the adaptor-ligated tester DNA to driver DNA were performed as follows: 1µl of each of the adaptor-ligated tester DNA samples were mixed individually with 2µl *Rsa*I digested driver DNA (120ng/µl) and 1µl 4X hybridisation buffer. The samples were overlayed with one drop of mineral oil (molecular biology grade) and centrifuged briefly. The mixtures were incubated at 98°C for 90 seconds and 63°C for a further 90 minutes, in a Thermo Hybaid thermal cycler.

In a sterile tube, a further 1µl *Rsa*I-digested driver DNA (120ng/µl) was mixed with 1µl 2X hybridisation buffer. One drop of mineral oil was added and the mixture was briefly centrifuged and incubated at 98°C for 90 seconds in a thermal cycler. The two completed first hybridisation samples were mixed with the denatured driver DNA, taking care not to allow the two first hybridisations samples to mix before they were in contact with the fresh denatured driver DNA. After mixing, the second hybridisation was carried out overnight at 63°C in a thermal cycler. After this period, 200µl Dilution Buffer was mixed with to the hybridised samples and the mixture was heated for a further 7 minutes at 63°C to eliminate non-specific hybridisation.

The hybridised samples were used as template DNA for two rounds of PCR-amplification. The first round utilises a PCR primer complementary to both ends of the two adaptors. The second round employs nested primers, complementary to areas within the adaptors between the binding sites for the primary PCR primer.

After the two rounds of amplification, the PCR products were cloned into pGEM-T vector using pGEM-T vector System II (Promega) according to the manufacturer's instructions. 3µl of the mixture of products from the secondary PCR were mixed with 1µl (50ng) pGEM-T vector, 5µl 2X Rapid Ligation Buffer and 1µl T4 DNA Ligase (Promega). The ligation mixtures were incubated at room temperature overnight. 50µl chemically competent *E. coli* JM109 cells (Promega) were defrosted on ice for each of the transformation reactions to be performed. 2µl of the ligation mixture was added to one 50µl aliquot of JM109 cells, which was mixed by vigorously tapping the tube and left on ice for 20 minutes. Cells were heat-shocked for 45-50 seconds in a water-bath set to 42°C and returned to ice for 2 minutes. 950µl room temperature SOC-medium was added and the mixture incubated at 37°C for 1.5 hours. 100µl aliquots were spread onto S-gal agar containing 50µg/ml ampicillin (Section 2.3.1.1) and incubated overnight at 37°C. For each of the test strains 250-300 colonies were picked from the resultant plates and transferred into either 50µl Instagene Chelex Matrix (BioRad) or 10ml LB broth. Those in LB broth were incubated overnight at 37°C with 200rpm shaking and the culture processed using the Wizard SV Plus Miniprep Kit (Section 2.6.1.4) to yield isolated plasmid DNA. Those picked into Instagene Chelex matrix were replica-plated onto LB agar containing 50µg/ml ampicillin for short-term storage and the tubes containing the

bacteria in Instagene Chelex Matrix were processed as Section 2.6.1.3 to prepare template DNA for PCR. Potential clones were screened for inserts using M13 universal primers. 1µl DNA (either isolated plasmid or Instagene-treated sample) included in a mixture comprising 0.3µM M13F and M13R primers (Table 2.2), 0.1mM of each dNTP, 1.5mM $MgCl_2$, 5µl GoTaq Flexi 5X Colourless PCR Buffer, 0.2µl GoTaq Flexi DNA Polymerase (Promega). The cycling protocol comprised 1 cycle of 5 minutes at 94°C, then thirty cycles of the following: 94°C for 15 seconds, 51°C for 30 seconds and 90 seconds at 72°C. This was followed by a final elongation step at 72°C for 10 minutes. The resulting fragments were analysed by agarose gel electrophoresis. Any clones yielding no amplicon or more than one amplicon were removed from further analysis.

2.6.8.1 Verification of subtracted library by Southern Hybridisation.

The DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) was used according to the manufacturer's instructions:

2.6.8.1.1 DNA Labelling

1µg of the DNA to be used as the probe in a volume of 16µl was denatured in a boiling water bath for 10 minutes; then immediately chilled on ice for 2 minutes. 4µl DIG-High Prime was added, mixed briefly by vortexing and the tube centrifuged briefly to collect the contents at the base. This mixture was incubated overnight at 37°C. Subsequently 2µl 0.2M EDTA (pH 8.0) was added and the mixture heated to 65°C to stop the reaction. Labelling efficiency was determined by preparing a serial dilution of the mixture and transferring 1µl spots of diluted samples to Whatman Supercharged Nytran Nylon Membranes. Membranes were fixed by UV treatment for 2 minutes each side in a UV crosslinker, while kept moist by filter paper soaked in 10X SSC buffer. Membranes were rinsed in distilled water and allowed to air-dry. Once dry, the membranes were incubated for 2 minutes in Maleic Acid Buffer (0.1 M Maleic acid, 0.15 M NaCl; adjusted to pH 7.5 using solid NaOH) followed by 30 minutes in 1X Blocking Buffer (prepared by diluting 10X Blocking Buffer in Maleic Acid Buffer) then for 30 minutes in Antibody Solution (Anti-Digoxygenin-Alkaline Phosphatase Conjugated Antibody diluted 1 in 10,000 in Blocking Buffer). Membranes were washed twice for 15 minutes in Washing Buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20) and equilibrated in Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). Each membrane was placed in an exposure cassette and 1ml CSPD (chloro-5-substituted adamantyl-1,2-dioxetane phosphate) solution was applied to the surface of each membrane. Membranes were covered with transparent plastic and the CSPD was spread across the surface. Excess CSPD was squeezed out gently and removed using tissue. Membranes were incubated at room temperature for 5 minutes. X-ray film was exposed to the membrane and developed using an X-ray developer. Labelling efficiency was considered sufficient if spots of 1pg/µl DNA were visible after 40 minutes of exposure.

2.6.8.1.2 Hybridisation

5µl aliquots of the PCR products generated by M13 PCR (Section 2.6.8) were combined with 5µl aliquots of 0.6M NaOH. 2µl amounts of this mixture were spotted onto Whatman Supercharged Nytran Nylon Membranes. Membranes were neutralised in 0.5M Tris HCl (pH 7.5) for 4 minutes, then washed in distilled water. Membranes were allowed to dry briefly and exposed to UV light in a UV light-box. Dry membranes were stored at 2-8°C between two sheets of filter paper. DIG Easy Hyb (DEH) solution was preheated to 65°C in a hybridising oven. Membranes were prehybridised in prewarmed DEH solution for one hour with constant agitation. DNA probes (Section 2.6.8.1.1) were denatured for 5 minutes in a boiling water bath and transferred immediately to ice. 10µl probe was added to 25ml prewarmed DEH solution. Liquid was removed from the membranes and replaced with the probe in DEH solution. Membranes were incubated in probe/DEH mixture at 65°C overnight in sealed containers with constant agitation. Washing was subsequently carried out, firstly using ample 2X SSC containing 0.1% (w/v) SDS at room temperature with one change of buffer and secondly, using ample prewarmed 0.5X SSC containing 0.1% (w/v) SDS at 68°C for 5 minutes. All subsequent steps were carried out at room temperature. Membranes were washed briefly in Wash Buffer at room temperature then transferred to 50ml 1X Blocking Buffer. Membranes were incubated in Blocking Buffer for 3 hours at room temperature after which time the liquid was removed and replaced with 20ml Antibody Solution. Antibody Solution was removed and replaced with Wash Buffer and two 15 minute washes were carried out. Washing Buffer was replaced with Detection Buffer and incubated for 5 minutes at room temperature with agitation. Membranes were placed in film cassettes, overlaid with CSPD solution. A transparent plastic sheet was placed on the surface of the membrane and air bubbles were carefully smoothed out. X-Ray film was exposed for 1-24h and developed using an X-ray developer. Images were scanned using a GelDoc Imager and converted to tagged image format files (.tif). Resulting images were imported into the Total Lab Array (Progenesis) program. Grids were fitted to the spots and pixel intensity was calculated and normalised against background. Data generated was exported to Microsoft ExCel. Spots representing each clone in the library were ranked according to their intensity and the lowest intensity spots were chosen for sequencing.

2.6.8.2 Sequence-analysis of subtracted libraries

DNA-sequencing was performed using DNA prepared using the Wizard SV Plus Miniprep Kit (Section 2.6.1.4). Sequencing was carried out at MWG Biotech, using the T7 universal forward primer. Database searches were performed using the Basic Local Alignment Tool (BLAST) (Altschul *et al.*, 1997;Zhang *et al.*, 2000). Gene accession numbers and Protein ID of genes or predicted genes covered in the most significant BLAST-N hit were recorded. If no significant hits were returned by BLAST-N, BLAST-X searches were performed to predict any translated proteins from the region identified.

2.7 Competition analysis

2.7.1 Preparation of soft agar lawns of bacteria

LB containing 0.7% Agar No.1 (Oxoid) was melted and cooled to 40˚C. 65µl overnight culture of the sensitive strain was added to 4ml molten soft agar and poured onto preset hard nutrient agar plate (Oxoid) and allowed to set. 10µl spots of bacterial supernatant or lysate, filter paper discs (prepared as described in the following sections) or section of polyacrylamide gel, were gently placed on the surface of the lawn. Plates were incubated overnight at 37˚C.

2.7.2 Preparation of filter paper disc containing bacterial lysate

Overnight streak plates of *E. coli* isolates were prepared on LB agar. Filter paper discs of 7mm diameter were soaked in chloroform for 10 seconds and then laid onto a single, well-isolated colony. Chloroform was allowed to evaporate and discs were kept at 4°C until used as detailed in Section 2.7.1.

2.7.3 Mitomycin C-induced supernatant/lysate preparation

An adaptation of the method described by (Gordon *et al.*, 1998) was employed to recover bacterial products containing potential inhibitory substances. Briefly, overnight culture was diluted 1:10 in LB and incubated at 37˚C, 200rpm shaking for 1hr. Mitomycin C was added to a final concentration of 0.1-0.2µg/ml and incubated for a further 4hr at 37˚C, 200rpm shaking. Culture was centrifuged at 10 000g for 5mins and the supernatant from this was added to 1/20 volume of chloroform to kill viable cells. Alternatively, to recover a cell lysate bacterial culture without prior centrifugation was added to 1/20 volume of chloroform then centrifuged for 2 minutes at a minimum of 5000g. 100µl aliquots were plated onto a sterile LB plate to ensure no viable organisms were present. In order to recover bacterial products at higher concentrations, an Amicon Ultra-15 filter (Ultra-Cel 5kDa or 3kDa cut-off, Millipore) was

used according to manufacturer's instructions to give a 60-fold concentration. Supernatants were stored at 4˚C until required.

2.7.5 Bacterial competition assays

At each time-point in the competitive assays described in this section (competitive growth, competitive exclusion with supernatants and competitive adherence) samples containing live bacteria were removed and used to prepare serial 1 in 10 dilutions in sterile PBS. Three 100µl aliquots of each dilution were used to prepare three spread-plates on SMAC or TBX medium. Plates were incubated overnight at 37˚C.

2.7.5.1 Competitive Growth Assay

Overnight LB cultures of the two strains to be co-cultured were prepared. OD_{600} was determined to ensure both cultures had grown to the same extent. 50µl of each culture was added to 10ml of LB and incubated continually at 37˚C, 200rpm shaking.

2.7.5.2 Competitive exclusion with supernatant

Supernatant, prepared as described in Section 2.7.3, was filtered with a 0.22µm filter unit (Millex GV Millipore). 50µl of MCI 0200 was added to 10ml of a 1:1 mixture of test supernatant and LB and incubated at 37˚C, 200rpm shaking.

2.7.5.3 Competitive adherence

EBL cells were grown to confluence in 12-well tissue culture plates. 24hr prior to challenge, the cell culture medium was removed and replaced with fresh culture medium with or without serum. 1hr prior to challenge, the volume in each well was adjusted to 500µl. Overnight cultures of strains to be tested for competitive adherence were prepared in 10ml aliquots of MEM-HEPES (Section 2.3.1.1). Cultures were diluted tenfold in sterile MEM-HEPES, prewarmed to 37˚C and were subsequently incubated at 37˚C, 200rpm shaking until OD_{600}=0.35, which was determined to be approximately $3.85x10^7$cfu/ml (± $2.39x10^6$cfu/ml;

n=56). 250µl aliquots of culture of each of the two strains to be assayed for competition were added to each of two wells. This translated to a total multiplicity of infection of 100 bacteria per cell (MOI=50 bacteria of each strain per cell). Plates containing bacteria and cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Wells were washed three times with 1ml PBS to remove non-adherent bacteria. 100µl of TritonX-100 (0.05%) was added to lyse the cells and the monolayers were solubilised, aided by scraping with a 1ml rubber syringe insert. Lysates were used to prepare serial tenfold dilutions and spread-plates as detailed in Section 2.7.5.

2.8 Data analysis

Histograms were prepared using Microsoft ExCel, showing the mean ± standard error of the mean (unless stated otherwise). Values in the text are also given as mean ± standard error of the mean. Assessments of significant differences between means were carried out by performing a Student's *t*-test using the Minitab software, where a *P*-value less than 0.05 is considered significant.

Chapter 3: Analysis of EHEC-secreted factors and their interaction with the host

3.1 Introduction

As explained in Section 1.3, *E. coli* O157:H7 poses a significant threat to human health but may exist in the bovine host without clinical signs of disease. Work presented in this chapter was designed to investigate the differential interactions of factors exported by *E. coli* O157:H7 with the bovine and human host, generally employing cultured epithelial cell lines for this purpose. Detection of differential responses of epithelial cells to EHEC could lead to further explanation of the different outcomes of EHEC colonisation of the bovine and human host and could have implications for the control and treatment of EHEC infection. Within the bovine host *E. coli* O157:H7 is generally described as a commensal organism, in that it normally does not cause clinical symptoms and is frequently isolated from healthy cattle (Griffin and Tauxe, 1991). Nevertheless, it has been noted that EHEC induces the formation of A/E lesions in the bovine gastrointestinal tract (Phillips *et al.*, 2000;Naylor *et al.*, 2005) which is a function associated with pathogenicity in humans (See Section 1.4.1).

In general, bacterial components which are exported from the bacterial cell cause the most profound and measurable effects in the host. Exported molecules are the portion of the bacterium which is most accessible to recognition by the innate immune system since they come into direct physical contact with the host. Enterohaemorrhagic *E. coli* is no exception since it exports a wide variety of proteins with diverse biological functions. Exported proteins may be presented on the cell surface, secreted into the extracellular medium or injected into the host cell. EHEC secretes certain enzymes, toxins and effector proteins which may have specific effects upon host defence mechanisms. Proteases, such as EspP and StcE, may alter the functionality of host proteins involved in innate immunity (Brunder *et al.*, 1997;Lathem *et al.*, 2004). Certain bacterial exported proteins may target signal transduction pathways, subverting cellular responses to the bacterium, such as those introduced by the T3SS and shiga-like toxin (described in Sections 1.4.1 and 1.4.2 respectively). In addition to secreted or injected factors many exported proteins are structural. These may be embedded in the membrane and include the components which make up complex extracellular appendages such as flagella and fimbriae. Proteins which make up extracellular appendages are often found in the extracellular medium as mechanical shearing of these structural extremities is commonplace.

Proinflammatory responses to EHEC during human infection are well characterised. Bacterial disruption of the epithelium causes proinflammatory cytokine release, especially via TLR5 stimulation with bacterial flagellin and subsequent infiltration of neutrophils to the mucosal surface (Berin *et al.*, 2002;Dahan *et al.*, 2002;Miyamoto *et al.*, 2006). Proinflammatory responses during colonisation of the bovine gastrointestinal tract are yet to be fully determined, although disruption of the epithelial layer by the formation of A/E lesions is likely to induce some level of proinflammatory cytokine release

Production of proinflammatory cytokines is an important part of the host immune response to bacterial infection. Interleukin-8 (IL-8) is a stereotypical proinflammatory cytokine, which acts to recruit neutrophils to the site of infection and promotes their infiltration (Savkovic *et al.*, 1996). IL-8 is produced in response to many cellular stressors, such as heat shock, irradiation, oxidative stress, cytotoxic drugs, reactive oxygen species and cellular injury. Bacterial products may cause IL-8 production by activation of pattern recognition receptors (PRRs) as described in Section 1.5.3. Bacteria may also induce inflammation as a consequence of cellular injury (Naito and Yoshikawa, 2002) and further inflammation may occur due to secondary infection in regions of barrier disruption caused by the primary pathogen. The release of proinflammatory cytokines, such as TNFα, induces production of proinflammatory cytokines by other cells and thereby the stress signal is amplified.

Flagellin has an established role as a major inducer of epithelial IL-8 secretion through the TLR5 pathway, as described in Section 1.5.3. This response has been shown both *in vitro* and *in vivo* to enteric pathogens, including EHEC, EPEC, EAggEC and *Salmonella enteritica* Serovar Typhimurium (Gewirtz *et al.*, 2001b;Berin *et al.*, 2002;Zhou *et al.*, 2003;Khan *et al.*, 2004;Miyamoto *et al.*, 2006;Gobert *et al.*, 2008). Flagellin from *S. enteritica* Serovar Typhimurium has been recently shown to inhibit production of IL-10 by mouse macrophages (Vicente-Suarez *et al.*, 2007). The epithelial proinflammatory response to live bacteria is unlikely to be solely dependent on flagellin, especially as IL-8 production in Caco-2 cells in response to both *C. rodentium* and EPEC is upregulated by treatment with Δ*fliC* bacterium, albeit to a lesser extent than the response elicited by flagellated bacteria (Khan *et al.*, 2008). Nevertheless, the dominance of flagellin as an elicitor of IL-8 secretion highlights the potential importance of flagellin in the progression of EHEC induced disease, particularly as levels of serum IL-8 have been shown to positively correlate, while levels of IL-10 negatively correlate,

with the risk of development of HUS in children affected by EHEC (Westerholt *et al.*, 2000). Conversely, constant low level stimulation of PRRs, such as TLR5, by bacteria of the gastrointestinal microflora has been shown to be important in the maintenance of a healthy gut (Rakoff-Nahoum *et al.*, 2004).

It may be beneficial to the bacterium to limit proinflammatory responses by reducing infiltration of neutrophils and thereby reduce the proportion of bacteria eliminated by phagocytosis. The action of T3SS in EHEC and EPEC results in inhibition of IL-8 secretion in human IECs (Zhou *et al.*, 2003;Rogers *et al.*, 2003;Hauf and Chakraborty, 2003;Sharma *et al.*, 2005). In this way, inflammation can be described as a "net effect" of bacterial pro- and anti-inflammatory stimuli and highlights the antagonism of the bacterium and the host response.

Thus, the proinflammatory response is strongly linked to the action of bacterial flagellin during enteric infection and there is some antagonism between pro- and anti-inflammatory molecules produced by EHEC. It is not yet determined whether the reasons for decreased pathogenicity of EHEC O157:H7 in the bovine host compared with the human host may include differential proinflammatory responses between the two species although this remains a possibility.

Many EHEC-exported components have been shown to exert specific effects upon the host (discussed in detail in Section 1.4). The StcE protease is an example of a secreted protein with roles in the subversion of host immunity. StcE was first identified by Paton & Paton (2002) in their analysis of the immunodominant antigens of *E. coli* O157:H7 by screening an *E. coli* O157:H7 cosmid library against sera from HUS patients. One of the antigens discovered had not previously been functionally characterised. The antigen was termed "TagA" for its homology with ToxR-regulated lipoprotein TagA encoded by *Vibrio cholerae*. The immune recognition of this protein during human infection has been confirmed (John *et al.*, 2005), but was not identified in a screen for *E. coli* O157:H7 immunogens in colonised cattle (Kudva *et al.*, 2006).

Lathem *et al.* (2002) identified that the TagA homologue was responsible for the aggregation of Jurkat cells, using a minitransposon mutagenesis screen and further related the effect to the

protein's ability to cleave C1-esterase inhibitor. The TagA homologue was henceforth termed secreted protease of C1 esterase inhibitor from EHEC (StcE). Cleavage of C1-INH by StcE was later found to increase the complement inhibition mediated by C1-INH (Lathem *et al.*, 2004). C1-INH is an inhibitor of the complement cascade, which is a group of proteases whose activation triggers a cascade of protease activation by cleavage, resulting in the formation of the membrane attack complex which facilitates the killing of infected cells (Janeway *et al.*, 2001). Increased inhibition of complement would prolong the survival of infected cells, hence allowing the bacterium to persist on the mucosal surface.

StcE also cleaves human mucin 7 and gp340 and decreases the viscosity of salivary mucous (Grys *et al.*, 2005;Grys *et al.*, 2006). Cleavage of mucins could allow the bacterium to access the epithelium, where it binds and forms microcolonies necessary for successful colonisation. StcE is well conserved among *E. coli* O157:H7 strains and homologues have been identified in a number of members of the Gammaproteobacteria, including *E. coli* serotypes O55 and O103, *Shigella boydii*, *Shewanella baltica*, *Aeromonas* spp. and *Photobacterium profundum* (Lathem *et al.*, 2003;Vezzi *et al.*, 2005;Seshadri *et al.*, 2006); publicly available genome sequences stored on the NCBI database). The StcE homologue in *Aeromonas hydrophila* shares only 65% amino acid identity with StcE yet it retains activity against C1-INH (Pillai *et al.*, 2006).

The main objective of this work was to further investigate the roles of extracellular factors which may benefit *E. coli* O157:H7 in its interactions with the host, focusing on extracellular factors which modulate host innate immunity and on the epithelial proinflammatory response to such factors. Another objective was to characterise the response of bovine epithelial cells to *E. coli* O157:H7 compared with the response to other bovine commensal *E. coli*. In order to perform this analysis, cell lines representing the human and bovine epithelium were chosen. At the time that this study was conducted no cell line model for the bovine intestinal epithelium was available. Therefore, for the purposes of this analysis embryonic bovine lung (EBL) cells were utilised as a bovine epithelial cell line model. A related objective of this work was to characterise the function of the StcE protease in interactions with the epithelium and to establish a role for StcE in interactions with the bovine host.

3.2 Results

3.2.1 Analysis of bacterial modulation of the epithelial proinflammatory response

3.2.1.1 Modulation of interleukin-8 production by the T84 cell line by bacterial extracellular products

Bacterial supernatants from a wild type strain of a naturally occurring verotoxin-negative *E. coli* O157:H7 (MCI 0024) and an isogenic *fliC* mutant of this strain (MCI 0025) were prepared according to Section 2.3.4 and used to stimulate cultured epithelial cell line monolayers for 24 hours. Initial results, presented in Figure 3.1, suggest that in the absence of flagellin, bacterial supernatant (obtained at late-log growth phase) suppressed the production of IL-8 in T84 cells to levels below that induced by sterile medium. Preliminary data also suggest that supernatant from the *fliC* mutant could suppress TNFα-induced IL-8 production, when TNFα was included at 25ng/ml (Figure 3.2). At a concentration of 50ng/ml TNFα appeared to overcome the suppressive effect. This suggests that the effect is dose-dependent. However, in this instance, supernatant from MCI 0024 did not upregulate IL-8 production above the basal level.

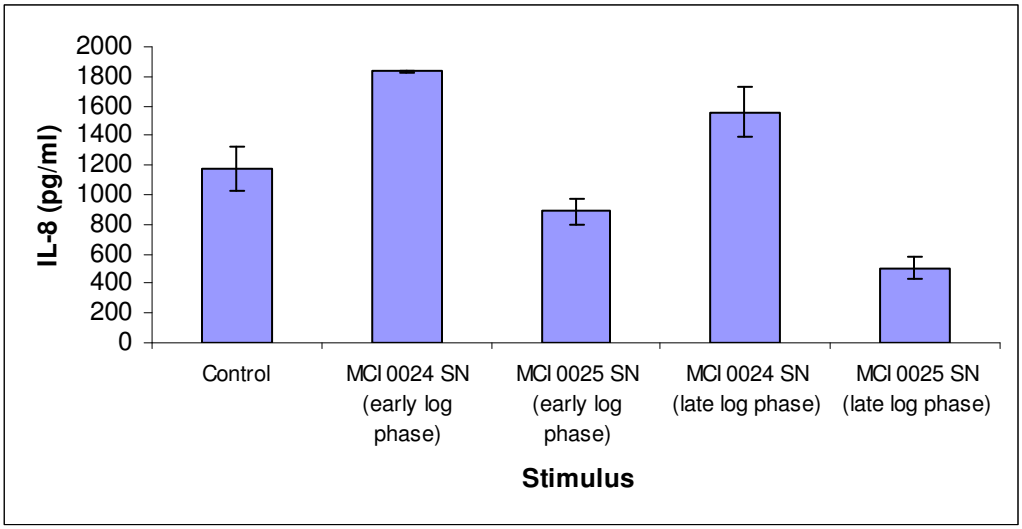


Figure 3.1: IL-8 response of T84 cells to bacterial supernatants: Preliminary data shows that in wells treated with bacterial supernatant from the FliC mutant, IL-8 production is below the level seen in those treated with sterile MEM-HEPES medium alone (n=3, P=0.025). IL-8 induction appears to be greater in response to early log phase culture supernatants, although this difference is not statistically significant.

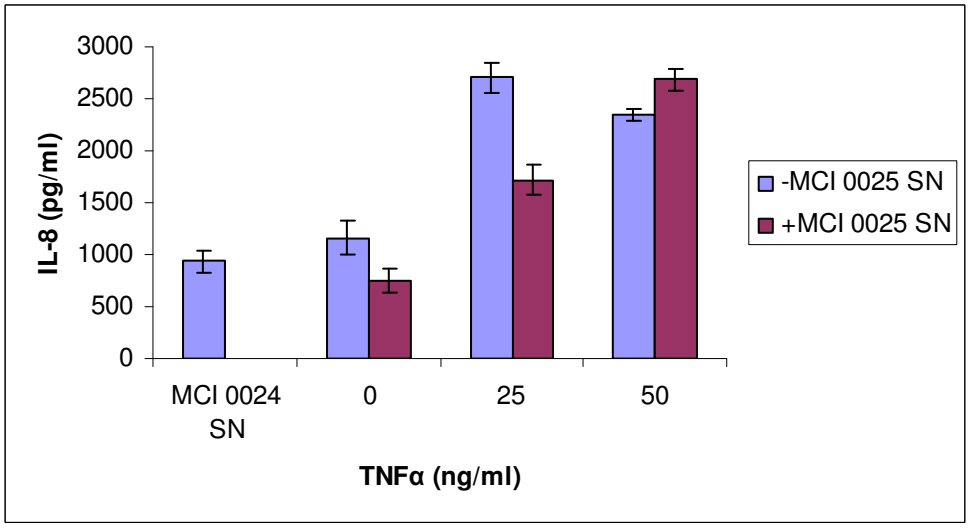
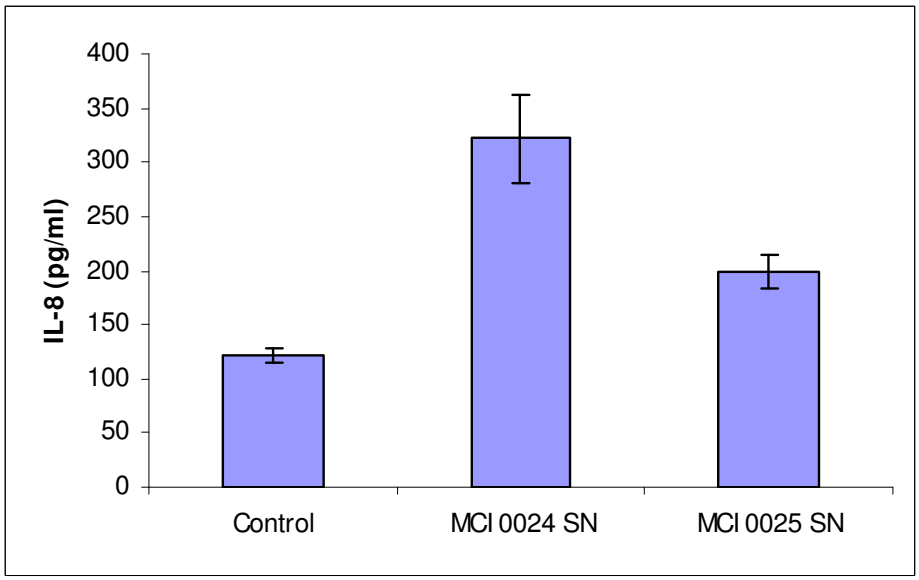


Figure 3.2: Effect of MCI 0025 supernatant on TNFα-induced IL-8 production: In the presence of supernatant from the FliC$^-$ bacterium, 25ng/ml TNFα was seen to induce IL-8 production to a significantly decreased level (n=3 P = 0.015). IL-8 levels in response to MCI 0024 were not significantly greater than the basal level or that induced by MCI 0025 supernatant.

Given the potential biological significance of the apparent suppressive effect indicated in Figures 3.1 and 3.2, the initial experiment was repeated using a greater number of replicates (n=6). Results are presented in Figure 3.3(A). In this instance, supernatant from MCI 0024 significantly upregulated IL-8 production compared with unstimulated cells (P=0.001). MCI0025 supernatant also caused significant upregulation of IL-8 production compared with unstimulated cells (P=0.001) but to a significantly reduced extent (P=0.016). Hence, the apparent suppressive effect shown in Figure 3.2 was not observed in further replication of the initial experiment. The results presented in Figure 3.3(B) show that MCI 0025 supernatant has no measurable effect on TNFα-induced IL-8 production, contrary to the initial result presented in Figure 3.3.

A



B

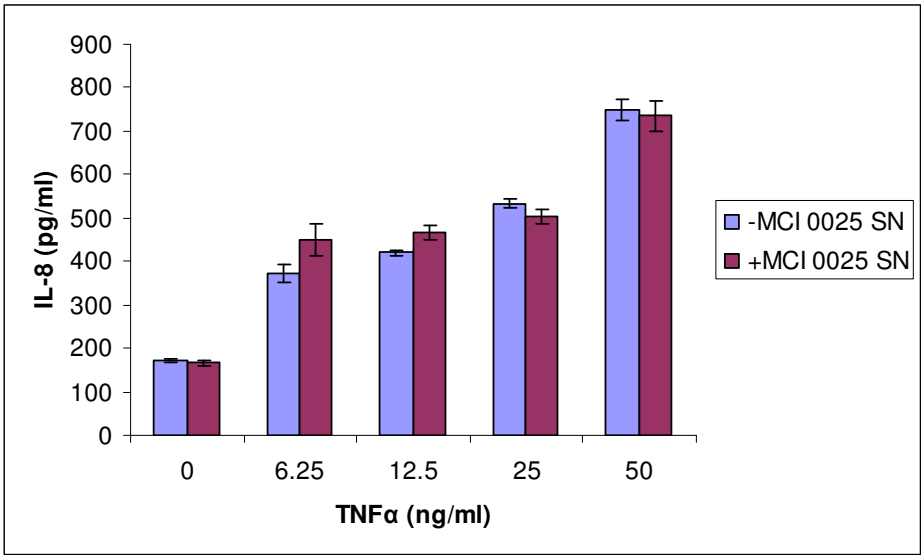


Figure 3.3: IL-8 response to bacterial supernatants: **(A)** Both MCI 0024 and MCI 0025 supernatant significantly increase IL-8 production above the basal level (n=6, P<0.05) **(B)** MCI 0025 supernatant did not appear to suppress TNFα-induced IL-8 production in this instance.

As the initial results apparently indicating a suppressive effect of bacterial supernatants on IL-8 production were not reproduced in subsequent experiments, possible technical reasons were examined for these potentially anomalous results. One possible reason for the observed suppression of IL-8 production is that the basal IL-8 levels of the control were misleadingly high. This could be a positional effect, as no effort was made to randomly distribute the replicates of different stimuli on the plate in these initial assays. To assess the potential positional effect, a negative control of sterile serum-free culture medium (DMEM:Hams F12, 2mm L-glutamine) was added to 4 corner wells and 2 centre wells on each of 6 plates and analysed by the IL-8 ELISA kit after 24 hours to assess edge-effects and plate-to-plate variation. IL-8 production by cells in corner wells was observed to be significantly greater than the IL-8 production by cells in wells at the centre of the plate (P=0.05; Figure 3.5). This compromises the validity of the initial results shown in Figures 3.1 and 3.2, as in earlier experiments it was assumed that basal IL-8 production does not vary significantly between regions of the tissue culture plate. Treatment and control wells were organised in a regular order in these initial experiments, with the control wells always placed in the first three wells of the plate (one corner well and two edge wells); whereas, in the experiments performed to generate data presented in Figure 3.3, a greater number of replicates were performed and treatments were positionally distributed about the tissue culture plate (according to the pattern displayed in Figure 3.4(B). This may have caused an unusually high apparent basal level of IL-8 production observed in Figures 3.1 and 3.2. This finding calls into question the validity of previous conclusions.

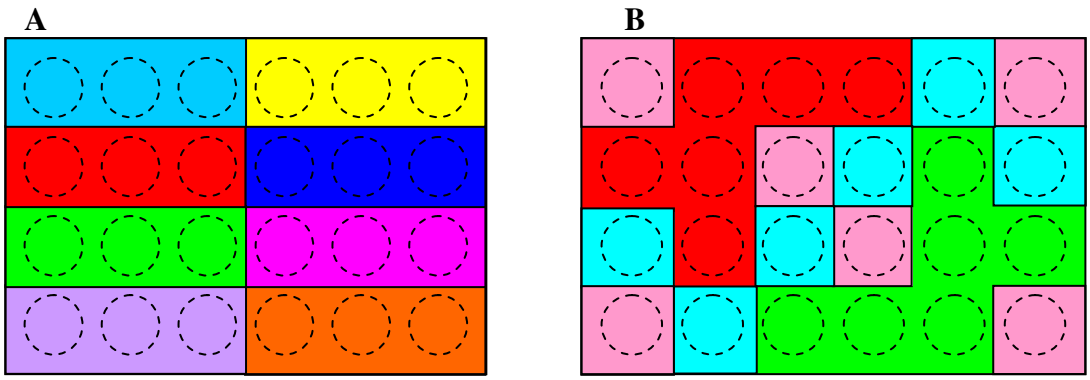


Figure 3.4: Setup method for IL-8 ELISA challenges: (A) In initial challenges, treatment groups were arranged in blocks of 3. Control wells were always the first three on the plate (blue). **(B)** In later challenges, treatment groups (n=6) were arranged in the pattern shown (blue=control, red=treatment #1, blue=treatment #2). The 4 corner wells and two of the centre wells (pink) were used to assess edge effects with the plate and were not exposed to stimuli.

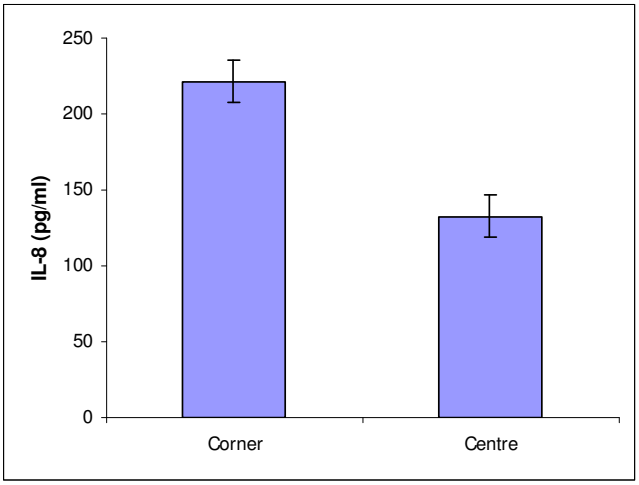


Figure 3.5: Edge-effect on IL-8 production/secretion: T84 cells in corner wells of 24-well tissue culture plates (n=24) were found to secrete significantly more IL-8 than centre wells (n=12, P=0.05).

3.2.1.2 Proinflammatory responses of EBL cells to EHEC and bovine commensal *E. coli*

The data presented in Section 3.2.1.1 provides further evidence of the role of flagellin in the initiation of a proinflammatory response. As yet unpublished data suggest that live *E. coli* O157:H7 *fliC*- (MCI 0025) induces IL-8 gene transcription to a lesser extent than the wild type (MCI 0024) and that IL-8 gene transcription is decreased in response to a bovine commensal strain, designated *E. coli* MCI 0105, compared with that initiated by *E. coli* O157:H7 in EBL cells (Paton N.; personal communication). In order to validate these findings using a proteomic technique, similar experiments were carried out using the human IL-8 ELISA Duoset (R&D Systems).

Confluent EBL-cell monolayers were challenged with live bacteria, including wild-type *E. coli* O157:H7 (MCI 0024), the *fliC*-mutant (MCI 0025) and a bovine faecal isolate (MCI 0105) at an approximate MOI of 100 bacteria per cell, according to the method detailed in Section 2.3.6. Culture medium was removed at each timepoint and analysed using the IL-8 ELISA kit, as set out in Section 2.5.2. None of the bacteria elicited a significant IL-8 response above the basal level. These data are not shown, as cells had totally lifted from the cell culture plate after 6 hours, suggesting that they had lost viability prior to producing detectable levels of IL-8. To protect cells from this effect an alternative protocol was adopted based on the gentamicin protection assay (Tang *et al.*, 1993). This method consists of a brief bacterial challenge, followed by removal and eradication of residual bacteria by the addition of gentamicin (detailed in Section 2.3.6).

The results presented in Figure 3.6 show that at a MOI of 100, MCI 0025 induced IL-8 production to the greatest extent in the 1 hour-challenge procedure. In the 1 hour-challenged procedures and the 3 hour-challenged/MOI=100 procedure, all bacteria upregulated IL-8 production above the control ($P<0.05$) except that the difference between the level induced by MCI 0024 (MOI=500/1h) and control was not significant, possibly due to the inherent variability displayed at the low absorbance values observed. No bacterium caused significant IL-8 upregulation compared with control in the 3 hour-challenged/MOI=500 procedure.

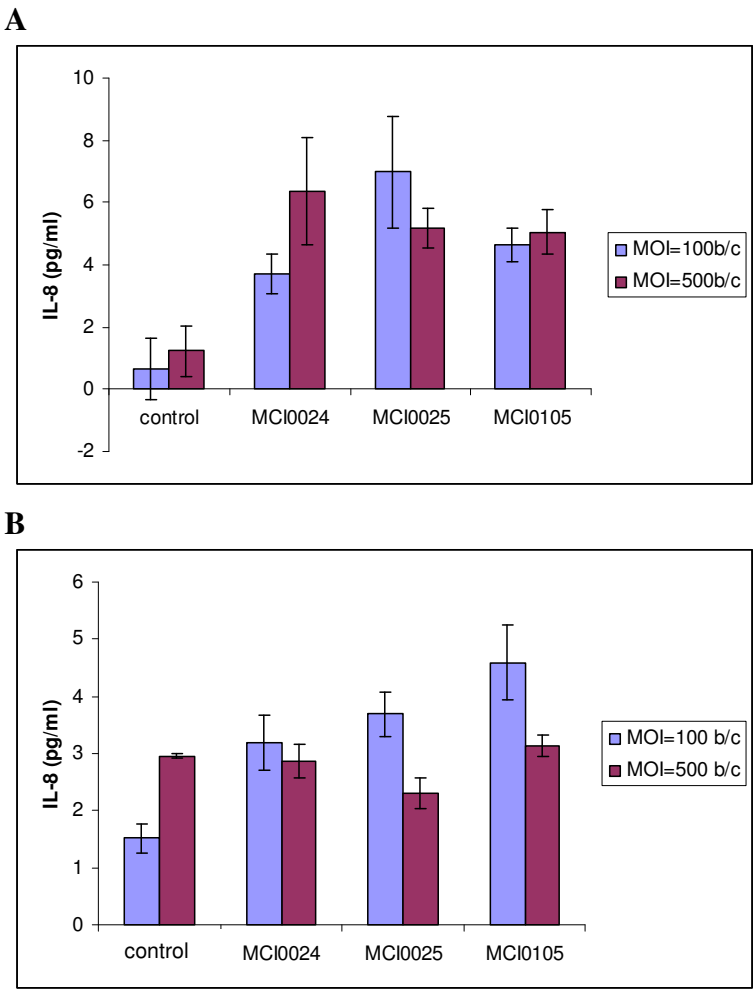


Figure 3.6: IL-8 responses of EBL cells to brief challenge with live bacteria followed by removal of the bacterial stimulus using gentamicin: (A) 1h bacterial challenge: IL-8 production appeared to be upregulated in response to all bacteria at both MOI tested, compared to control, except that the difference between MCI 0024 (MOI=500) and control was not significant (n=6 in each case) **(B) 3h bacterial challenge:** IL-8 production was significantly upregulated in response to each bacterium except MCI0024 compared to control after 3h pulse for the MOI of 100b/c (P<0.05) but no significant upregulation was observed at MOI of 500b/c (n=3 in each case).

To assess the effects of bacterial secreted factors on IL-8 production by EBL cells, 24 hour challenges using bacterial supernatants were performed. Supernatants were prepared at the late log growth phase in MEM-HEPES, as detailed in Section 2.3.4 and incubated with confluent EBL cell monolayers as set out in Section 2.3.5. Samples were analysed by IL-8 ELISA as described in Section 2.5.2. Data presented in Figure 3.7 suggest that supernatant from each bacterium significantly upregulated IL-8 production above the basal level ($P<0.05$ for each strain tested). Supernatant from the bovine commensal strain (MCI 0105) was observed to induce IL-8 production to a significantly lower level than supernatant from the *E. coli* O157:H7 strains. There was no significant difference in the level of IL-8 induced by supernatants from the wild type and *fliC* mutant *E. coli* O157 strains. This could suggest that *E. coli* O157:H7 possesses factors other than flagellin which are capable of initiating an IL-8 response in EBL cells, which may not be produced by *E. coli* MCI 0105. It is equally possible that MCI 0105 may produce factors which inhibit IL-8 production of EBL cells.

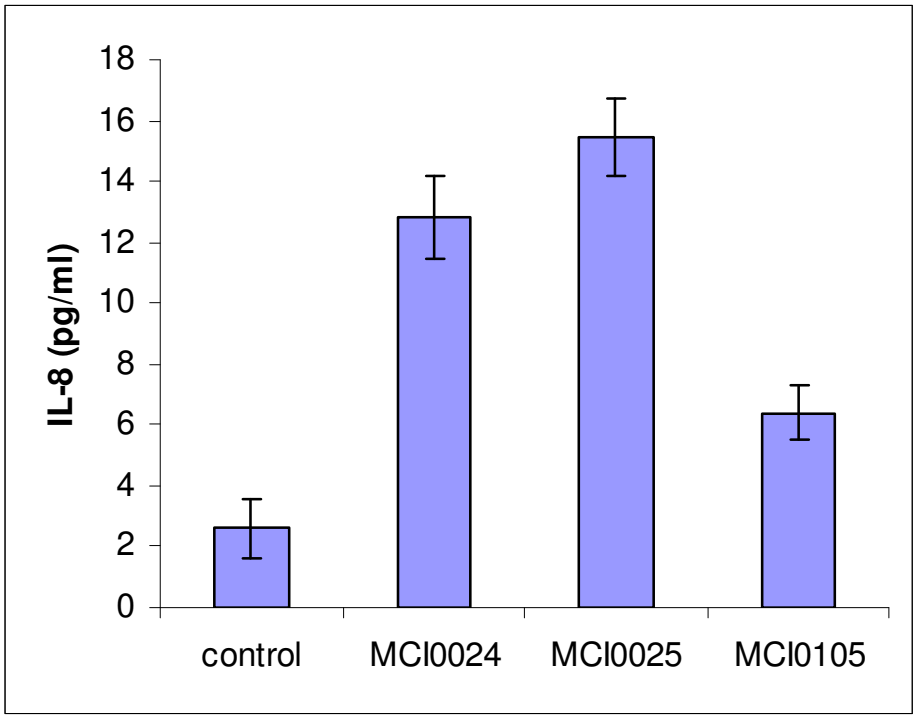


Figure 3.7: IL-8 responses of EBL cells to bacterial supernatants: IL-8 production was significantly upregulated in response to supernatants prepared from each bacterium. IL-8 production was upregulated to a significantly lower level by MCI 0105 than by MCI 0024 (P=0.002).

3.2.2 Functional analysis of the StcE protease

The aim of the following experiments was to confirm and further establish a role for StcE in interactions with the host, focussing on the intestinal epithelium and to determine whether StcE has a role in interactions with the bovine host. For all experiments, recombinant StcE (rStcE) provided by Rodney Welch (University of Madison, Wisconsin) was utilised.

3.2.2.1 Confirmation of activity of purified StcE protease

The activity of the purified rStcE was confirmed by digestion of its known substrate: C1-Esterase Inhibitor. The experiment was carried out as previously reported in Lathem *et al.* (2002) Briefly, 16µg purified human recombinant C1-INH (Europa Bioproducts: CP2041U) was incubated in 480µl AD buffer (20mM Tris, 100mM NaCl, 10% v/v Glycerol 0.01% v/v Tween-20 pH 7.5). To this, 4.8µg StcE or an equivalent volume of sterile PBS (11µl) was added. Aliquots (30µl) were removed at each timepoint and subjected to SDS-PAGE (Section 2.4.1). A Western blot was performed by the method set out in Section 2.5.1, using the anti-C1 Esterase Inhibitor primary antibody and the HRP-conjugated Rabbit anti-Goat IgG secondary antibody. As shown in Figure 3.8, levels of a degradation product (60-65kDa) were seen to increase over time. Further bands of lower molecular weight can be seen when exposure time is increased, which could suggest that StcE may cleave C1-INH at more than one site. These results confirm activity of the purified protease against its known substrate.

In an attempt to reproduce the effects of StcE on human salivary mucins and further confirm the activity of the purified protein, the procedure set out in Grys *et al.* (2005) was carried out. If the effects demonstrated by Grys *et al.* could be confirmed by this method it was envisaged that further analysis of the effects of StcE on bovine mucins would be assessed. A 100µl aliquot of human buccal saliva was collected from the author and incubated with 5µg rStcE in a total volume of 115µl for 4 hours at room temperature. A control sample, in which rStcE was replaced with an equal volume of sterile PBS (15µl) was prepared and treated identically. Samples (5-20µl) were subjected to SDS-PAGE and stained using first the Glycoprotein Staining Kit (Pierce) and subsequently with Colloidal Coomassie (as described in Section 2.4.4). No bands were visualised after glycoprotein staining (data not shown) suggesting either

that the saliva contained no salivary glycoproteins or that these glycoproteins were not mobilised under the electrophoresis conditions employed. The resultant image produced after Coomassie staining is presented in Figure 3.9. No clear differences were observed in the protein profiles between rStcE-treated and untreated saliva samples, except for 2 bands present in rStcE treated profiles. These were excised and both analysed by MALDI-TOF to determine if either of these bands may be degradation products of a larger salivary protein affected by StcE; however, results of MALDI-TOF analysis and subsequent Mascot database search suggest that both bands are fragments of the StcE protease itself and that the smaller of the two bands is StcE lacking the C-terminal domain, as no peptides covering this region were detected (Appendix I). This could suggest that StcE could undergo cleavage or be expressed in both full length and truncated forms. It is not yet determined whether the shorter form has similar activity to the full length StcE protease. A similar doublet of bands was noted by Lathem *et al.* (2002) in a Western blot image after probing *E. coli* O157:H7 supernatants with an anti-StcE antibody. The authors termed this band "non-specific" but presented no evidence to confirm this assumption.

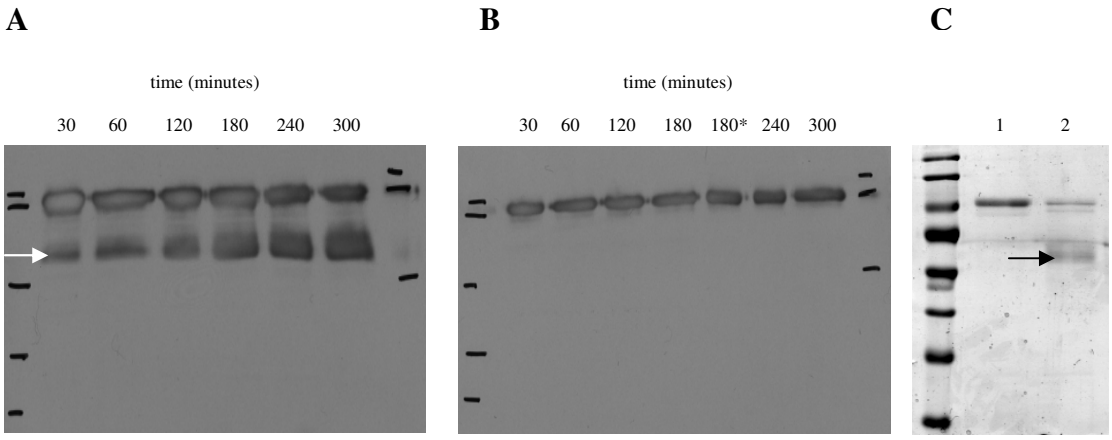


Figure 3.8: Confirmation of activity of rStcE: (A) C1-INH degradation is exhibited in the production of a 60 kDa band (denoted by a white arrow) increasing with time when incubated with rStcE. **(B)** A control confirms that rStcE is necessary for the pattern shown (* - the sample for 180 minutes was mistakenly loaded twice in this gel. **(C)** The effect is visualised on coomassie stained SDS-PAGE gel. A band at approximately 60 kDa is present in lane containing C1-INH treated with StcE for 300 minutes (lane 2) which is absent in the lane containing untreated C1-INH (lane 1).

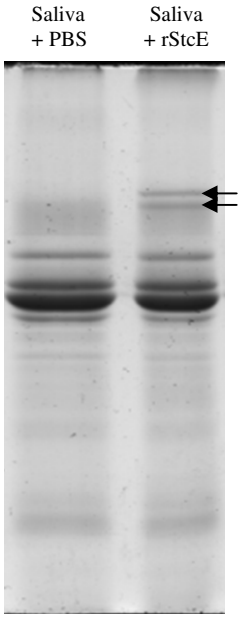


Figure 3.9: Treatment of saliva with rStcE: Lathem *et al.* (2002) presented data showing cleavage of salivary proteins by StcE. A similar experiment was conducted in order to attempt to reproduce this work and further confirm the activity of the recombinant StcE protein. No clear difference was observed in the protein profiles between StcE treated and untreated saliva samples except for 2 bands, denoted by the arrows, present in rStcE-treated profiles. These were excised and both confirmed by MALDI-TOF as the StcE protease (Appendix I).

3.2.2.2 Affinity of anti C1-INH antibody for rStcE

To determine if StcE affects bovine C1-INH, StcE was incubated with foetal bovine serum (FBS) which theoretically should contain the bovine C1-esterase inhibitor homologue. 2.5µg rStcE (or an equivalent volume of PBS) was incubated with 20µl FBS and made up to 127.5µl using AD buffer. The experiment was continued as described in Section 3.2.2. The resultant Western blot images (using the anti-C1 Esterase Inhibitor primary antibody HRP-conjugated Rabbit anti-Goat IgG secondary antibody) are shown in Figure 3.10. The success of this experiment was dependent upon the presence of bovine C1-INH in FBS and also that the antibody may have affinity for bovine C1-INH. In the untreated FBS, the anti C1-INH antibody appears to have bound to a protein band less than 60kDa, which is unlikely to be C1-INH. Bovine C1-INH primary sequence contains deletions totalling 32 amino acids (6.4% of the total length) when compared with human C1-INH. Therefore, bovine C1-INH in its intact glycosylated form (assuming that the glycosylation of bovine and human C1-INH is similar) is likely to have a molecular mass of approximately 93kDa. Presence of strong bands in the FBS incubated with rStcE suggests that the antibody binds to rStcE. The cross-specificity was confirmed in a separate blot in which rStcE alone was subjected to SDS-PAGE and Western blot, using the anti-C1 Esterase Inhibitor primary antibody HRP-conjugated Rabbit anti-Goat IgG secondary antibody. The resultant image is shown in Figure 3.10 (C).

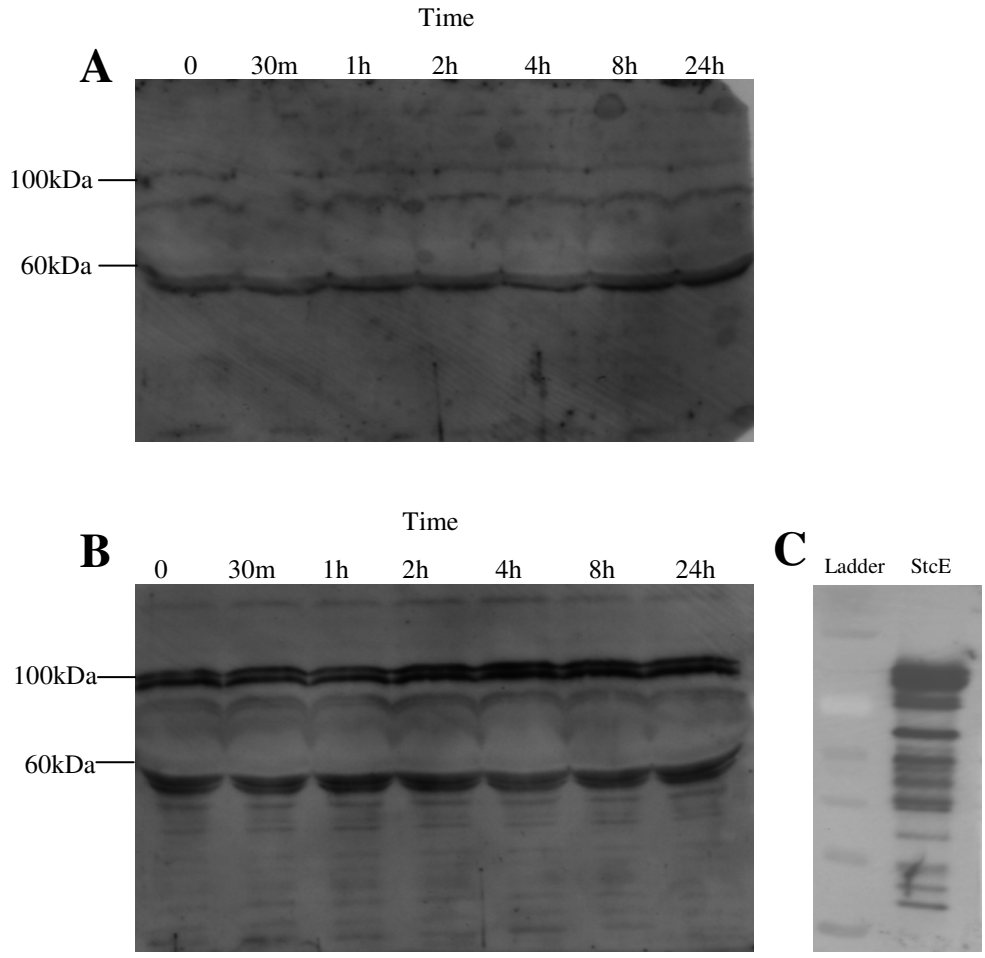


Figure 3.10: Anti C1-INH Western blots showing treatment of foetal bovine serum with rStcE:
(A) FBS treated with PBS alone: the strongest band visible is below 60kDa. **(B)** FBS treated with StcE: the band below 60 kDa remains unchanged. Two strong bands were visible at approximately 100kDa, close to the predicted size of rStcE (105kDa). Visible distortion of this gel is presumably due to the large amount of bovine serum albumin present in FBS. **(C)** A sample of rStcE alone subjected to SDS PAGE and Western blot probed with anti C1-INH antibody. The anti C1-INH antibody appears to bind to rStcE.

The apparent affinity of the anti-C1INH antibody with rStcE could be an artefact due to impurity of the primary antibody (whole goat antiserum). The anti-C1-INH antibody used by Lathem *et al.* (2002) was not available at the time that this study was conducted. It is possible that the antiserum could include anti-StcE antibodies as goats may be effectively colonised by EHEC (Heuvelink *et al.*, 2002). Another possibility is that C1-INH has sufficient structural similarity with StcE for the antibody to have affinity for both proteins. However, this is unlikely, as the two proteins share only 5% amino acid identity as determined by ClustalW alignment (Larkin *et al.*, 2007).

3.2.2.3 Bovine Rectal Mucosal Immune Response to StcE

To determine if StcE elicits a bovine mucosal immune response *in vivo*, a Western blot was carried out to establish the presence of IgA antibodies against StcE in mucosal material from a calf challenged with *E. coli* O157:H7. Samples of rStcE, H21 and H7 flagellin (1µg each) were subjected to SDS-PAGE in duplicate as described in Section 2.4.1. Western blotting was then carried out. Blots were probed using bovine mucosal material followed by anti bovine IgA-HRP secondary antibody, as described in Section 2.5.1. Mucosal material and flagellin samples were provided by Arvind Mahajan (University of Edinburgh).

Results suggest that anti-StcE and anti-H7 IgA antibodies were present in the mucosal sample, whereas anti-H21 antibodies were not detected. A parallel Coomassie stained SDS-PAGE gel confirms the presence of each protein in sufficiency. The presence of anti-StcE IgA antibodies suggests that StcE is expressed at the mucosal surface and recognised by the bovine host. Negative controls whereby a similar blot would be probed with mucosal scrapings from an unchallenged animal and the same blot probed only with the secondary antibody would aid the confirmation of this effect.

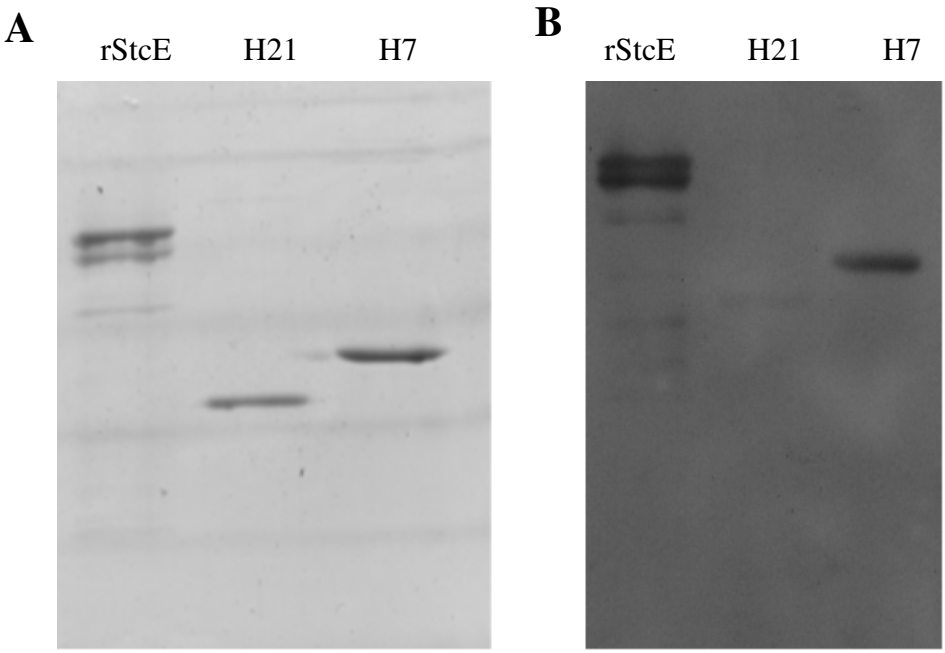


Figure 3.11: Rectal mucosal scrapings from *E. coli* O157:H7 (stx-)-challenged calf contains anti-StcE IgA: 1µg each of rStcE, H21 flagellin (negative control) and H7 flagellin (positive control) was subjected to SDS-PAGE **(A)** Colloidal Coomassie stained gel confirms that each of the analysed proteins is present in abundance. **(B)** Western blot probed with mucosal material and anti bovine IgA suggests that bovine anti-StcE IgA is present in the mucosal material. Anti H7-flagellin IgA but not anti-H21 flagellin IgA is detected.

3.2.2.4 Assessment of StcE activity against Caco-2 Whole Cells

Data presented in Grys *et al.* (2005) suggest that StcE affects the epithelial proteome. This observation is yet to be confirmed or characterised in detail, but could suggest that StcE affects proteins expressed by epithelial cells, either by proteolytic action or induction of a response from the cell, altering the protein expression profile. StcE is unlikely to activate the proinflammatory signalling pathway leading to a general stress response as treatment of epithelial cells with rStcE does not lead to degradation of IκBα or phosphorylation of p38 MAPK (Cameron P. unpublished observations) and also does not lead to activation of protease-activated receptors (PARs) (Plevin R. unpublished observations). The following experiment was conducted to attempt to characterise the effects of StcE on the epithelial proteome.

Three day confluent Caco-2 cells were treated with StcE protease according to Section 2.3.7 and 2D gel electrophoresis was carried out as detailed in Section 2.4.3. Microscopic examination of rStcE-treated cells revealed no obvious morphological alterations compared with untreated cells (not shown). The resulting gel images are presented in Figure 3.12. No gross differences between rStcE-challenged and control cell proteomes were observed. Although good separation of protein spots was achieved, consistent differences between proteomes of StcE-treated cells compared with untreated cells were not detected. Variation in spot intensities between the two replicates was considerable (observed using the Phoretix 2D Software) meaning that an impractical number of replicates of the procedure would be required to identify consistent differences.

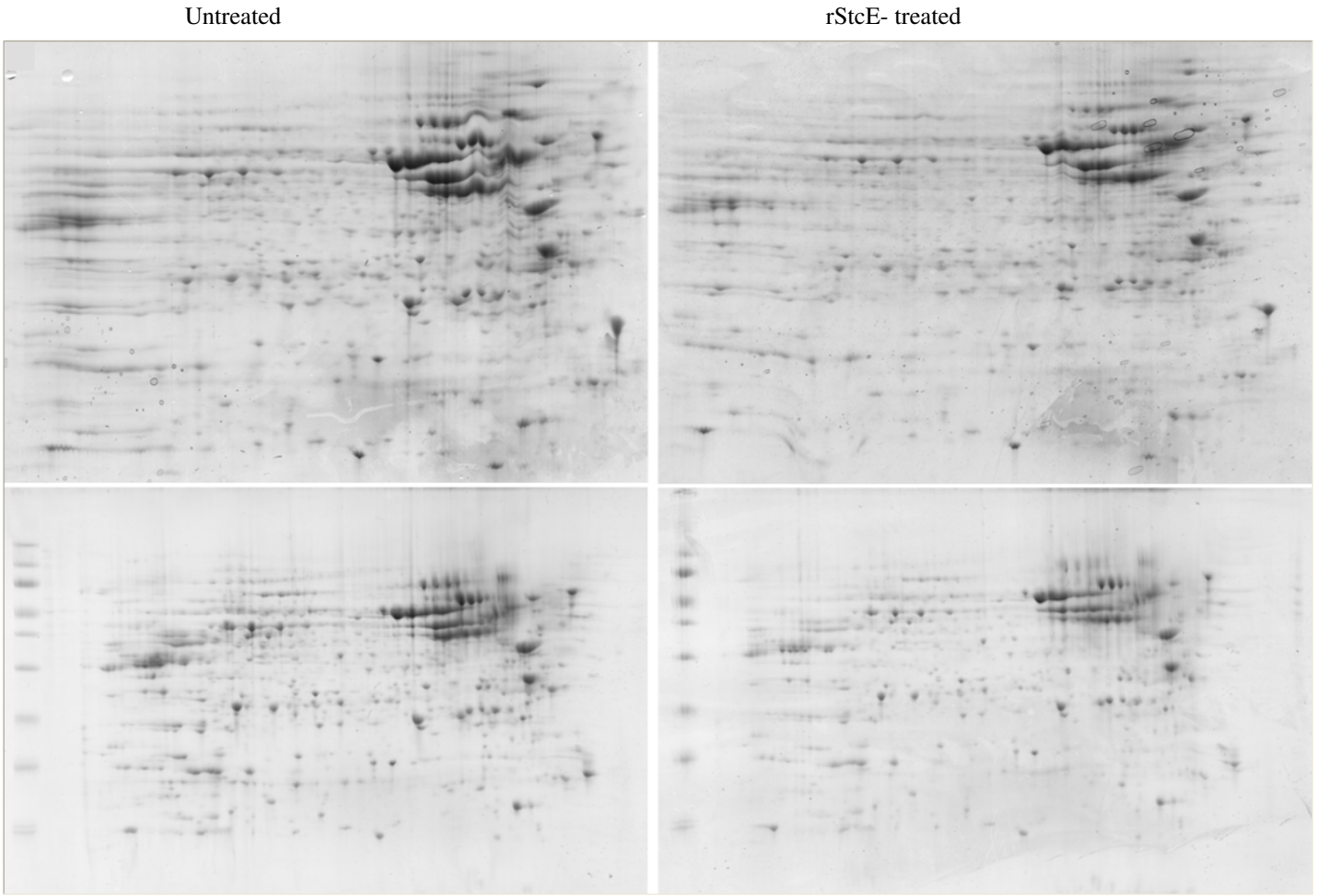


Figure 3.12: Analysis of effects of StcE the Caco2 cell proteome: Caco2-cells were treated with rStcE or an equivalent volume of sterile PBS. 2 replicates of rStcE treated (right) and untreated (left) Caco2 cell proteomes separated by 2D gel electrophoresis are shown. Inconsistencies in resultant gel patterns between replicates, such as those caused by horizontal streaking or poorly resolved spots meant that an impractical number of replicates would have been required to detect subtle differences.

3.3 Discussion

Data presented here provides further evidence that flagellin is the major proinflammatory stimulus among exported proteins of *E. coli* O157:H7 recognised by the human cultured epithelial T84 cell line. The inflammatory response of the epithelium to bacterial flagellin has been reported by others (Gewirtz *et al.*, 2001b;Berin *et al.*, 2002;Zhou *et al.*, 2003;Khan *et al.*, 2004;Miyamoto *et al.*, 2006).

Suppression of inflammatory cytokine production in response to flagellin-free bacterial supernatants was initially suspected. Suppression of proinflammatory signalling has been noted as a function of the T3SS of both EPEC and EHEC (Zhou *et al.*, 2003;Hauf and Chakraborty, 2003). On further investigation, the initial result was shown to be anomalous and it was shown that supernatant from *fliC* mutant actually upregulated IL-8 production in T84 cells to above the basal level. Exported proinflammatory stimuli other than flagellin have also been detected by others in EPEC supernatants, although the additional stimuli responsible for this effect have not been identified (Sharma *et al.*, 2005;Khan *et al.*, 2008).

Analysis of the effect of position in 24-well tissue culture plates on IL-8 secretion by T84 cells lead to the discovery that cell medium from corner wells of the plate contained significantly more IL-8 than those at the centre of the plate. This is an effect which was likely to be responsible for the early anomalous results and should be considered in any further experimental design where this cell culture system was used. The effect could be due to different levels of gaseous exchange at the two positions on the plate, resulting in different oxidative environments.

Evidence presented here does not suggest that the IL-8 response of EBL cells to bacteria and bacterial products depends on flagellin, contrary to the effects shown for the response of T84 human epithelial cell lines. This could suggest that there may be other, as yet unidentified, secreted factors capable of IL-8 induction in EBL cells present in bacterial supernatants.

Bacterial supernatants of a bovine commensal *E. coli* isolate (MCI 0105) induced IL-8 production to a lesser extent than *E. coli* O157:H7. Genomic analysis of this isolate, presented in Chapter 4, suggest that this isolate resembles *E. coli* K12, in that it lacks much of the

genomic content possessed by *E. coli* O157:H7. *E. coli* O157:H7 could produce additional factors capable of stimulating IL-8 production in EBL cells or the commensal strain could produce factors which may counteract the effect or both. There was no identifiable difference in the response of EBL cells to any of the live bacteria tested, including the bovine commensal isolate. This suggests that the IL-8 response of this epithelial cell line to live bacteria is independent of flagellin and is not a specific to EHEC strains; however, there may be a differential response to the extracellular products of EHEC versus commensal strains. The factor(s) responsible for this differential response remain to be elucidated.

It has not yet been determined whether EBL cells respond in a similar way to bacteria as intestinal epithelial cells. Human IECs are unresponsive to TLR2 and TLR4 ligands and only express TLR5 at the basolateral surface (described in detail in Section 1.5.2). It remains to be established whether bovine IECs share this pattern of TLR responsiveness. The presentation of PRRs by EBL cells has not yet been characterised; therefore, it cannot be determined whether EBL cells would be expected to respond in a similar manner to bacterial products as intestinal epithelial cells. Further analysis of the responses of bovine intestinal epithelial cells should be conducted using a system which is more likely to represent gastrointestinal epithelial cells, such as an immortalised intestinal epithelial cell line. The responses of the lung epithelium (generally an almost sterile environment) are likely to differ significantly from those of the intestinal epithelium, especially as *E. coli* is not generally tolerated in the airways of healthy animals and man and can cause pneumonia in susceptible hosts (reviewed in Russo & Johnson 2000). However, HeLa cells (derived from the cervix) and HEp-2 cells (derived from the larynx) have been heavily relied upon for the modelling of EHEC intestinal colonisation (Sperandio *et al.*, 2003;Grys *et al.*, 2005;Rendon *et al.*, 2007) and it was envisaged that EBL cells could provide an accessible model with which hypotheses may be generated for testing in more characteristic (but more complex to implement) model systems representing the bovine gastrointestinal epithelium more accurately. Primary bovine rectal epithelial cells are one such possibility for this purpose.

In every case, the levels of IL-8 induction in EBL cells were at the very minimum detectable by the human IL-8 ELISA kit, which highlights a major limitation in this analysis. The antibodies provided with the human kit are likely to have a decreased affinity for bovine IL-8 (human and bovine IL-8 share only 77% amino acid identity, as determined by ClustalW

alignment of the two sequences). No antibodies against bovine IL-8 are currently commercially available.

An objective of this analysis was to examine further the function of the StcE protease, especially its role in interaction with the epithelium and to determine if StcE has a role in interaction with the bovine host. Observations presented in this chapter suggest that cattle challenged with *E. coli* O157:H7 produce IgA antibodies against StcE. Although the results presented here do not resolve the function of StcE within the bovine host, the production of IgA antibodies to StcE suggest that StcE is expressed and recognised during colonisation. However, further confirmatory examination is required in order to verify this result (as set out in Section 3.2.2.3).

The activity of purified recombinant StcE was confirmed by digestion of its established substrate (human C1-INH). However, alterations to the proteomic profile of salivary proteins (as reported by Grys *et al.*, 2005) were not detected when visualisation was attempted using SDS-PAGE; however, visualisation of the digestion of mucin 7 and gp340 by immunoblotting was not performed. An attempt was made to determine whether StcE affects bovine C1-INH. The assay for this relied upon both the affinity of anti human C1-INH antibodies for bovine C1-INH and the presence of C1-INH in commercially available foetal bovine serum. This cross specificity was not observed but undesirable was affinity was observed between the C1-INH antibody and recombinant StcE, making the resultant Western blot image more difficult to interpret. If purified bovine C1-INH can be sourced the effect of StcE on bovine C1-INH may be established. Lathem *et al.* (2002) noted that the aggregative effects of StcE on human T-cells, attributed to the cleavage of C1-INH, occurred when either human or bovine serum was present, which suggests that StcE does indeed have the same effect of C1-INH. It remains to be established if StcE affects bovine T-cells in the same way.

An effort was made to determine whether StcE affects proteins expressed by a human epithelial cell line using 2D electrophoresis. This technique did not yield potential substrates, meaning that analysis by this method was aborted due to the variation in spot intensity and specific areas of poor resolution on the gels. New substrates may only be identified using this technique if StcE was to affect one of the more abundant proteins in the sample, which must also be within the resolvable molecular weight range of the technique (<150kDa). As proposed

StcE substrates described to date are in the high molecular weight range, it is unlikely that the method above will allow easy identification of any further StcE substrates.

In summary, data presented here reaffirms that flagellin elicits a significant proinflammatory cytokine response in T84 cells. The IL-8 response of EBL cells to bacteria and bacterial products does not appear to be flagellin-dependent. The role of StcE protease in the bovine host is not yet fully characterised. Observations presented here suggest a secretory antibody response at the mucosal surface, suggesting that the effects of this protein during colonisation of the bovine gastrointestinal tract are worthy of further investigation. Although no definitive conclusions may be drawn from this analysis, the data presented here support the multifactorial basis of *E. coli*-host interactions.

Chapter 4: Isolation and characterisation of *E. coli* from the bovine gastrointestinal tract

4.1 Introduction

As discussed in detail in Chapter 1, the prevalence of EHEC in ruminant hosts facilitates transmission of EHEC to humans (Borczyk *et al.*, 1987;Griffin and Tauxe, 1991;Gansheroff and O'Brien, 2000). *E. coli* O157:H7 has been found to exhibit preference for an area of lymphoid-follicle dense mucosal tissue, proximal to the recto-anal junction (RAJ) and is restricted to this area when the organism persists in the bovine host (Naylor *et al.*, 2003). The understanding of the factors involved in *E. coli* tissue tropism could lead to the realisation of interventions for control of pathogenic *E. coli* in ruminants.

E. coli O157:H7 also exhibits specific tissue tropism during the course of human infection. The bacterium has been observed to colocalise with Peyer's patches, which are lymphoid follicle-dense regions of the terminal ileum, as has been noted for other enteropathogenic bacteria (Jensen *et al.*, 1998;Phillips *et al.*, 2000;Vazquez-Torres and Fang, 2000;Chong *et al.*, 2007). It is not yet determined what role (if any) the lymphoid follicle-dense nature of bovine rectal mucosal tissue has on the enhanced colonisation at this site, although the cellular surface molecules presented at lymphoid follicle dense sites are different to those presented at non-lymphoid sites, especially due to the presence of large aggregations of cells involved in host immunity (described in Section 1.5.2). It is also possible that the physiochemical environment at the RAJ is optimal for bacterial growth and persistence. Aeration of the site could increase bacterial growth, as *E. coli* growth rate in culture is increased with greater aeration. Aeration has been shown to cause upregulation of adhesin genes in *E. coli* O157:H7 and *E. coli* O157:H- (James and Keevil, 1999;Musken *et al.*, 2008). Consequently, greater levels of oxygen at the RAJ may aid both proliferation and adherence of the bacterium.

The prevalence of *E. coli* of EHEC-related genotypes in ruminants has been investigated extensively. This has generally been by detection of EHEC-related genes, such as those encoding verotoxin, intimin and enterohaemolysin and detection of EHEC-associated serotypes among bovine isolates. Extensive studies using such methodology has been carried out in a number of geographical locations (Cobbold and Desmarchelier, 2000;Aktan *et al.*, 2004;Blanco *et al.*, 2004;Fukushima and Seki, 2004;Blanco *et al.*, 2005;Aidar-Ugrinovich *et al.*, 2007;Ishii *et al.*, 2007). These approaches selectively target EHEC-related organisms without comparing this to the *E. coli* population as a whole.

Sparse information is available on the specific gastrointestinal localisation of *E. coli* subtypes other than *E. coli* O157:H7. Colonisation of the ovine host by *E. coli* O26 was examined by experimental infection of weaned lambs (Aktan *et al.*, 2007). The organism was found to be most prevalent at sites in the upper GIT, including the ileum and persistence at these sites was observed up to 38 days post inoculation. An organism identified as *E. coli* ONT:H25, which encodes *eae* type β, *stx* type 1 and enterohaemolysin, was found to show preference for the bovine terminal rectum in healthy cattle (Sheng *et al.*, 2005). Thus, there is evidence that there may be strain-specific differences in colonisation patterns in ruminants; however, to date analysis has largely focussed on the tropism of EHEC-related organisms while knowledge is sparse regarding the distribution of other *E. coli* within the bovine GIT.

Several factors produced by EHEC have been linked to adherence to cells and hence have potential roles in colonisation and persistence in the bovine host. Bacterial outer membrane protein intimin has an established role in gastrointestinal colonisation of host animals, especially in concert with the translocated intimin receptor (Tir) (Tzipori *et al.*, 1995;Fitzhenry *et al.*, 2002;Cornick *et al.*, 2002;Vlisidou *et al.*, 2006;Sheng *et al.*, 2006b). Intimin-exchange studies suggest that intimin γ produced by *E. coli* O157:H7 defines both host specificity and tissue tropism of *E. coli* O157:H7 (Phillips and Frankel, 2000;Hartland *et al.*, 2000;Fitzhenry *et al.*, 2002). Given the established role of intimin as an adhesin, it is logical to predict that the subtypes of intimin may be involved in tissue tropism of intimin-encoding bacteria. It has been noted that *E. coli* O157:H7 forms A/E lesions at the bovine terminal rectum, dependent on the T3SS (Naylor *et al.*, 2005).

Besides intimin other bacterial molecules have been proposed to aid adherence or define the preference shown by *E. coli* O157:H7 for the bovine terminal rectum. Long polar fimbriae have been suggested to define *E. coli* O157:H7 colonisation of human ileal Peyer's patches and therefore could be important in defining localisation to lymphoid follicles (Fitzhenry *et al.*, 2006), although LPF have not yet been shown to be important for tissue tropism of EHEC in the bovine host. A study by Dziva *et al.* (2004), employing signature tagged mutagenesis, reaffirmed the role of LEE-encoded T3SS-effector proteins and revealed an array of additional factors influencing colonisation of experimentally infected calves including non-LEE-encoded putative T3SS effector proteins and putative fimbrial loci. A similar study revealed an

analogous group of factors aiding gastrointestinal colonisation by *E. coli* O26:H- (van Diemen *et al.*, 2005). Factors encoded on pO157 have also been implicated in bacterial colonisation of the bovine GIT (Sheng *et al.*, 2006b) *et al* 2006). The EspP protease has been established as a determinant of adherence to cultured bovine rectal epithelial cells (Dziva *et al.*, 2007). The action of verotoxin has also been proposed to aid gastrointestinal colonisation. Robinson *et al.* (2006) determined that verotoxin increases the surface expression of nucleolin by enterocytes in mice. As nucleolin is proposed to be a cellular receptor for bacterial intimin (Sinclair and O'Brien, 2002), it is by this mechanism that Stx is proposed to increase EHEC adherence to enterocytes. Although several candidate factors have been proposed, no single factor has been definitively linked to colonisation of the bovine terminal rectum above other sites in the bovine GIT.

The objectives of this line of investigation were two-fold: Firstly, to perform genomic characterisation of *E. coli* of the bovine GIT, with a view to prediction of diversity and potential pathogenicity of bovine resident *E. coli*. This was performed using molecular analysis by PCR to detect virulence, putative adhesin-encoding genes and to assign isolates to phylogenetic groups. To complement this approach comparative genome-indexing by microarray was also performed on selected isolates. The second major objective was to determine the colonisation patterns of resident *E. coli* of the bovine gastrointestinal tract, by sampling from selected sites and characterisation of strains recovered from different sites. Five gastrointestinal locations were selected for this purpose (Figure 4.12). Enumeration data was combined with molecular typing results in order to deduce links between specific genotypes and colonisation patterns. If colonisation patterns of bovine resident *E. coli* could be established, those which exhibit a similar colonisation pattern to *E. coli* O157:H7 may have applicability as probiotic agents (discussed further in Chapter 5) as they may be more adept at competition with *E. coli* O157:H7 at its preferred site of colonisation. A major goal of this analysis was to establish whether *E. coli* O157:H7 is unique in its specific tissue tropism or if other *E. coli* share this site-preference. This type of analysis would allow *E. coli* O157:H7 to be placed in context among *E. coli* of the bovine gastrointestinal tract and further elucidate the complex interactions between *E. coli* and the bovine host.

4.2 Results

4.2.1 Molecular analysis of bovine *E. coli* isolates

4.2.1.1 Bacterial strains

For each of the molecular techniques used in this study, reference strains of known genotype were utilised. These strains are summarised in Table 2.1 (Materials and Methods).

A prototype panel of bovine resident *E. coli* was provided by Neil Paton (MRI) and Stuart Naylor (SAC). These had been isolated by 3 methods, as set out in Section 2.3.2, namely: (i) faecal "free catch", (ii) recto-anal mucosal swab and (iii) PBS-washing of necropsy sections. Strains provided by N. Paton were originally isolated from 7 animals by method (i) and 12 different animals by method (ii), using CT-SMAC as a selective medium. Pooled colonies from TBX plates (Section 2.3.2) were provided by S. Naylor. They were prepared by method (iii) and were originally derived from 14 animals which had been orally challenged with *E. coli* O157:H7 (Δ*stx*, MCI 0010) but were not apparently effectively colonised by this organism (S. Naylor personal communication). There was a possibility that the challenge strain was out-competed by bovine intestinal bacteria and that among the *E. coli* isolates derived from these animals there may be a strain which is able to compete effectively with *E. coli* O157:H7 *in vivo*, that may have applicability as a probiotic agent (investigated further in Chapter 5), assuming that intra-*E. coli* competition was the reason for poor colonisation by the challenge strain in these animals. From each sweep, individual colonies were selected at random for analysis for molecular serotyping (Section 4.2.1.2). A full list of isolates is included in Appendix II.

Three isolates were selected from each animal for molecular H-typing except from animals 7 and 493 where only 2 and 1 isolates were provided respectively. Molecular O-typing was subsequently performed on all isolates, except for the isolate derived from animal 493 (MCI 0354), one colony from animal 740 (MCI 0369) and one colony from animal 583 (MCI 0379). In these cases, molecular H-type distinguished these isolates from others derived from that

animal (MCI 0369 and MCI 0379) and from all others among that group of animals (MCI 0369).

4.2.1.2 Molecular serotyping of bovine *E. coli* isolates

As highlighted in Section 1.2.1, *E. coli* may be categorised into over 180 different O-serotypes and more than 50 H-serotypes. Hence, there are more than 9000 combinations of O/H types which are theoretically possible. This means that serotyping is a powerful method for subspecific classification of *E. coli*. As serological methods of O and H antigen determination remain laborious and expensive, molecular methods were adopted to group *E. coli* isolates by serotype.

Molecular H-typing (H-RFLP) by the method of Fields *et al* (1997) and Ramos Moreno *et al.* (2006) (described in Section 2.6.4.1) was performed on each of the bovine resident *E. coli* isolates described in Section 4.2.1.1. Examples of H-RFLP and O-RFLP patterns are presented in Figures 4.1 and 4.2 respectively and results for all isolates analysed are given in Appendix III.

In the majority of cases, H-RFLP patterns were matched to expected patterns described in Ramos Moreno *et al.* (2006) or to theoretical patterns predicted by *in silico* digestion of all full length *E. coli fliC*-gene sequences available on the NCBI database, using the University of Alberta Sequence Manipulation Suite: http://www.ualberta.ca/~stothard/javascript/rest_digest.html. A table of predicted restriction fragment patterns is included in Appendix III.

From the 32 animals from which isolates were derived (representing 96 isolates in total) 14 different H-RFLP patterns were identified, with 3 amplified but unmatchable and 3 not amplified by the PCR, suggesting that these isolates were H- or carry a *fliC* gene of a variant type not amplifiable using the method described here. Patterns matching H35 and H21 were most prevalent, found in isolates from 10 animals each. A pattern matching H19NM was exhibited by isolates from 4 animals, H4 and H8 in 3 animals and H31 in 2 animals. All other patterns were observed in single animals.

Molecular O-typing (O-RFLP) was performed using the method of Coimbra *et al.* (2000). O-RFLP patterns obtained could not be related to the O-patterns set out in Coimbra *et al.* due to extensive variance in actual patterns compared with the schematic representation presented in that paper as illustrated in Figure 4.2 (B). Hence, it was not possible to derive a meaningful database of reference patterns from the information included in the paper. However, O-RFLP patterns could be matched to each other (where multiple representatives of one unknown O-pattern were obtained). It was also possible to assign a theoretical O-type to isolates which yielded an O-RFLP pattern which matched that of a reference isolate of serotype previously determined using conventional serological methods. Where isolates yielded an O-RFLP pattern which could not be matched to a known serotype, an arbitrary "Or" number was assigned (see Table 4.2) which should not be confused with the "R" patterns given in Coimbra *et al.* It was interesting to note that by this method, an isolate designated O5 by serological O-typing (MCI 0052) exhibited the same pattern as an isolate designated O113 (MCI 0247). The patterns more closely resemble that of O113 given in the paper. Confirmation by serotyping would be required to eliminate the possibility that the original serotype-designation was at fault.

From the 32 animals analysed, 28 distinct O-RFLP patterns were observed, with Or11 found in 4 animals; Or4, Or7, Or14, Or17 and a pattern matching that of serologically typed MCI 0319 (O146) each appeared in 3 animals. Or14 matched an isolate of serotype O45, while Or4, Or7 and Or17 matched to isolates which were untypeable by the serological method. All other types occurred in single animals only, with five isolates not yet defined (ND) the H-types of which suggested that they differed from other isolates from the same animals.

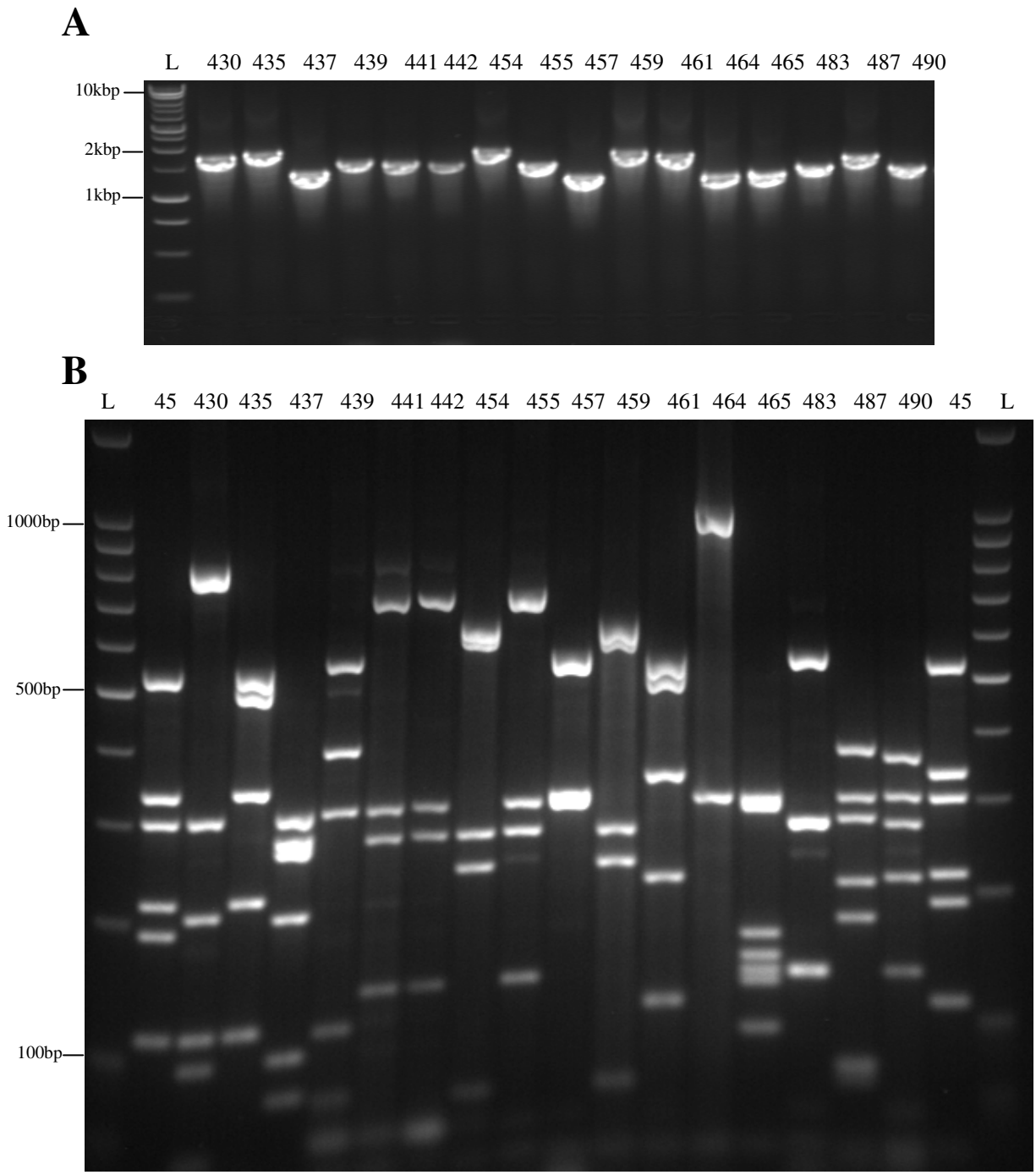


Figure 4.1: Example image showing H-RFLP analysis: (A) Uncut *fliC* gene amplicons were between 1000-2000bp in length. **(B)** H-RFLP patterns after *Rsa*I treatment. This image is an example showing a subset of the H-RFLP types observed. Numbers shown correspond to arbitrary MCI reference numbers for each strain (Tables 2.1, 4.2 and 4.5). MCI 0045 (*E. coli* O157:H7 EDL933) was included in each gel as a reference of known H-type (H7) H-RFLP profiles for all bovine isolates are presented in Appendix III.

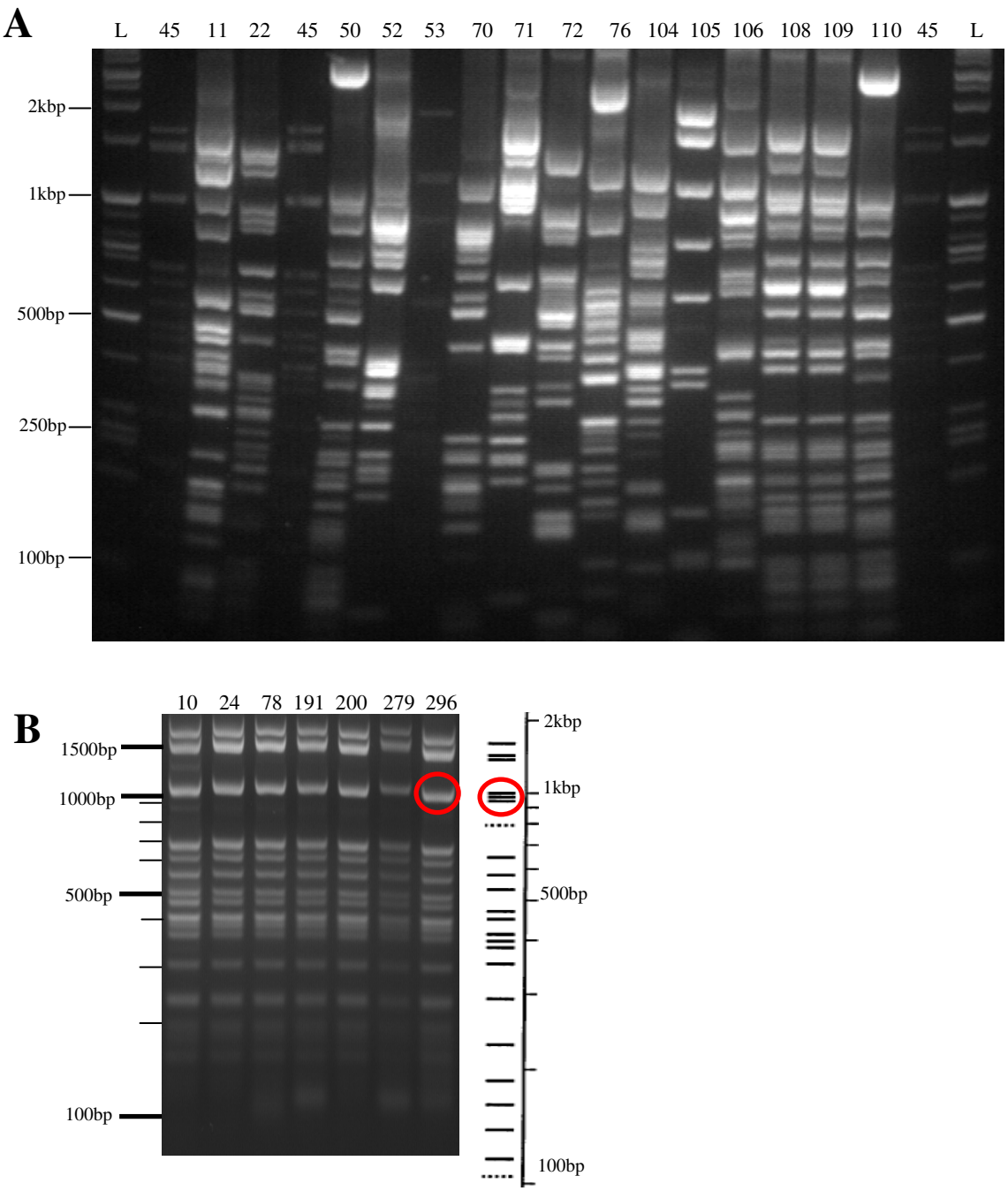


Figure 4.2: O-RFLP analysis: (A) Example of restriction patterns obtained from reference isolates and bovine isolates of unknown serotype. The bands for MCI 0045 (*E. coli* O157:H7) and MCI 0053 (*E. coli* O111:H21) appear fainter due to lower concentration of template DNA was lower than expected in this instance. Resultant images for all isolates are presented in Appendix III. **(B)** The image (left) shows the O-RFLP pattern obtained from 7 *E. coli* O157 isolates (MCI reference numbers correspond to those given in Table 2.1) compared with the expected pattern from Coimbra *et al.* (2000). The bands circled in red highlight the difficulties faced when attempting to match patterns to the published reference patterns.

4.2.1.3 PCR analysis of selected genes

From the isolates described in Section 4.2.1.1, one representative isolate of each O/H-RFLP pattern combination from each animal was further characterised by PCR-based screening for selected factors which were chosen on the basis that the detection of each would allow placement of the organism into a phylogenetic group or aid assignation of a predicted pathotype, allowing anticipation of the potential hazard to humans and animals posed by each strain. PCR primers were chosen based on published studies and are summarised in Table 2.2 (Materials & Methods).

Detection of the gene encoding intimin (*eae*) was performed using a method adapted from Aranda *et al.* (2004). This was performed in concert with detection of the *aat* gene, indicative of enteroaggregative adherence, present in 90% of human EAEC isolates (Schmidt *et al.*, 1995b) and the gene encoding bundlin (*bfpA*), the major structural subunit of the bundle forming pilus indicative of localised adherence exhibited by "typical" human EPEC (Donnenberg *et al.*, 1992). In optimising the assay, it was found that the *eae* primers would amplify the EPEC *eae* (α1) gene to a lesser extent than *eae* (γ) of EHEC, even though the primers matched identically with the primer sites given in the *E. coli* E2348/69 genome sequence (ftp://ftp.sanger.ac.uk/pub/pathogens/Escherichia_Shigella/E2348_array_chromo.embl). A possible reason for the faint band observed could be that there is some interference between the the eae primers and other primers or amplified products in the reaction, possibly due to the amplification of the *bfpA* gene which is also encoded by this EPEC strain. Nevertheless, the band for *eae* (α1) was visible and the assay was considered sufficient for the detection of these genes (see Figure 4.4). A triplex PCR, also adapted from Aranda *et al.* was used to detect genes encoding IpaH associated with the invasive mechanism of EIEC and *Shigella* spp. (Sethabutr *et al.*, 1993), along with *stx*1 and *stx*2 genes, encoding shiga-like/vero-toxins. The method of Lin *et al.* (1993) was adopted as a complementary approach for *stx*-gene detection, allowing further subtyping of *stx* genes, according to the method presented in Bastian *et al.* (1998). PCR-RFLP was employed to determine the subtype of intimin encoded using the method of Ramachandran *et al.* (2003).

Assays to detect genes encoding two key components of the T3SS; *escN* and *espA*, by the methods of Kyaw *et al.* (2003) and China *et al.* (1999) respectively, were assessed. The *escN* PCR assay was successfully employed to amplify the gene from a range of diverse T3SS encoding *E. coli* strains. The *espA* PCR failed to amplify the *espA* gene from EPEC (MCI 0278) and was therefore discarded.

Plasmid-borne EHEC-encoded putative virulence factor-encoding *ehxA* and *espP* genes were detected using previously published methods (McNally *et al.*, 2001;Cookson *et al.*, 2007). The method for *ehxA* gene detection included an RFLP-typing scheme. It must be noted that the *ehxA* restriction patterns could not be related to those presented in Cookson *et al.* (2007) possibly due to incomplete digestion of amplified fragments. However all *ehxA* positive *E. coli* isolates exhibited one of two patterns (designated "106*" and "319*", after MCI 0106 and MCI 0319 respectively) distinct from the pattern yielded by *E. coli* O157:H7 MCI 0045 (Figure 4.5). The method of Brunder *et al.* (1999) allowed *espP* typing to be performed. To date, 4 main groups of EspP protein have been determined (designated α, β, γ and δ). EspP of types α and γ are proteolytically active, while EspP of types β and δ have been found to be inactive against pepsin A (Brockmeyer *et al.*, 2007).

Genes encoding components of cytolethal distending toxin (*cdtB*), cell necrotising factor (*cnf*), F5 (K99), F17 (*gafD*) and F41 fimbriae were detected according to published methods (Blanco *et al.*, 1996;Bertin *et al.*, 1996b;Franck *et al.*, 1998;Janka *et al.*, 2003). Cytolethal distending toxin (CDT) causes cytopathic effects on human endothelial cells, by triggering cell cycle arrest (Comayras *et al.*, 1997). Functional CDT has been found to be produced by ExPEC of both the human and bovine host and diarrhoeagenic *E. coli* including sorbitol-fermenting *E. coli* O157:H- (Pickett *et al.*, 1994;Scott and Kaper, 1994;Peres *et al.*, 1997;Janka *et al.*, 2003;Toth *et al.*, 2003;Bielaszewska and Karch, 2005). Cell necrotising factors, CNF-1 and CNF-2 induce rearrangement of the actin cytoskeleton, possibly facilitating endocytosis of bacteria (De Rycke *et al.*, 1990;Oswald *et al.*, 1994). CNF, along with CDT, is produced by the majority of ExPEC strains (reviewed in De Rycke *et al.*, 1999). The role of CNF in enteric pathogenesis remains unclear as CNF has been found in *E. coli* isolated from both healthy and diarrhoeic cattle (Blanco *et al.*, 1998a;Blanco *et al.*, 1998b;Orden *et al.*, 1999). The elaboration of F17 fimbriae is a common feature of CNF-2 producing bacteria. F17 fimbriae producing *E. coli* have been related to bovine neonatal diarrhoea and septicaemia (Shimizu *et*

al., 1987;Lintermans *et al.*, 1988). K99 (F5) and F41 fimbriae have been related to ETEC which cause diarrhoea in cattle (Acres, 1985;Güler *et al.*, 2008).

Multiplex PCRs described by Nowrouzian *et al.* (2001) were also performed in order to detect genes encoding several adhesins (*papC*, *fimA*, *sfaD/E*, *draA* and *iutA*) and capsular antigen-related proteins (*neuB* and *kfiC*). The *papC* gene encodes the P-fimbrial usher protein, a chaperone which aids the formation of P-fimbriae (Dodson *et al.*, 1993). The *neuB* and *kfiC* genes are encoded by the K1 and K5 operons respectively. P-fimbriae as well as K1 and K5 capsular antigens have been identified in persistent colonists of the human intestine (Tullus *et al.*, 1992;Adlerberth *et al.*, 1998;Nowrouzian *et al.*, 2001). The *fimA* gene encodes the structural subunit of Type I fimbriae which are encoded by the majority of *E. coli* strains and are proposed to enhance adherence to a variety of tissues (Krogfelt *et al.*, 1990).

The *sfaD/E* genes (carried on the operon encoding S-fimbriae), the *draA* gene, which encodes the Dr haemagglutinin and *iutA*, which encodes the aerobactin receptor (which aids iron acquisition), are commonly found in ExPEC. Each of these factors has been put forward as enhancers of cellular adherence and have been identified as markers of persistence in the human intestine (Adlerberth *et al.*, 1995;Nowrouzian *et al.*, 2001). Neither their role in interactions of *E. coli* with the bovine host nor their carriage among bovine *E. coli* isolates have been examined in detail.

The triplex PCR described by Clermont *et al.* (2000) was used to assign isolates to the 4 the four most common phylogenetic groups (A, B1, B2 and D, although groups C and E also exist) of those determined by MLST and MLEE (see Section 1.2) based on the carriage of three genomic loci: *chuA*, *yjaA* and TSPE4.C2;. Virulent extra-intestinal strains are reported to belong mainly to group B2 and D (Bingen *et al.*, 1998;Picard *et al.*, 1999;Johnson and Stell, 2000). The majority of virulent EHEC belong to Group A or Group E although the majority of other STEC are members of group B1 (Escobar-Paramo *et al.*, 2004;Girardeau *et al.*, 2005). *E. coli* O157:H7 belongs to Group E, but would be identified as belonging to Group D by the method of Clermont *et al*.

PCR assays were verified before proceeding with the analysis, using at least one positive and negative control (listed in Table 2.1, Materials & Methods). Example images of the results of

PCR-characterisation are shown in Figures 4.3-4.5. Results for all isolates are summarised in Table 4.2 and images supporting these results are included in Appendix IV.

Of the 46 isolates analysed, 34 isolates were found to encode the *fimA* gene. 14 isolates were found to encode one or more verotoxin gene. Aerobactin receptor gene (*iutA*) was amplified from 16 isolates. 13 isolates were found to encode *gafD* and *cdtB* was also detected in 13 isolates, of which 12 also encoded *cnf*. Intimin and *escN* genes were detected in 6 isolates. The enterohaemolysin gene (*ehxA*) was detected in 7 isolates and 4 isolates were found to encode the *espP* gene. These data are included in a summary of *E. coli* genotypes, discussed further in Section 4.2.3.

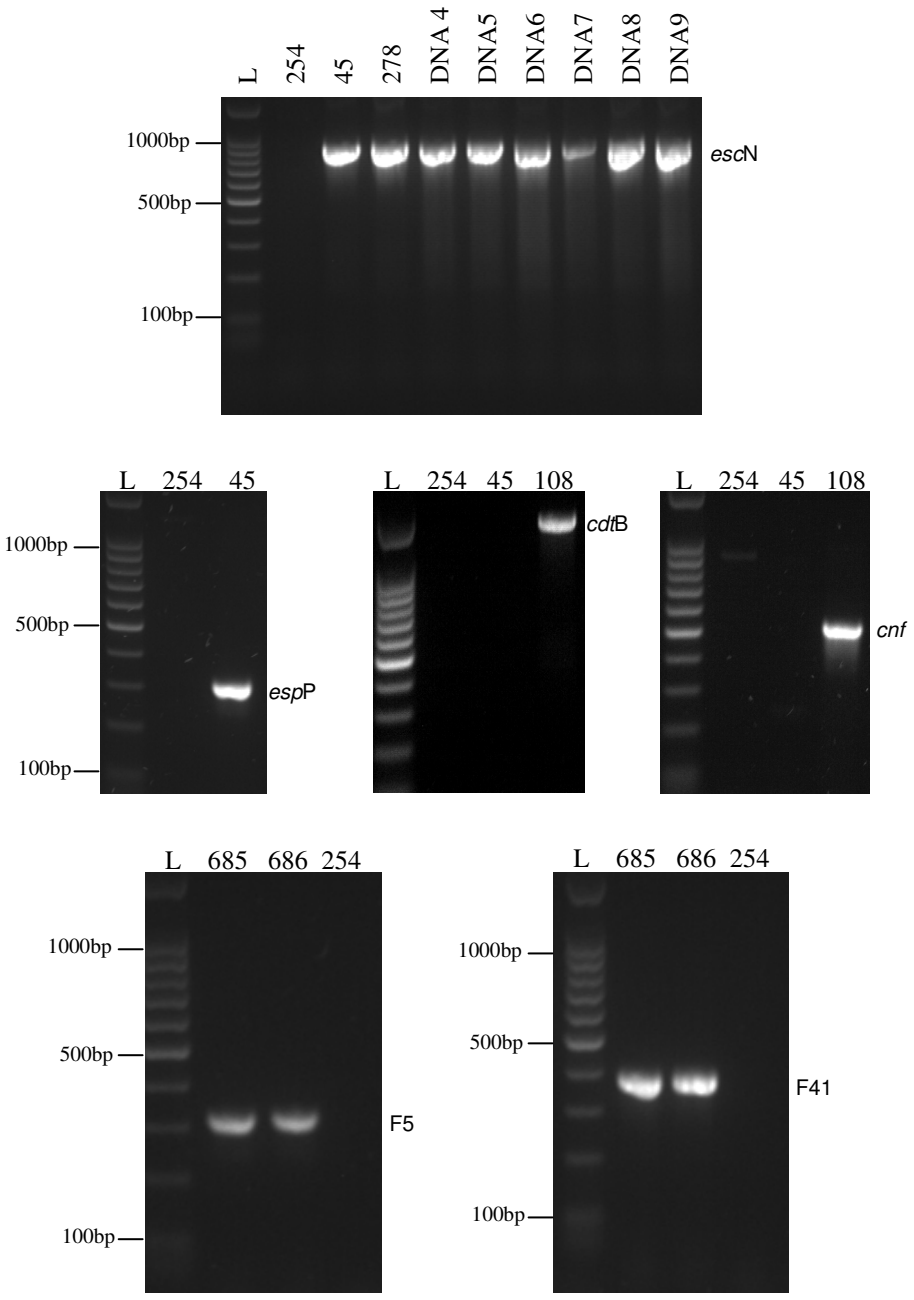


Figure 4.3: Verification of single-factor PCR assays: Reference strains of known genotype were used to assess the sensitivity and specificity of PCR-based gene detection assays. In all cases shown, amplification of single genes from reference isolates occurred as expected, while bands were absent from negative controls. No positive control was available for *gafD* prior to commencing this work. Numbers correspond to MCI reference numbers given in Table 2.1 (Materials & Methods). Results for all strains are provided in Appendix III.

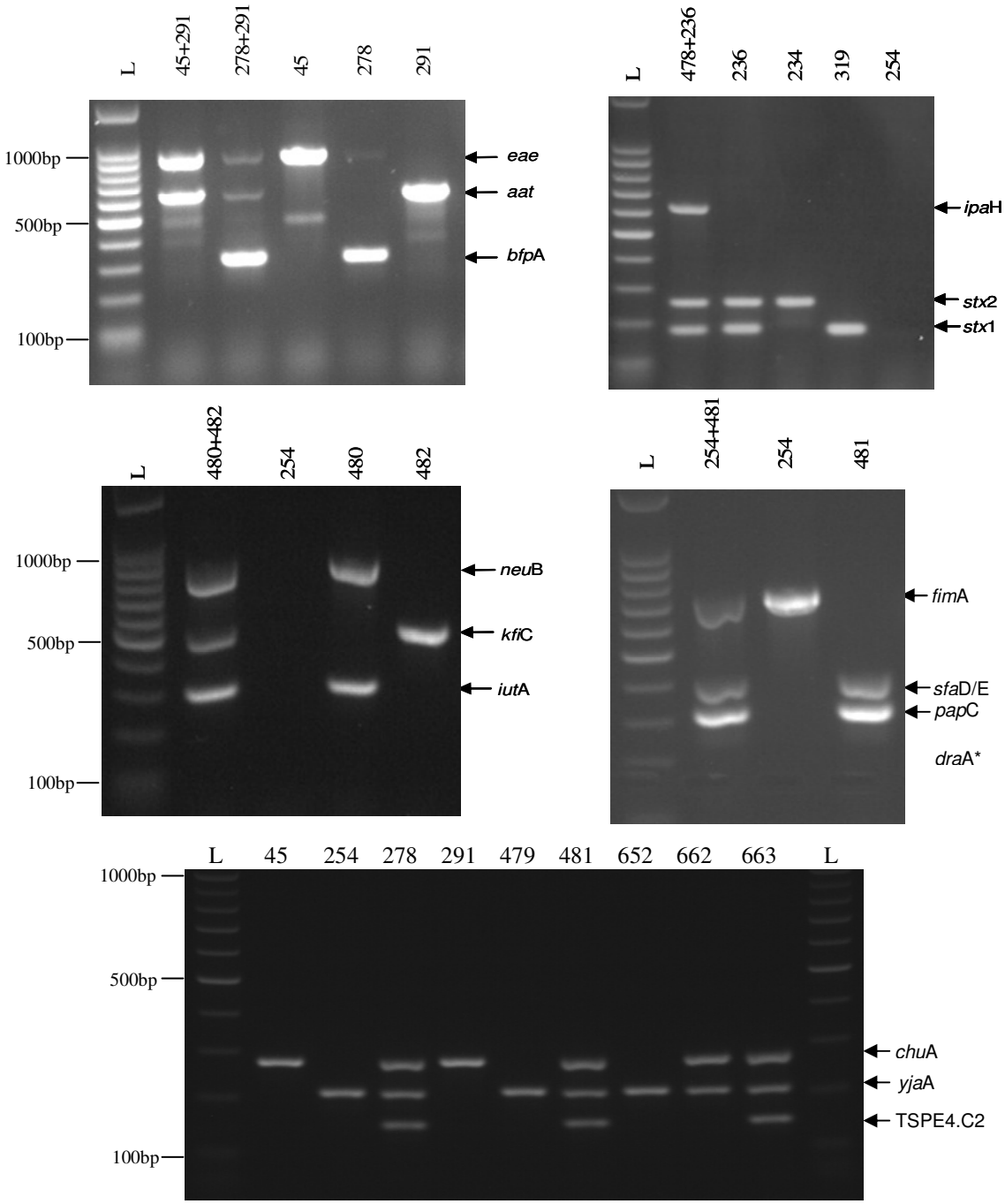
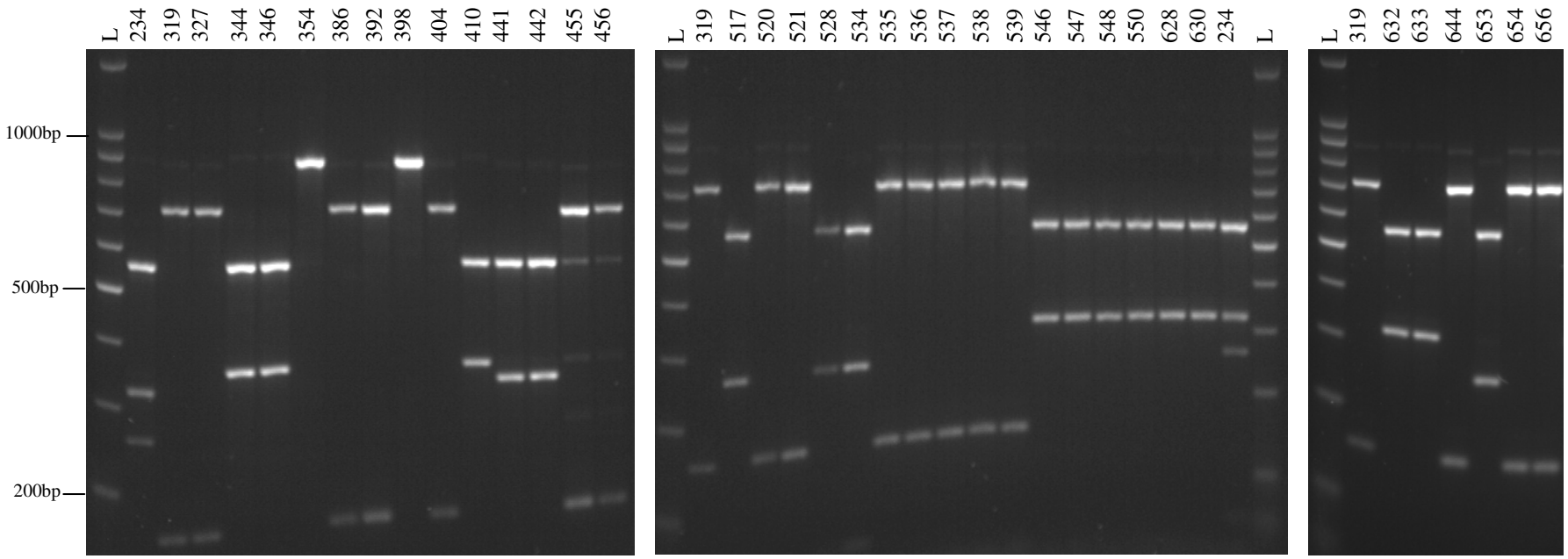


Figure 4.4: Verification of multiplex PCR assays: Multiplex PCR assays were carried out using template DNA from reference isolates with known genotype. In all cases, PCR amplification resulted in bands at the expected sizes, without non-specific amplification. No positive control for the *draA* haemagglutinin gene was available during this study. Images of PCR detection of genes from all bovine isolates are included in Appendix III. Numbers correspond to MCI reference numbers given in Table 2.1 (Materials & Methods).

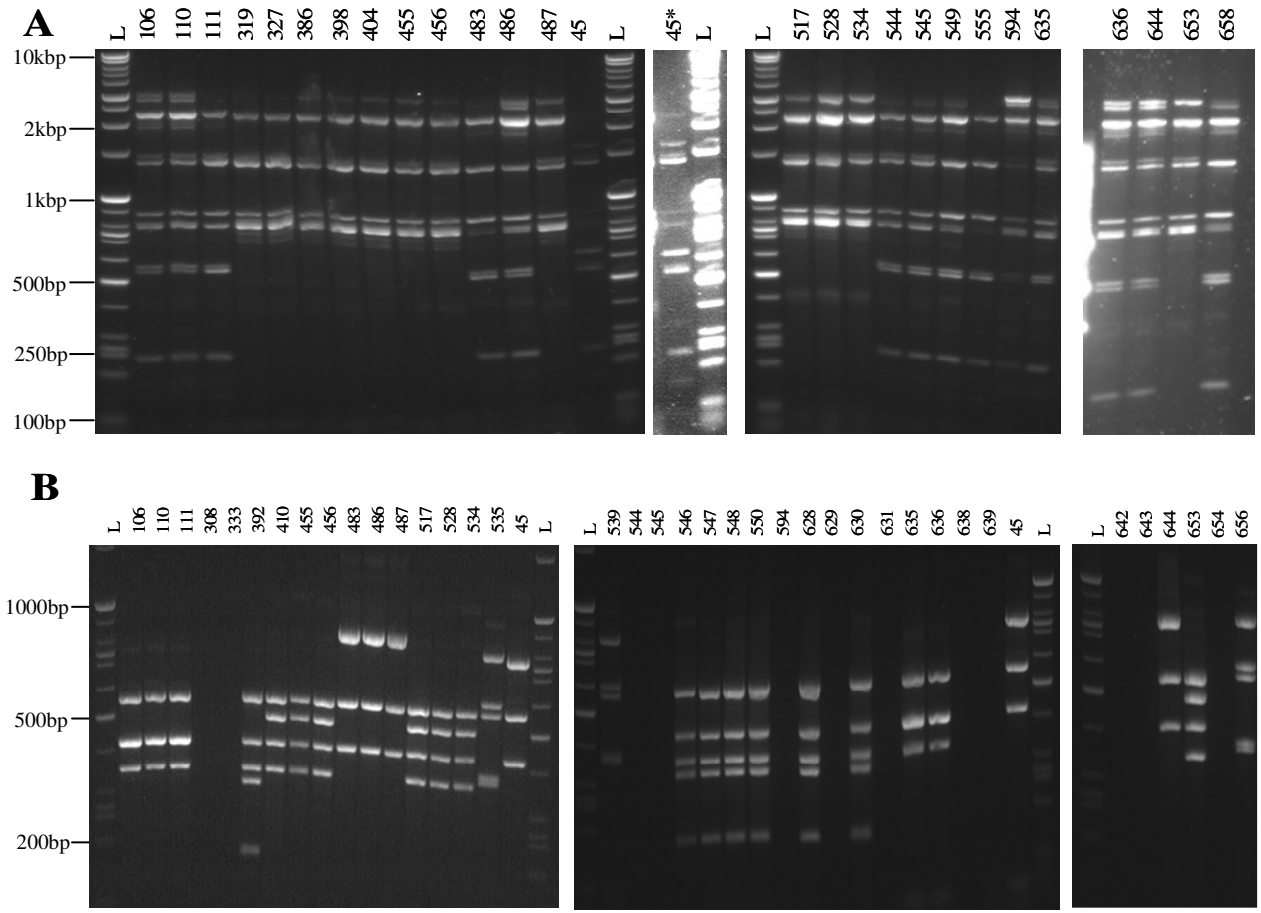
A



B

Types	Fragment sizes (bp)	Pattern
*stx*1	705, 158, 32	A
*stx*2	555, 262, 62	B
*stx*2$_c$/*stx*2$_d$	555, 324, 16	C
*stx*2$_e$	555,340	D
*stx*2$_{vO111}$/*stx*2$_{vOX392}$	880, 15	E
*stx*2$_e$/*stx*2$_f$	521,374	F
*stx*2$_g$	902 (uncut)	G

Figure 4.5: PCR-RFLP analysis of stx genes: (A) *Hinc*II-digested RFLP patterns obtained from *stx*-encoding bovine *E. coli* isolates. MCI 0319 (*stx*1) and MCI 0234 (*stx*2 and *stx*2$_{c/d}$) are included as reference patterns. Numbers correspond to MCI reference numbers given in Tables 4.2, 4.5 and Appendix II **(B)** Expected patterns, adapted from Bastian *et al.* (1998) and updated by *in silico* digestion using sequences available from the NCBI and the Sequence Manipulation Suite: http://www.ualberta.ca/~stothard/javascript/rest_summary.html



C

espP type	Expected fragment sizes (bp)	Associated serotypes
A (αO157)	773, 677, 420, 57, 21.	O157:H7
B (αO26)	677, 420, 422, 351, 48, 30	O26:H11/ H-, O145:H-, O111:H-.
C (β)	677, 498, 422, 351	O22:H8, O69:H-, Orough:H11.
D (γO77/δ)	849, 677, 422	O55:H-, O52:H19, O8:H19.
E (γO128)	819, 677, 422, 30	O128:H8.
F	1778, 170	ONT:H14.
G	677, 420, 360, 350, 220	O98:H8.
αO157 #2	422,420, 400, 351, 48,30	O157:H7.
vO113	887, 516, 400, 145	O113:H21.

Figure 4.6: PCR-RFLP analysis of plasmid-encoded EHEC virulence factors: (A) *Taq*I restriction digestion of *ehxA* amplicons yielded two distinct patterns, designated "106" and "319". MCI 0045 was included as a reference pattern, but gave a fainter pattern. The central panel shows the MCI 0045 pattern more clearly with increased exposure **(B)** *Alu*I restriction digestion of *espP* amplicons yielded patterns which were matched to those given by Brunder *et al.* (1999). Numbers correspond to MCI reference numbers given in Tables 4.2, 4.5 and Appendix II. **(C)** Expected RFLP patterns of *espP* subtypes, along with the associated serotypes given by Brunder *et al.* (1999) and formulated by *in silico* restriction analysis using the sequence manipulation suite: (http://www.ualberta.ca/~stothard/javascript/rest_digest.html).

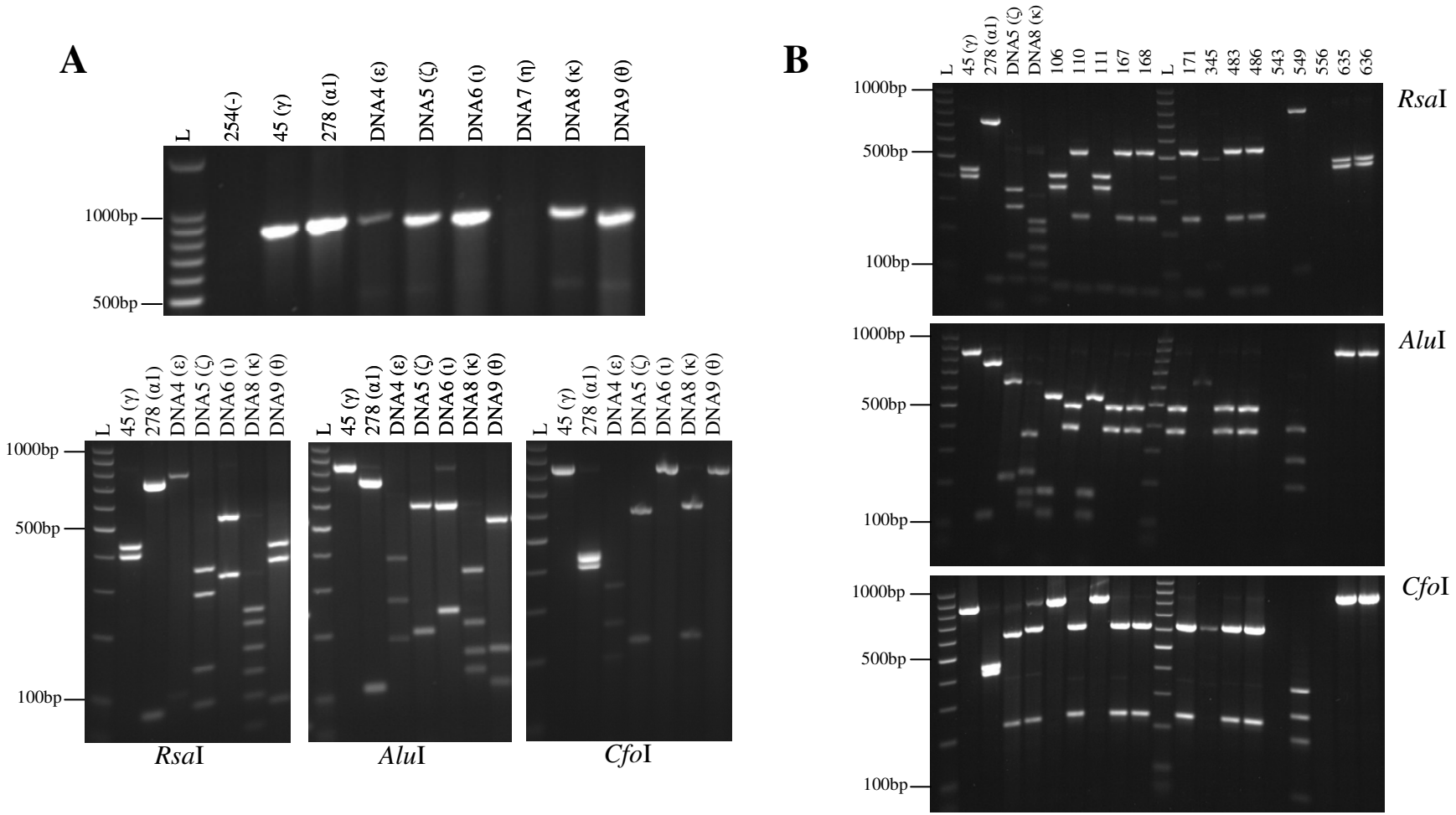


Figure 4.7: PCR-RFLP analysis of intimin-encoding genes: (A) Verification of *eae*-RFLP protocol: The primers designed by Ramachandran *et al* (2003) amplified gene fragments from all of the reference types available apart from *eae* type η. All yielded expected patterns, as described by Ramachandran *et al.* (2003) **(B)** Analysis of *eae* genes carried by bovine *E. coli* isolates. Deduced *eae* types are included in Tables 4.2 and 4.5.

Ref. No.	Animal	Isolation method	O-type/O-RFLP type	H-RFLP-type	Lineage	*ipaH*	*stx*	*espP*	*escN*	*eae*	*cdtB*	*cnf*	*fimA*	*papC*	*iutA*	*ehxA*	*gafD*
MCI 0104	7	FFC	Or1	H35	D	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0105	9	FFC	ONT (Or2)	H34	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0106	10	FFC	ONT (Or3)	H-/NT	A	-	-	B	+	θ	-	-	+	-	-	106*	-
MCI 0108	8	FFC	ONT (Or4)	H4	B1	-	-	-	-	-	+	+	-	-	+	-	+
MCI 0109	11	FFC	ONT (Or4)	H4	B1	-	-	-	-	-	+	+	-	-	+	-	+
MCI 0110	14	FFC	O26	H11	B1	-	A	B	+	β	-	-	+	-	+	106*	-
MCI 0114	11	FFC	Or5	H4	B1	-	-	-	-	-	+	+	-	-	+	-	+
MCI 0120	13	FFC	Or6	H35	B1	+/-	-	-	-	-	-	-	+	-	-	-	-
MCI 0121	9	FFC	ONT (Or7)	H+(NT)8	D	-	-	-	-	-	+	-	+	-	-	-	-
MCI 0122	7	FFC	O116	H25	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0160	100742	RS	Or9	H31	B1	-	-	-	-	-	+	+	+	+	+	-	+
MCI 0162	400211	RS	O43 (Or10)	H35	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0164	500348	RS	Or11	H14	B1	-	-	-	-	-	+	+	+	-	+	-	+
MCI 0165	400396	RS	Or11	H-/NT	B2	-	-	-	-	-	+	+	-	-	+	-	+
MCI 0167	400385	RS	Or12	H35	B1	-	-	-	+	β	-	-	+	-	-	-	-
MCI 0168	400211	RS	Or10	H35	B1	-	-	-	+	β	-	-	+	-	-	-	-
MCI 0169	500340	RS	Or13	H35	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0170	300741	RS	Or11	H21	B1	-	-	-	-	-	-	-	+	-	-	-	+
MCI 0171	300741	RS	ONT (Or4)	H35	B1	-	-	-	+	β	-	-	+	-	-	-	-
MCI 0172	400376	RS	Or11	H-/NT	B2	-	-	-	-	-	+	+	-	-	+	-	+
MCI 0173	500340	RS	O45 (Or14)	H35	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0174	600341	RS	ONT (Or7)	H21	B1	-	-	-	-	-	+	+	+	-	+	-	+
MCI 0176	300395	RS	O45 (Or14)	H35	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0177	600341	RS	ONT (Or7)	H21	B1	-	-	-	-	-	+	+	+	-	+	-	+
MCI 0179	600747	RS	Or15	H19NM	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0181	600747	RS	O45 (Or14)	H30	A	-	-	-	-	-	-	-	-	+	-	-	-
MCI 0182	300744	RS	Or16	H21	B1	-	-	-	-	-	-	-	+	-	-	-	-

Ref. No.	Animal	Isolation method	O-type/O-RFLP type	H-RFLP-type	Lineage	*ipaH*	*stx*	*espP*	*escN*	*eae*	*cdtB*	*cnf*	*fimA*	*papC*	*iutA*	*ehxA*	*gafD*
MCI 0308	824	TS	ONT (Or17)	H8	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0311	540	TS	Or18	H-/NT	A	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0319	540	TS	O146	H21	B1	-	A	-	-	-	-	-	+	-	+	319*	-
MCI 0327	593	TS	O146	H21	B1	-	A	-	-	-	-	-	+	-	+	319*	-
MCI 0344	541	TS	Or19	H32	A	-	D	-	-	-	-	-	+	-	-	-	-
MCI 0345	541	TS	Or18	H35	A	-	-	-	+	+(NT[a])	-	-	-	-	-	-	-
MCI 0354	493	TS	ND	H29	B1	-	G	-	-	-	-	-	-	-	-	-	-
MCI 0356	710	TS	O45 (Or14)	H21	B1	-	A+D	-	-	-	+	+	-	-	+	-	+
MCI 0362	739	TS	Or20	H21	B1	-	-	-	-	-	+	+	-	-	+	-	+
MCI 0368	740	TS	Or21	H48	B1	-	A	-	-	-	-	-	+	-	-	-	-
MCI 0369	740	TS	ND	H31	D	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0373	824	TS	Or22	H8	B1	-	D	-	-	-	-	-	+	-	-	-	-
MCI 0377	583	TS	ONT (Or17)	H-/NT	B1	-	-	-	-	-	-	-	+	-	-	-	-
MCI 0378	583	TS	Or23	H21	B1	-	D	-	-	-	+	+	-	-	+	-	+
MCI 0386	587	TS	O146	H21	B1	-	A	-	-	-	-	-	+	-	+	319*	-
MCI 0392	566	TS	Or24	H21	B1	-	A	G	-	-	-	-	-	-	-	-	-
MCI 0398	586	TS	Or25	H19NM	B1	-	G	-	-	-	-	-	+	-	-	319*	-
MCI 0404	825	TS	Or26	H19NM	B1	-	A	-	-	-	-	-	+	-	-	319*	-
MCI 0410	826	TS	Or27	H19NM	B1	-	D	C	-	-	-	-	+	-	-	-	-

Table 4.2: Results of molecular typing of *E. coli* isolates: Isolates which yielded a band of similar molecular weight and intensity to the reference strains are noted with a '+'. Those yielding a band of lower intensity, suggesting either artifactual non-specific amplification or polymorphisms in the primer-binding site are denoted '+/-'. RFLP-patterns are included for *eae*, *stx*, *espP* and *ehxA*. No isolates were found to carry genes encoding *aat*, *bfpA*, F5, F41, *sfaD/E*, *kfiC*, *neuB* or *draA* and hence, these genes are omitted from the table.

Notes: "Lineage"= Phylogenetic group determined by method of Clermont *et al.* (2000)..ND=not determined, NT=Untypeable. Isolation method: FFC: faecal free-catch, RS: recto-anal mucosal swab TS: tissue section from terminal rectum.

For *stx*, *espP*, *eae* and *ehxA*, RFLP patterns are also given. For *ehxA*: type 106* and 319* were assigned to isolates which exhibited *ehx*-RFLP patterns indistinguishable from MCI 0106 and MCI 0319 respectively.

a: an amplicon of the expected size was produced by the method of Aranda *et al.* (2004) but not by the method of Ramachandran *et al.* (2003). Therefore this isolate may be intimin positive, but it is untypeable by the methods described here.

4.2.1.4 Extended Genotyping of *E. coli* isolates using microarrays

To obtain extended genotypic information and relate this to PCR-based characterisation, five bovine commensal *E. coli* were selected (based on their pathotype, as predicted by virulence factor PCR) for extended spectrum virulence gene detection using a miniaturised DNA microarray (ArrayTube system, Clondiag) designed and tested by Anjum *et al.* (2007) (described in Section 2.6.7). Genes that were detected using the array are summarised in Table 4.3. The full list of oligonucleotides included in the ArrayTube is given in Appendix V. For each of the genes represented in both the nanoarray and the PCR-screen, the results of gene-detection by these methods were in agreement.

Isolate	**Factors detected by PCR**	**Factors detected by nanoarray**	**Predicted pathotype**
E. coli ONT (Or2):H34 (MCI 0105)	*fimA*.	*gad*, *rrl*.	Non-pathogenic
E. coli ONT (Or3):H-/NT (MCI 0106)	*eae*θ, *espP*, *fimA*, *ehxA* (type 106).	*rrl*, *eae*, *ehxA*.	Atypical EPEC
E. coli ONT (Or4):H4 (MCI 0108)	*cdtB*, *cnf*, *iutA*, F17 (*gafD*)	*gad*, *rrl*, *cdtB*, *cnf*1, F17-G, *iss.*	ExPEC
E. coli O26:H11 (MCI 0110)	*stx*1/*stx*1$_v$, *eae*β, *escN*, *espP* (type B), *ehxA* (type 106), *iutA*, *fimA*.	*rrl*, *cba*, *eae*, *espB*, *ehxA*, *iss*, *stx1*.	EHEC
E. coli O146:H21 (MCI 0319)	*stx*1/stx1$_v$, *iutA, ehxA* (type 319), *fimA*.	*gad, rrl*, *celB*, *ehxA*, *iss*, *mchB*, *mchC*, *mchF*, *senB*, *stx*1.	STEC*

Table 4.3: Gene detection using the ArrayTube nanoarray compared with gene detection by PCR: In all cases, virulence factors detected by nanoarray were in agreement with those detected by PCR. * MCI 0319 is designated as a predicted STEC (shiga-toxigenic *E. coli*) as it encodes genes for Shiga-like toxin not genes encoding intimin and proteins associated with a functional T3SS. Shiga-toxigenic intimin/T3SS-negative *E. coli* strains have been noted to cause haemorrhagic colitis and HUS (Banatvala *et al.* 2001).

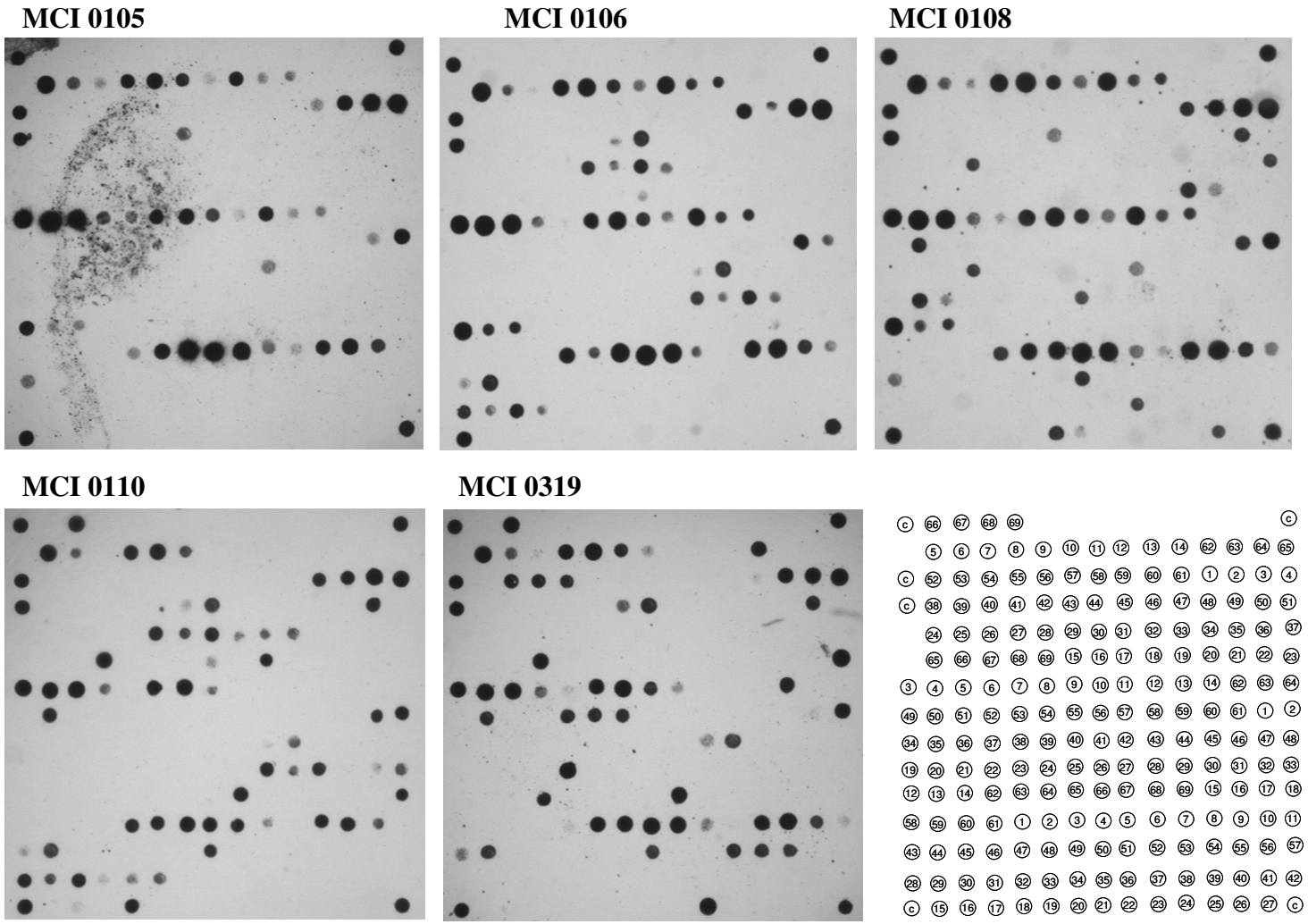


Figure 4.8: Images resulting from virulence gene nanoarray: Oligos described in Appendix V were included in triplicate. Bottom left: The grid shown corresponds to the positions of the oligonucleotides, the numbers shown referring to those shown in Table A5 (c = HRP control spots). Where areas of smudging occurred, due to particulate matter, results for oligonucleotides affected were omitted from subsequent analysis.

In order to obtain a broader view of the genomes of the strains analysed by nanoarray, a pan-genomic microarray was utilised, as described in Section 2.6.6, which comprised oligonucleotides representing the genomes of two sequenced *E. coli* O157:H7 isolates (EDL933 and Sakai) and that of prototypic laboratory isolate *E. coli* K12 MG1655. Examples of resultant array images are presented in Figure 4.9. A schematic diagram summarising the microarray results is presented in Figure 4.10. As shown in Figure 4.10 the backbone component, common to *E. coli* O157:H7 and *E. coli* K12 MG1655, is conserved among the strains analysed. Greater heterogeneity is observed in the portion of the genome present in *E. coli* O157:H7 but not *E. coli* K12, including oligonucleotides representing regions which are designated "O-islands". The presence and absence of each O-island is examined in more detail in Figure 4.10.

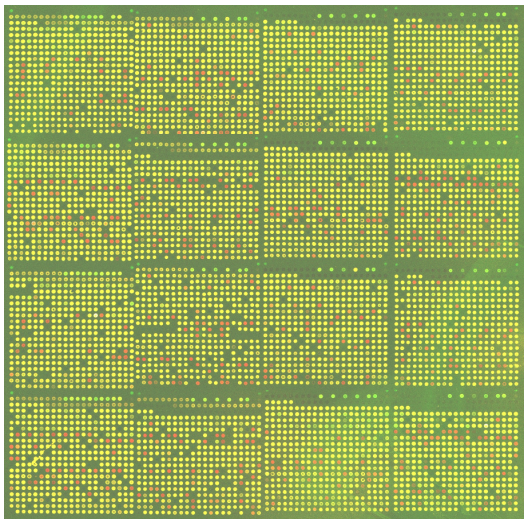
It can be seen from this analysis that isolates encoding EHEC-related genes (MCI 0106, MCI 0110 and MCI 0319) carry the largest proportion of the O157-specific sequences of the isolates analysed here. MCI 0108 (ExPEC-like strain) was found to be devoid of almost all of the genes encoded on the major O-islands. MCI 0105, which was predicted to be non-pathogenic (as it was negative for all of the virulence factor-encoding genes), was positive for more than 50% of the oligonucleotides representing O-islands 7, 30, 36 44, 102 and 115, although the functions of the genes encoded on these regions are not defined. As would be expected of intimin-encoding strains, MCI 0106 and MCI 0110 were positive for more than 50% of the oligonucleotides representing the LEE and also O-islands 71 and 122. These islands are conserved among pathogenic EHEC and both encode T3SS-effector proteins (Karmali *et al.*, 2003;Tobe *et al.*, 2006;Coombes *et al.*, 2008). MCI 0110 and MCI 0319, which both encoded *stx*1, were both positive for the majority of oligonucleotides representing O-islands 51, 52 and 76. Once again, the functions of the genes encoded on these islands are largely unknown. Although MCI0319 encodes *stx*1, it was negative for much of the oligonucleotides representing the *stx*1 bacteriophage. This highlights the diversity of bacteriophages which carry *stx* genes, as has been noted by others, especially among those carried by bovine STEC (Besser *et al.*, 2007). MCI 0110 (predicted EHEC as it encodes *eae* and *stx*) was the only strain tested which carries a significant proportion of O-island 50. Again, this island is largely composed of genes of unknown function. As the bovine isolates were observed to only encode small parts of many islands, this suggests that many of the O-islands may be mosaic, as noted by others (Perelle *et al.*, 2003;Shen *et al.*, 2004;Bielaszewska *et al.*,

2007a;Bielaszewska *et al.*, 2007b), suggesting that some of the larger O-islands are the product of multiple smaller acquisitions, rather than wholesale acquisitions of entire genomic islands.

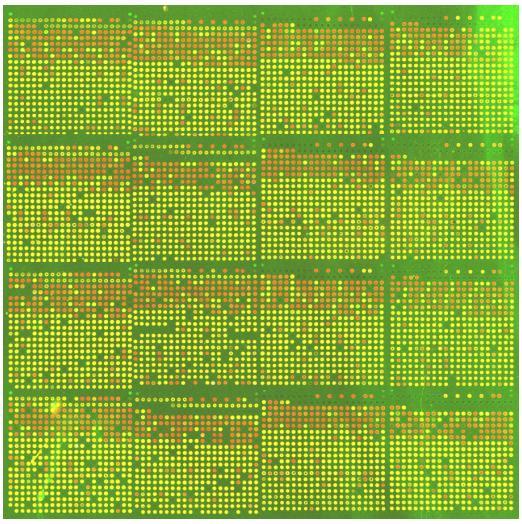
Although MCI 0106 and MCI 0110 were both found to encode *espP* and *ehxA* genes by PCR (both of these genes are carried on pO157 in *E. coli* O157:H7), these strains were only positive for a small proportion (approximately one quarter: Figure 4.11) of the oligonucleotides representing pO157. Both strains were positive for *kat*P and oligonucleotides representing genes flanking *espP* (locus tags L7013-L7029). The probes representing *espP* were ambiguously positive in MCI 0106, suggesting some diversity in the *espP* gene encoded by this strain. Operons encoding enterohaemolysin were detected in MCI 0106, 0110 and 0319 which corroborates the results of the *ehxA* PCR.

The isolates analysed herein were positive for many of the oligonucleotides representing the K-islands, which comprise the portion of *E. coli* K12 genome which is absent from *E. coli* O157:H7 (Perna *et al.*, 2001). MCI 0108, MCI 0110 and MCI 0319 were each positive for approximately 40% of these oligonucleotides, whereas MCI 0105 and MCI 0106 were each positive for approximately 30%. K12-specific regions conserved in all strains include parts of K-islands 77, 121, 142, 166, 172, 183 and 192, which contain genes which have only putative or unknown functions assigned, K-island 108 which encodes genes involved in molybdate metabolism, and K-island 153 which encodes a malate synthase and glycolate oxidase genes. MCI 0108 and MCI 0110 were positive for a cluster of genes involved in citrate dependent iron transport.

MCI 0066



MCI 0105



MCI 0110

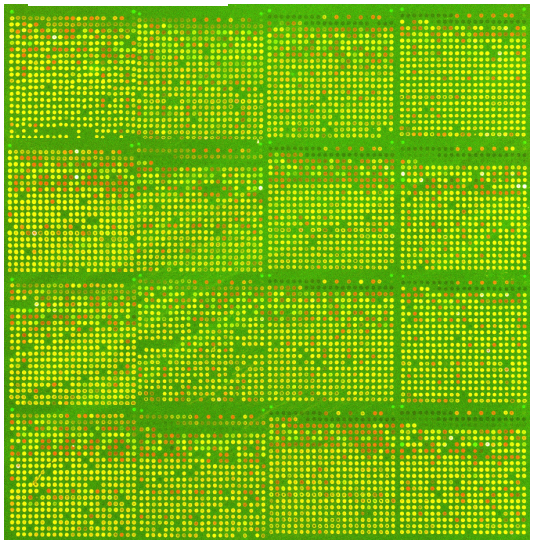


Figure 4.9: Example images resulting from genomic microarray hybridisations: Each panel shows one block of 5978 oligonucleotides. Three of these blocks are printed on each array, meaning that 3 replicates of each oligo are included on each slide. Grossly, it can be seen from this image that *E. coli* O157:H7 (MCI 0066) is positive for much of the O157-specific content, while predicted non-pathogenic strain MCI 0105 lacks many of these genes (evidenced by the propensity of red spots in the upper part of each block, where the O157-specific oligonucleotides were situated), whereas predicted EHEC-like strain (MCI 0110) is positive for many of these oligonucleotides.

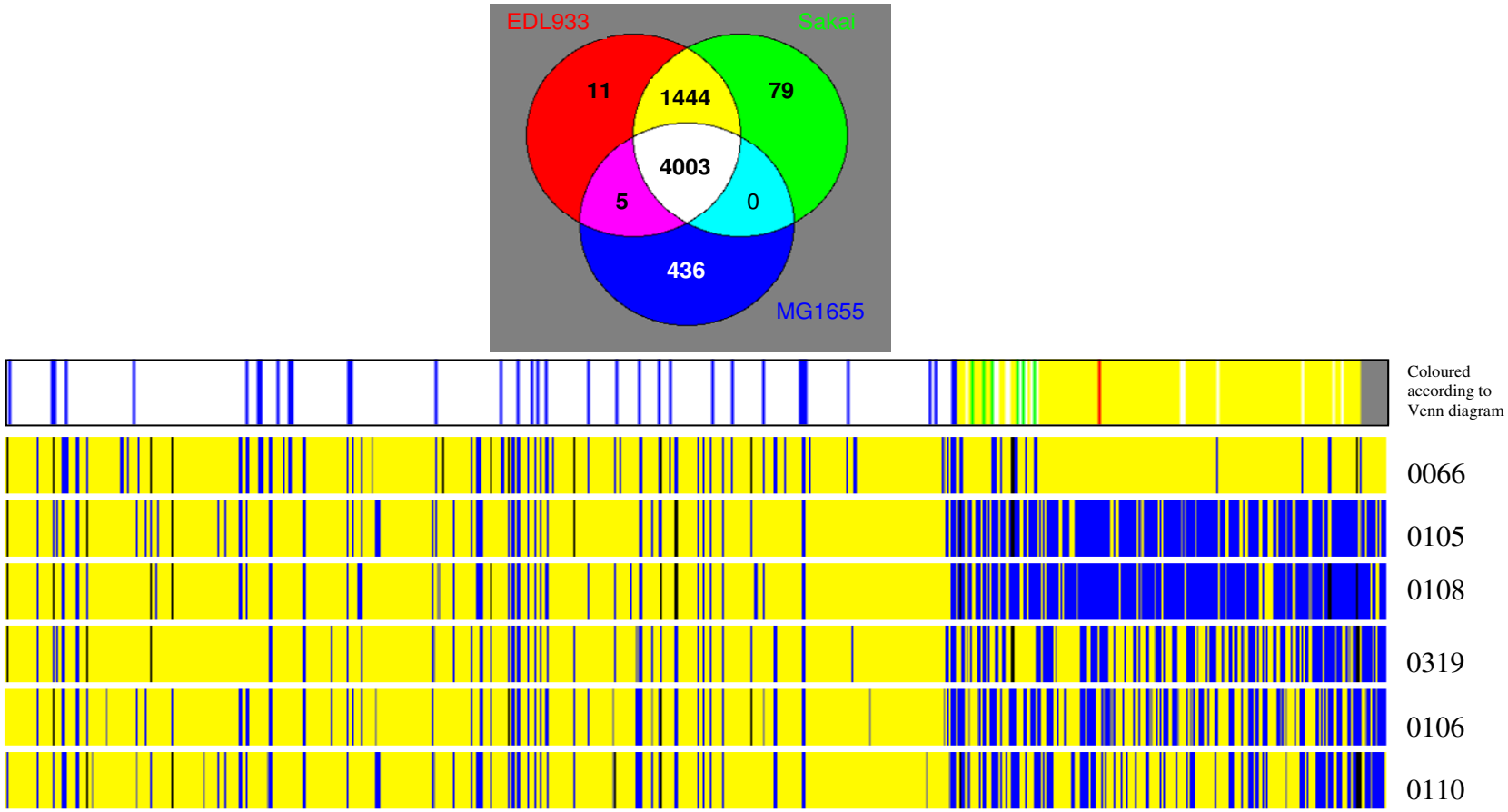


Figure 4.10: Results of O157/K12 microarray analysis of bovine commensal *E. coli*: the Venn diagram above shows the numbers of oligonucleotides specific to each of the three control strains represented on the array and those common to two or all three isolates. Each oligonucleotide is represented by a vertical bar, the topmost bar coloured according to the Venn diagram (the grey area at the rightmost end of the bar corresponds to the genes encoded on pO157) and subsequent bars coloured by presence or absence in each strain analysed (yellow: present, blue: absent, grey: ambiguous). It can be seen that the core genome component is broadly conserved in each strain, whereas the O157-specific component (the rightmost third of the bars) shows heterogeneous carriage across the bovine isolates analysed. The numbers to the right correspond to MCI reference numbers (Tables 2.1 and 4.2).

	O-island																						
	1	**7**	**8**	**9**	**14**	**17**	**20**	**28**	**30**	**35**	**36**	**44**	**45**	**47**	**50**	**51**	**52**	**55**	**70**	**71**	**76**	**79**	**84**
MCI 0066	100	100	100	100	100	100	100	100	100	100	100	100	80	100	100	94	100	100	100	95	100	94	91
MCI 0105	40	73	33	14	86	100	0	0	80	0	54	50	0	0	14	0	20	14	0	5	0	19	0
MCI 0106	40	67	17	14	0	0	0	0	80	0	58	63	83	0	29	35	32	14	0	75	4.2	38	0
MCI 0108	40	13	25	14	0	100	0	0	40	0	0	6.3	0	0	0	0	32	14	0	5	25	6.3	9.1
MCI 0110	40	43	8.3	0	0	100	0	0	40	0	83	75	12	0	71	82	60	14	0	85	79	0	9.1
MCI 0319	40	70	17	14	0	100	0	0	80	0	63	38	44	0	29	59	64	14	0	5	54	19	0
size (kbp)	6.5	36	23	6.1	5.3	5.3	8.1	25	12	14	39	45	62	32	47	16	54	6.7	7.9	58	21	45	14
oligos	5	30	12	7	7	6	5	5	5	14	24	16	41	27	21	17	25	7	7	20	24	16	11
													a							c			

	O-island																						
	90	**93**	**95**	**102**	**108**	**115**	**122**	**123**	**138**	**139**	**140**	**141**	**144**	**145**	**148**	**153**	**154**	**162**	**166**	**167**	**172**	**173**	**pO157**
MCI 0066	100	75	100	100	100	100	100	100	100	100	89	100	100	100	95	100	100	100	100	90	58	100	99
MCI 0105	0	6.3	0	33	0	50	12	0	0	20	11	0	100	38	0	17	0	100	0	10	8.3	0	23
MCI 0106	0	19	100	33	14	80	76	0	0	30	0	0	100	100	71	17	0	50	100	10	13	0	23
MCI 0108	0	6.3	100	33	0	0	24	0	0	100	0	0	100	38	0	33	0	100	0	10	8.3	0	21
MCI 0110	0	56	0	47	29	45	76	0	0	10	0	0	100	100	62	33	0	100	86	10	17	0	28
MCI 0319	0	25	0	33	0	50	18	0	0	30	0	0	100	100	2.4	17	0	100	0	0	13	0	18
size (kbp)	5.1	49	6.2	13	22	17	23	7.3	15	6.6	9.1	5.9	6.1	5.4	43	7.4	6.9	5.5	6.3	9.3	44	5.6	92
oligos	3	16	6	15	14	20	17	6	18	10	9	6	2	8	42	6	6	2	7	10	24	3	97
		b				c	c	c				e			d		e						

Figure 4.11: O-island scan of bovine commensal *E. coli*: For each of the O-islands of greater than 5kbp that were represented on the array (excluding all oligonucleotides predicted to hybridise to multiple locations on the genomes of any of the control strains), the proportion of oligonucleotides giving a positive result for each strain was determined. It can be seen that a typical human *E. coli* O157:H7 strain (WallaWalla1, MCI 0066) was positive for the majority of O-islands, while these genes are carried more variably by other bovine resident *E. coli*. (a: the *stx*2 encoding bacteriophage, b: *stx*1 encoding bacteriophage, c: sites containing genes encoding non-LEE-encoded T3SS-effectors, d: the LEE, e: operons encoding long polar fimbriae). pO157 is included in this scan as it is another significant genomic element present in *E. coli* O157:H7 but absent from the *E. coli* K12 MG1655 genome. Colours: blue: <25% oligos positive, light blue: 25-50% positive, green: 50-90% positive, yellow: >90% positive.

4.2.2 Distribution of bovine resident *E. coli* in the bovine GIT

In order to obtain a profile of the *E. coli* present at different sites in the bovine GIT, five sites were chosen for the isolation of mucosa-associated *E. coli* by necropsy sampling as set out in Section 2.3.2. The selected sites are highlighted in Figure 4.12 including **(1)** terminal rectum (TR), within 5cm of the RAJ, **(2)** the proximal rectum (PR) between 15 and 20cm from the RAJ, **(3)** a site in the distal colon (DC) approximately one metre from the rectum **(4)** a site at the proximal colon (PC) at a flexure close to the caecum, **(5)** the terminal ileum (IL), 10-20cm from the ileocaecal junction. In the initial animals, which were used to optimise techniques (described in Section 4.2.2.1), site 3 (colon) was not sampled. In the first animal, only sites 1, 2 and 4 were sampled. Sites 3 and 5 were added to give increased coverage of regions of the lower GIT in the animals for which enumeration data was included in the analysis of *E. coli* prevalence at each location.

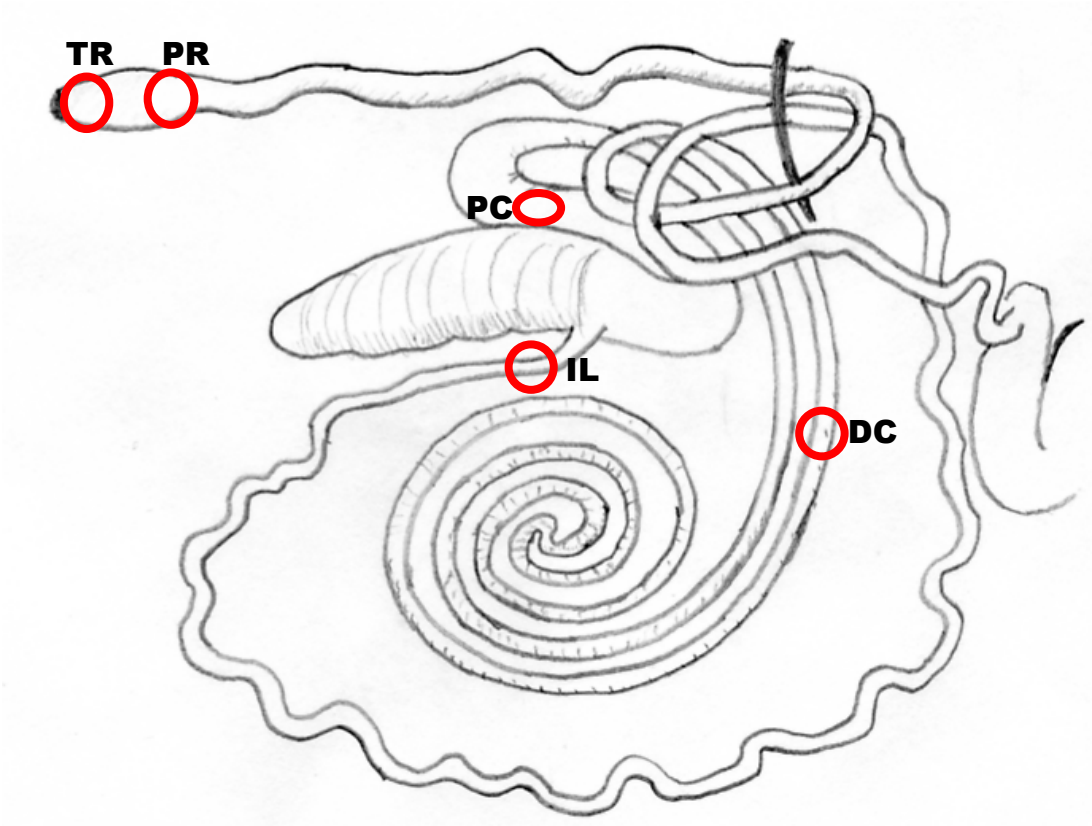


Figure 4.12: Representation of bovine gastrointestinal tract, showing the sites of *E. coli* necropsy sampling: Each of the sites analysed in this study is circled in red in the diagram above, including: TR: Terminal rectum, PR: Proximal rectum, DC: Distal colon, PC: Proximal colon and IL: Ileum (redrawn from Dyce *et al.*, 1987).

4.2.2.1 Optimisation of viable counts

Four animals (which showed no signs of diarrhoeal illness and had not been treated with antibiotics) which were to undergo routine disposal were analysed in order to assess and refine techniques for bovine *E. coli* isolation. The first was a milking cow of approximately seven years, used to pilot two techniques of isolating mucosa-associated *E. coli*. As well as the technique set out in Section 2.3.2, a second method was assessed for its applicability for this purpose, whereby a sterile swab was vigorously rubbed across a defined area (5cm x 5cm) of the mucosal tissue. The method set out in Section 2.3.2 proved more practical for this purpose as wrinkling of the tissue made measurement of the area to swab more difficult and saturation of the swab was not always even. For this first animal, count data was not obtained as insufficient countable replicates of the spread plates were prepared. However, this animal was used to provide isolates for analysis of the numbers of different *E. coli* serotypes present within one animal (see Section 4.2.2.3).

The three further animals (two milking cattle of approximately 29 months and a four week old male calf) were used to determine the expected range of *E. coli* colonisation density which could be expected to be found allowing refinement of the methodology.

The data obtained indicated that the two milking cows were moderately colonised at all sites sampled (in the range of 10^2-10^3cfu/cm^2). The young calf exhibited heavy colonisation at the rectum (10^5-10^6 cfu/cm^2) and low numbers of *E. coli* at the proximal colon and ileum (approximately 10^2 cfu/cm^2).

4.2.2.2 Enumeration of mucosa-associated *E. coli* at selected GIT sites in a panel of healthy weaned calves

Eleven calves, aged between 4 and 8 months which showed no signs of diarrhoeal disease and had not been treated with antibiotics were used to obtain mucosally-associated *E. coli*. Tissue sections from the five sites detailed in Figure 4.11 were taken and *E. coli* was isolated according to the method detailed in Section 2.3.2.

Animal 300710 was excluded from the analysis as the counts were universally low. Resultant bacterial counts from other animals were transformed by the dilution factor of the countable plates (those exhibiting less than 500 colonies) to give a value for cfu/100µl of the liquid in which the tissue was suspended, which would contain the bacteria removed from approximately $2mm^2$ of tissue surface by the vigorous vortexing (described in Section 2.3.2). The data was transformed using the following equation: $\log_{10}(c+1)$, where c=cfu/100µl. The addition of the constant (+1) allows zero values to be retained after the transformation. The data was imported into the GenStat program and a restricted maximum likelihood model (REML) was fitted to the data, using "site" as a fixed factor and "replicate tissue section within site" and "animal" as random factors. Site within animal was identified as having a significant effect on the number of *E. coli* recovered (F=0.050), meaning that colonisation was not uniform across the sites analysed. The terminal rectum and proximal colon appeared to be most heavily colonised of the sites analysed, although not to a significant extent over the other sites (Figure 4.14).

The terminal rectum and ileum were presupposed to be areas of lymphoid follicle-dense mucosal tissue, with the proximal rectum and colon likely to be less densely populated with lymphoid tissue. To validate this presumption and to affirm that sampling was performed at the same sites in each animal, $1cm^2$ pieces of tissue were fixed in formal saline, embedded in paraffin wax and sections of tissue taken for haematoxylin and eosin (H & E) staining (performed at Moredun Research Institute: Pathology Department). Images representative of each site are presented in Figure 4.13.

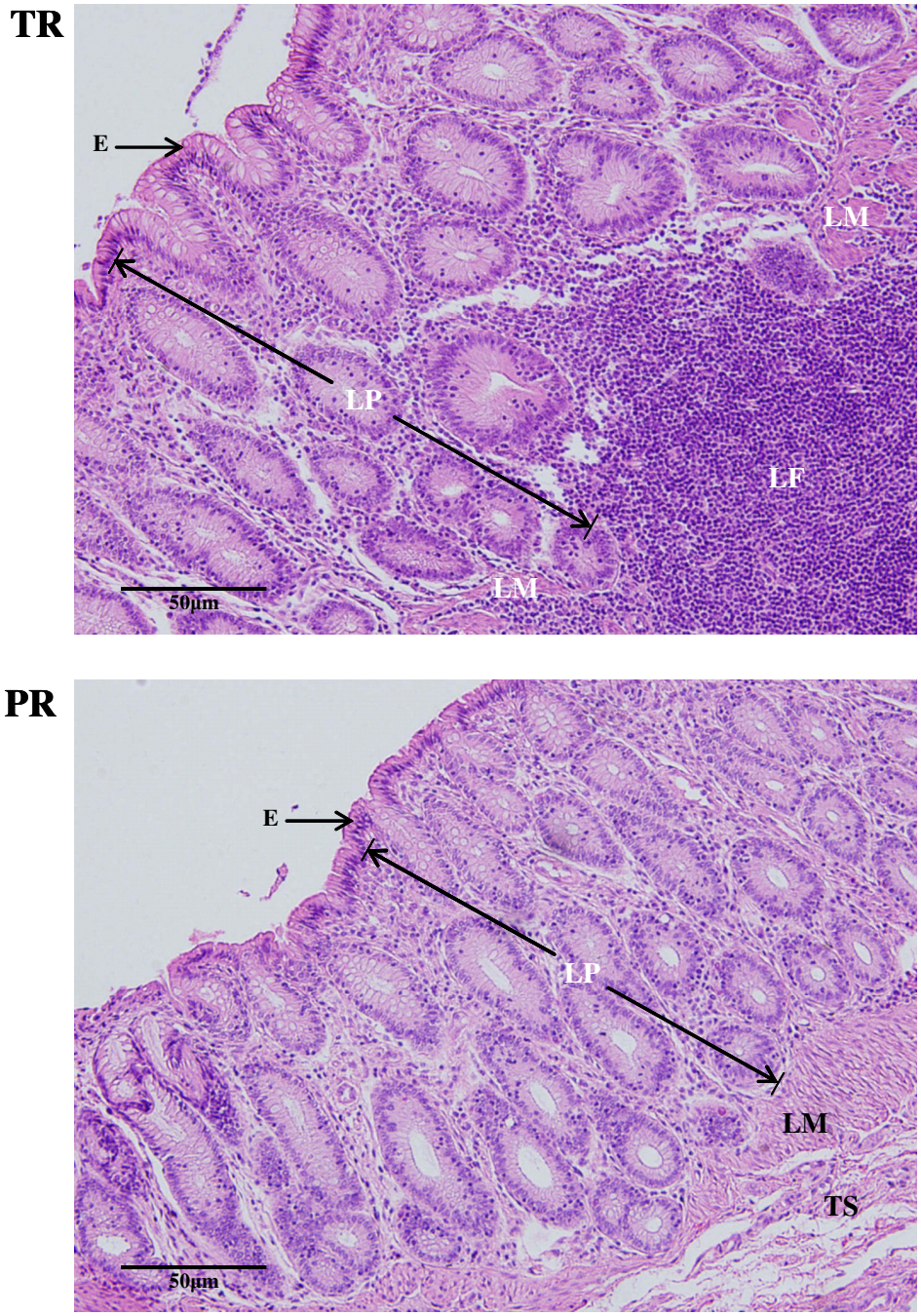


Figure 4.13: Mucosal morphology at sites selected for the isolation of resident *E. coli* (part 1): TR: terminal rectum, within 5cm of the recto-anal junction (RAJ): the principle site of colonisation for *E. coli* O157:H7. Lymphoid follicles (**LF**) are present at high density at this site. An LF is shown here extending through the *lamina muscularis* (**LM**) into the *lamina propria* (**LP**). **PR:** proximal rectum (15cm proximal to the RAJ), lymphoid follicles are less frequent at this site. **TS:** *tela submucosa*, **E:** epithelium.

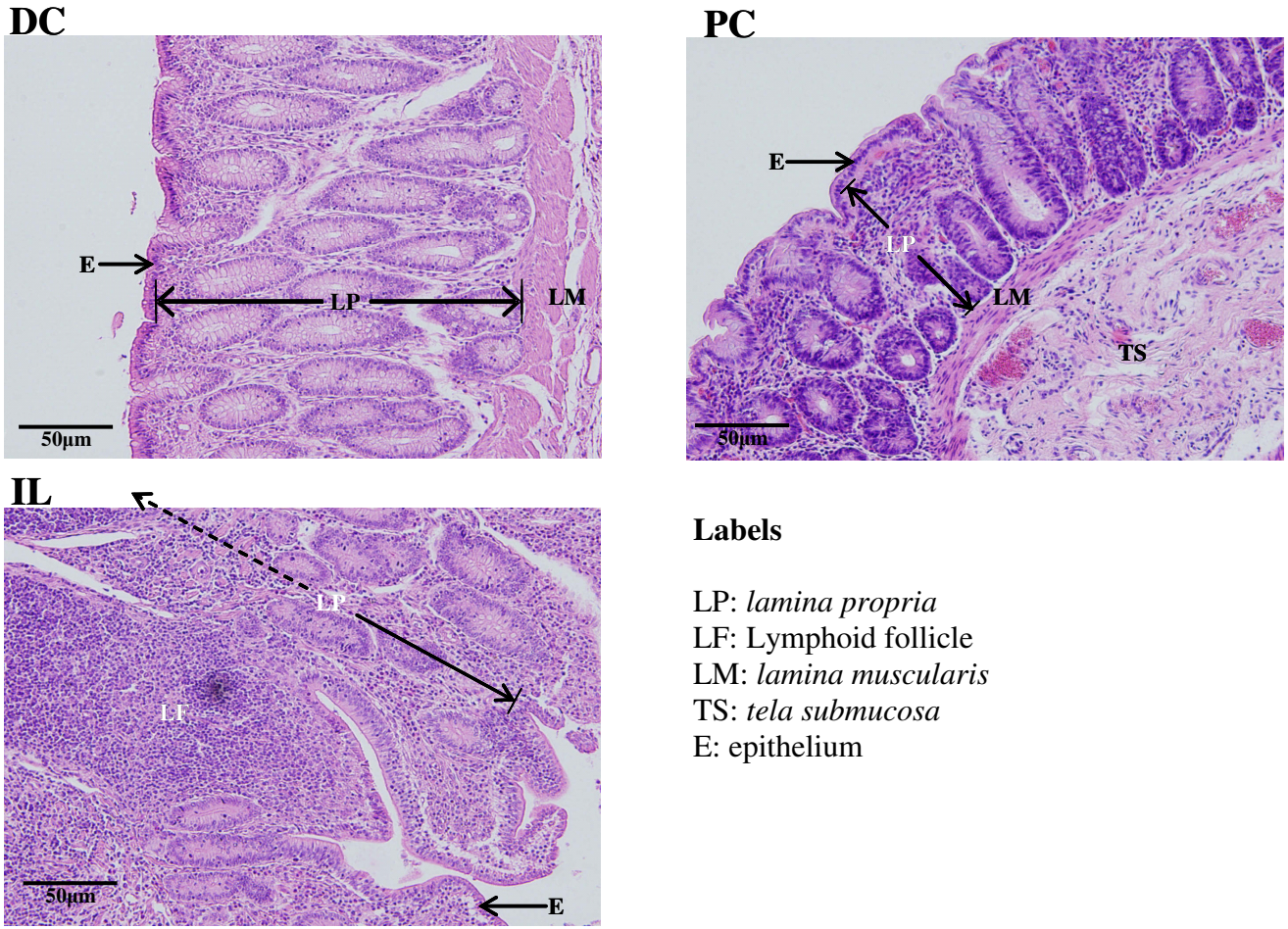


Figure 4.13: Mucosal morphology at sites selected for the isolation of resident *E. coli* (part 2): proximal colon (PC) and distal colon (DC) are both primarily involved in the absorption of water and solutes from the gut lumen. Similarly to the proximal rectum, these sites are not densely populated with LFs. The terminal ileum is a site of particularly high LF density, a site including ileal Peyer's patches. The image shows an LF which extends into the *lamina propria* to the mucosal surface.

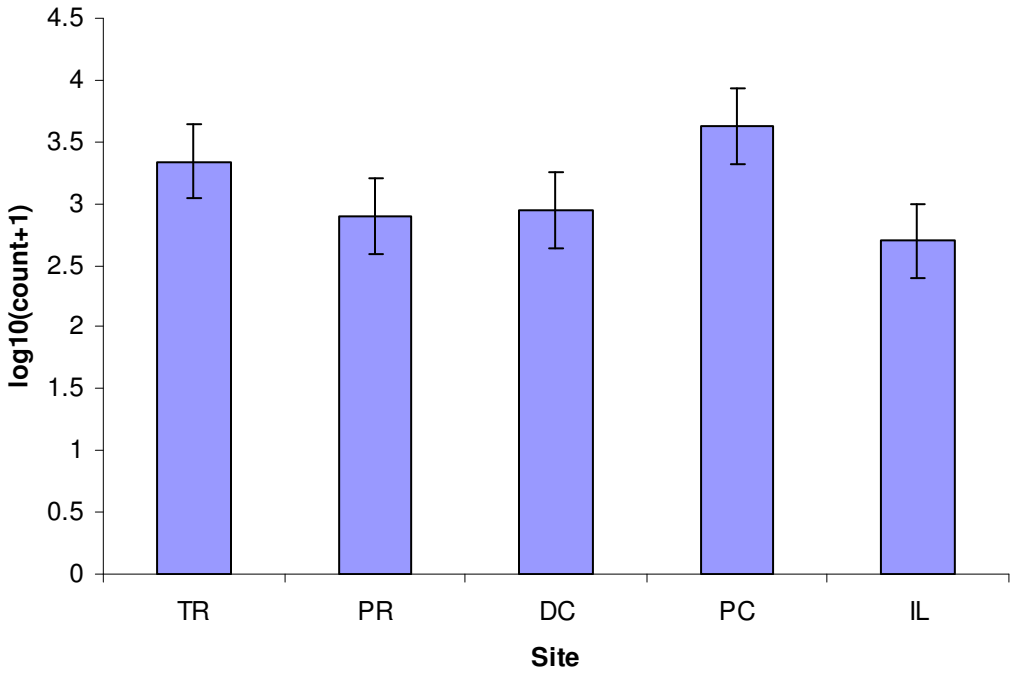


Figure 4.14: Viable counts of *E. coli* from each gastrointestinal site analysed: Mean ± SEM of count data from animals described in Section 4.2.2.2 (n=10) are shown as predicted by REML analysis using the GenStat program. These data suggest that *E. coli* may be generally more prevalent at the TR and PC than the other sites analysed. (TR: terminal rectum, PR: proximal rectum, DC distal colon, PC: proximal colon, IL: ileum).

4.2.2.3 Characterisation of mucosa-associated *E. coli* of the bovine gastrointestinal tract

The first animal studied was used to obtain an indication of the number of different *E. coli* serotypes that may be present within one animal, (Figure 4.9). From this animal, 54 colonies from each intestinal site were chosen for analysis by molecular H-typing.

From all other animals, 18 colonies were picked from plates representing each site, except in the cases where CFU counts from particular sites were so low that there were insufficient colonies available for selection (in such cases all available colonies were taken for analysis). Where blue (glucuronidase-positive) and white (glucuronidase-negative) colonies were present on TBX medium, proportions of blue:white colonies picked were made to reflect the proportions of these present on the plate. The full list of strains recovered from the animals analysed in this study is given in Appendix II.

When glucuronidase-negative (white colonies on TBX) organisms were selected for analysis, they normally exhibited no growth in LB. When the organism did grow in LB and sufficient DNA was obtained (determined by nanodrop), amplification of the *rfb* locus for O-RFLP failed in each case. It was therefore deduced that these were colonies of an organism other than *E. coli* and therefore discounted from the viable count data and no further characterisation of these organisms was performed. It was important to attempt to characterise these glucuronidase-negative organisms, considering that *E. coli* O157:H7 is generally glucuronidase-negative; however, the vast majority (96-97%) of *E. coli* strains are glucuronidase positive (Ratnam *et al.*, 1988).

For five of the total number of animals utilised in this study rectal swabs were taken and plated onto TBX agar 3 weeks prior to euthanasia. Three colonies from each animal were included in the molecular characterisation, to assess whether particular *E. coli* subtypes had persisted.

Molecular H-typing (H-RFLP) was used as a preliminary method used to predict the number of different serotypes present within an animal. This procedure was performed for each selected colony as set out in Section 2.6.4.1. For each of the H-RFLP-patterns found, three

colonies exhibiting this pattern from each gastrointestinal site were chosen for molecular O-typing (O-RFLP). When no amplicon was generated by H-RFLP, six of these colonies were chosen for O-RFLP when available. If the O-RFLP patterns of the three (or six) chosen of a particular H-RFLP type were indistinguishable, it was assumed that all isolates of that H-type bear the same O-type within that animal. Where more than one O-RFLP pattern was identified for one H-RFLP pattern in one animal, it was necessary to obtain O-RFLP patterns for all isolates bearing that H-RFLP pattern within that animal. O-RFLP patterns and H-RFLP patterns obtained from each animal are included in Table 4.5.

The molecular serotype analysis of *E. coli* isolated from the first animal is presented in Figure 4.15. It was observed that a single serotype was most prevalent at each of the sites analysed, making up 76-82% of the *E. coli* recovered from each site (79.6% of the total *E. coli* recovered from that animal). A second serotype was present at lower levels (13-17% at each site, 14.8% overall). In total, five different serotypes were identified among isolates from this animal, although three of these serotypes combined comprised less than 5% of the *E. coli* sampled from this animal. This was found to be typical of other animals analysed (Table 4.4). For the remaining animals analysed, molecular serotype data was combined with viable count data in order to examine the colonisation patterns for each serotype identified. Where isolates of a particular serotype were isolated from more than one colony from each animal, these were considered to be significantly present in the animal. Relative proportions of each serotype were predicted by multiplying total *E. coli* count (cfu/cm^2) by the proportion of recovered colonies exhibiting this serotype. These data are presented in Table 4.4. The mean number of *E. coli* types detected within an animal was calculated as 2.571 (± 0.453, n=14, min=1, max=7).

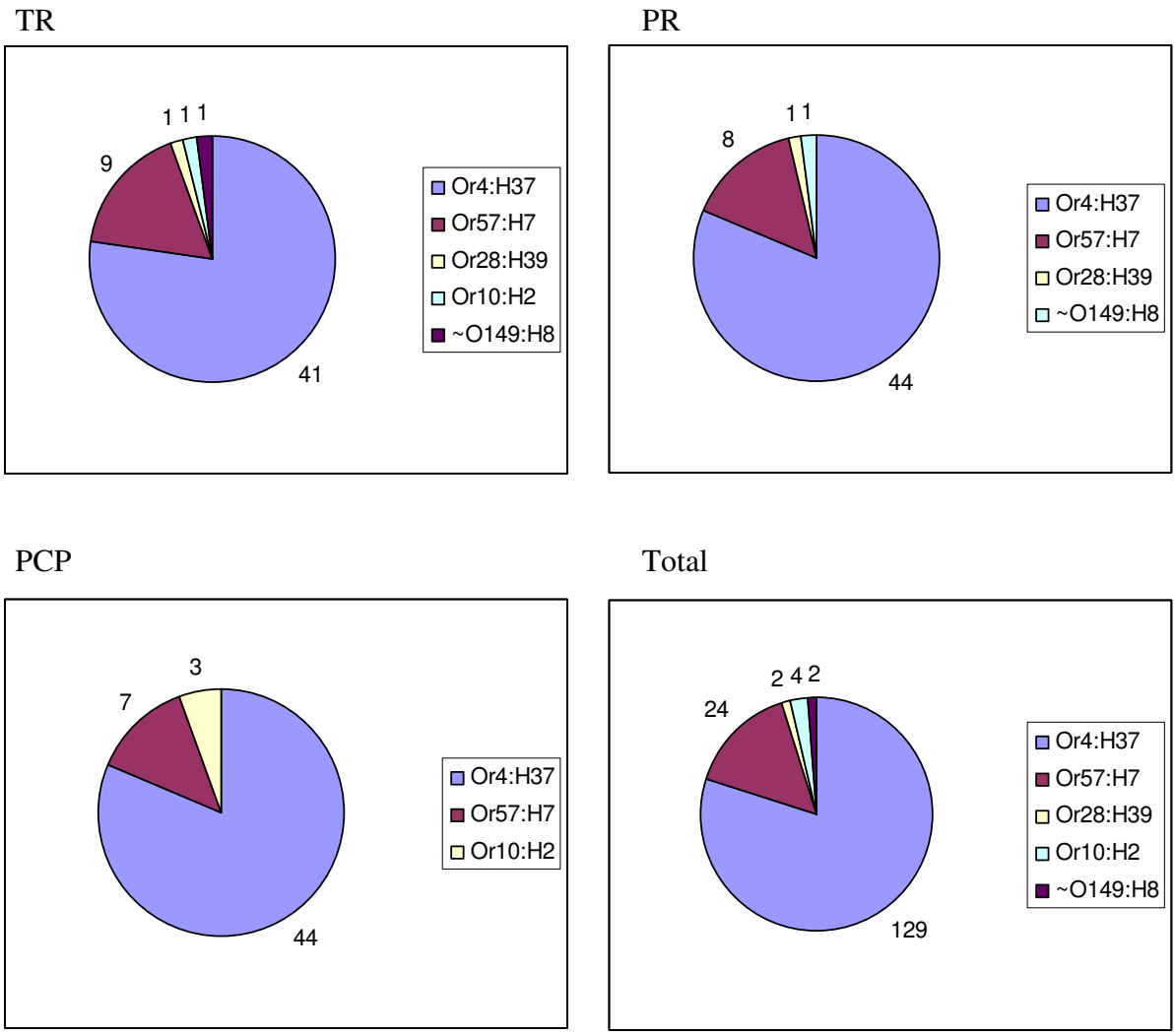


Figure 4.15: Pie charts showing distribution of *E. coli* subtypes within one animal: At each site analysed one serotype (ONT (Or4):H37, represented by isolate MCI 0430) predominates, with a second type (Or57:H7, represented by isolate MCI 0435) making up a smaller but significant proportion of the *E. coli* population. This is in agreement with the numbers of *E. coli* subtypes recovered from the rest of the panel of animals (Section 4.2.2.3 and Table 4.4).

ANIMAL	TYPE	TR	PR	DC	PC	IL	RT
04097	Or29:H19NM	352.9	846.6	*	2523	73.41	No
	Or30:H8	117.6	0	*	137.3	14.68	No
	TOTAL	500	888.9	*	2883	102.8	
02757	O103:H36	119	343.4	*	1455	357.1	No
	Or29:H19NM	47.62	156.1	*	783.3	642.9	No
	TOTAL	250	655.6	*	2350	1500	
600096	O26:H+(NT)1	2E+06	1E+06	*	138.9	50	Yes
	Or35:H20	5E+05	0	*	0	0	Yes
	TOTAL	2E+06	1E+06	*	138.9	50	
200927	Or7:H+(NT)2	58833	4728	3465	22891	0	No
	TOTAL	58833	4728	3850	24417	0	
601223	Or7:H+(NT)2	100	233.3	2317	438.9	1078	No
	TOTAL	100	233.3	2317	438.9	1078	
300710	Or7:H+(NT)2	27.78	22.22	50	38.89	27.78	No
	TOTAL	27.78	22.22	50	38.89	27.78	
300129	Or37:H+(NT)1	14383	0	0	0	0	Yes
	Or35:H49	9589	651.9	2261	30904	2050	No
	Or14:H-/NT	0	217.3	174	0	128.1	No
	TOTAL	28767	977.8	2783	32722	2178	
300136	Or37:H+(NT)1	6224	0	0	0	0	Yes
	Or14:H-/NT	4841	3932	687.5	9018	10144	No
	Or38:H+(NT)1	691.5	245.8	437.5	1932	2029	No
	TOTAL	11756	4178	1000	10950	12172	
700140	Or39:H+(NT)2	2201	2311	3080	1818	5906	No
	Or37:H+(NT)1	593.8	0	0	0	0	Yes
	Or36:H47	0	0	0	699.1	0	No
	TOTAL	2672	2311	3272	2517	6644	

* = site was not analysed

ANIMAL	TYPE	TR	PR	DC	PC	IL	RT
700217	Or41:H25	2296	68.06	0	0	0	Yes
	O103:H2	1722	68.06	0	0	0	Yes
	Or17:H8	1148	22.69	0	0	0	Yes
	Or7:H+(NT)2	0	45.37	36.36	1158	233.3	No
	~O147:H-/NT	0	22.69	0	0	0	No
	Or10:H2	0	45.37	27.27	2027	700	No
	Or42:H16NM	0	0	18.18	1737	933.3	No
	TOTAL	5167	272.2	100	5211	1983	
400354	Or7:H+(NT)2	848.1	524.5	7.407	6228	59.72	No
	Or45:H2	318.1	0	0	0	0	Yes
	Or47:H40	0	0	37.04	566.2	119.4	No
	TOTAL	1272	572.2	66.67	6794	238.9	
301437	Or47:H40	45804	5627	49238	41839	921.8	No
	TOTAL	48667	6378	59086	44300	1006	
401473	Or41:H25	305.6	0	0	0	0	Yes
	Or10:H2	55.56	0	0	774.1	0	No
	Or46:H2	27.78	686.3	127.8	1161	369.4	No
	Or52:H+(NT)2	0	915	553.7	3870	1355	No
	Or53:H9	0	114.4	0	774.1	0	No
	TOTAL	388.9	1944	766.7	6967	2217	
200522	Or7:H+(NT)2	2872	256.5	447.2	1662	117.3	No
	Or52:H+(NT)2	1045	615.7	511.1	1477	93.83	No
	TOTAL	4178	872.2	1150	3139	211.1	

	<10
	11-100
	101-1000
	1001-10000
	10001-100000
	>100001

Table 4.4: Distribution of *E. coli* in the bovine GIT: Proportion of each type of *E. coli* present at each site in each animal sampled was calculated and multiplied by the total count at each site to give predicted density (cfu/cm^2). Isolates which colonised at the rectum at greater than two-fold density at the rectum than other sites were considered to be candidate rectal tropic strains (RT).

PCR-screening for selected genes was carried out on two representatives of each molecular serotype from each animal; one isolate from two separate sites where strains of this serotype were isolated from more than one site in an animal. The pair of isolates chosen was always from the two sites where the organism was recovered that were spatially furthest away from each other (the terminal rectum and the ileum in most cases). PCR characterisation was carried out as Section 4.2.1.3 and example images are presented in Figures 4.3-4.7. Results of molecular characterisation of these isolates by PCR are summarised in Table 4.5 with supporting imaged included in Appendix IV. The genes detected in each of the paired isolates were identical in each case and for simplicity; results from one representative of each paired isolate are included in Table 4.5. The full list of isolates is given in Appendix II.

Ref. No.	Animal	Isolation method	O-type/O-RFLP type	H-RFLP-type	Lineage	*ipaH*	*stx*	*espP*	*escN*	*eae*	*cdtB*	*cnf*	*fimA*	*sfaD/E*	*neuB*	*iutA*	*ehxA*	*gafD*
MCI 0430	001	TS	ONT (Or4)	H37	A	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0435	001	TS	Or57	H7	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0437	001	TS	Or28	H39	A	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0439	001	TS	O43 (Or10)	H2	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0441	001	TS	O149*	H8	B1	-	stx2NT[d]	-	-	-	-	-	+	-	-	-	-	-
MCI 0454	04097	TS	O139 (Or29)	H19NM	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0455	04097	TS	Or30	H8	B1	-	A+D+B	C	-	-	-	-	+	-	-	-	319*	-
MCI 0457	04097	TS	Or31	H10	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0464	04097	TS	Or34	H25	B2	-	-	-	-	-	-	-	-	-	-	-	-	-
MCI 0465	02757	TS	O103	H36	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0468	02757	TS	ND	H-/NT	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0475	02757	TS	O139 (Or29)	H19NM	A	-	-	-	-	-	-	-	-	-	-	-	-	-
MCI 0483	600096	TS	O26	H+(NT)1	B1	+/-	-	D	+	β	-	-	+	-	-	-	106*	-
MCI 0487	600096	TS	O18ac (Or35)	H20	B1	-	A+D+B	D	-	-	-	-	+	-	-	-	319*	-
MCI 0490	200927	TS	ONT (Or7)	H+(NT)2	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0494	200927	TS	Or36	H47	B1	-	-	-	-	-	+	+	+	-	-	+	-	-
MCI 0496	601223	TS	ONT (Or7)	H+(NT)2	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0501	300710	TS	ONT (Or7)	H+(NT)2	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0512	300129	TS	O18ac (Or35)	H49	B1	-	-	-	-	-	+	-	+	-	-	-	-	-
MCI 0517	300129	TS	Or37	H+(NT)1	B1	-	B	C	-	-	-	-	+	-	-	-	319*	-
MCI 0518	300129	TS	O45 (Or14)	H-/NT	B2	-	-	-	-	-	+	+	-	-	-	+	-	+
MCI 0521	300129	TS	ONT (Or7)	H5	B2	-	A	-	-	-	+	+	-	+	+	+	-	-
MCI 0522	300129	TS	Or39	H+(NT)2	B1	-	A	+/-[c]	-	-	+	+	+	-	-	-	-	+
MCI 0653	300129	TS	Or57	H-/NT	B1	-	B	C	-	-	-	-	+	-	-	-	319*	-
MCI 0654	300129	TS	Or39	H-/NT	B1	-	A	+/-[b]	-	-	+	+	-	-	-	-	-	+
MCI 0655	300129	TS	O18ac (Or35)	H49	B1	-	-	-	-	-	+	-	-	-	-	-	-	-
MCI 0656	300129	TS	Or39	H49	B1	-	A	+/-[c]	-	-	+	+	-	-	-	-	-	+

Ref. No.	Animal	Isolation method	O-type/O-RFLP type	H-RFLP-type	Lineage	*ipaH*	*stx*	*espP*	*escN*	*eae*	*cdtB*	*cnf*	*fimA*	*sfaD/E*	*neuB*	*iutA*	*ehxA*	*gafD*
MCI 0523	300136	TS	O45 (Or14)	H-/NT	B2	-	-	-	-	-	+	+	-	-	-	+	-	+
MCI 0528	300136	TS	Or37	H+(NT)1	B1	-	B	C	-	-	-	-	+	-	-	-	319*	-
MCI 0529	300136	TS	O15 (Or38)	H+(NT)8	D	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0534	700140	TS	Or37	H+(NT)1	B1	-	B	C	-	-	-	-	+	-	-	-	319*	-
MCI 0535	700140	TS	Or39	H+(NT)2	B1	-	A	NEW[c]	-	-	+	+	+	-	-	-	-	+
MCI 0540	700140	TS	Or26	H+(NT)9	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0541	700140	TS	Or36	H47	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0543	700140	TS	Or40	H+(NT)3	B1	-	-	-	-	+/-[a]	+	+	+	-	-	+	-	+
MCI 0544	700217	TS	O43 (Or41)	H25	B1	-	-	+/-[b]	+	-	+	-	+	-	-	-	106*	-
MCI 0546	700217	TS	Or17	H8	B2	-	C	G	-	-	-	-	+	-	-	-	-	-
MCI 0549	700217	TS	O103	H2	B1	-	-	-	+	ε	-	-	+	-	-	-	106*	-
MCI 0551	700217	TS	ONT (Or7)	H+(NT)2	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0557	700217	TS	O43 (Or10)	H2	B1	-	-	-	-	-	-	-	+	-	-	-	-	+
MCI 0561	700217	TS	O118 (Or42)	H16NM	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0564	700217	TS	Or43	H+(NT)7	B1	-	-	-	-	-	-	-	+	-	-	-	-	+
MCI 0566	700217	TS	Or44	H8	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0657	700217	TS	~O147	H-/NT	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0658	700217	TS	O103	H2	B1	-	-	-	+	-	-	-	+	-	-	-	106*	-
MCI 0567	400354	TS	Or45	H2	B1	-	C	-	-	-	-	-	+	-	-	-	-	-
MCI 0568	400354	TS	ONT (Or7)	H+(NT)2	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0573	400354	TS	Or46	H2	B1	-	-	-	-	-	-	+	+	-	-	-	-	-
MCI 0574	400354	TS	O149	H12	D	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0575	400354	TS	O58 (Or47)	H40	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0578	400354	TS	Or48	H+(NT)4	B1	-	-	-	-	-	-	-	-	-	-	-	-	+
MCI 0579	400354	TS	-[e]	H5	B2	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0580	400354	TS	ONT (Or7)	H+(NT)2	B1	-	-	-	-	-	-	-	-	-	-	-	-	+

Ref. No.	Animal	Isolation method	O-type/O-RFLP type	H-RFLP-type	Lineage	*ipaH*	*stx*	*espP*	*escN*	*eae*	*cdtB*	*cnf*	*fimA*	*sfaD/E*	*neuB*	*iutA*	*ehxA*	*gafD*
MCI 0581	301437	TS	O58 (Or47)	H40	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0586	301437	TS	O149*	H12	D	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0589	301437	TS	Or49	H5	B2	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0590	301437	TS	Or50	H16NM	A	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0591	301437	TS	O45 (Or14)	H-/NT	B2	-	-	-	-	-	+	+	-	-	-	+	-	+
MCI 0593	301437	TS	Or51	H-/NT	A	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0594	401473	TS	O43 (Or41)	H25	B1	-	-	+/-[b]	+	-	+	-	+	-	-	-	106*	-
MCI 0595	401473	TS	O43 (Or10)	H2	B1	-	-	-	-	-	-	-	+	-	-	-	-	+
MCI 0597	401473	TS	Or46	H2	B1	-	-	-	-	-	-	+	+	-	-	-	-	-
MCI 0602	401473	TS	O147 (Or52)	H+(NT)2	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0606	401473	TS	O118 (Or42)	H16NM	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0608	401473	TS	Or46	H-/NT	B2	-	-	-	-	-	+	+	-	-	-	+	-	+
MCI 0611	401473	TS	Or53	H9	A	-	-	-	-	-	-	-	-	-	-	+	-	+
MCI 0613	401473	TS	O147 (Or52)	H-/NT	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0614	401473	TS	O5/O113	H-/NT	A	-	+[f]	-	-	-	-	-	-	-	-	-	-	-
MCI 0659	401473	TS	Or17	H8	B1	+/-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0615	200522	TS	ONT (Or7)	H+(NT)2	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0619	200522	TS	O147 (Or52)	H-/NT	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0621	200522	TS	O147 (Or52)	H+(NT)2	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0625	200522	TS	Or54	H25	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0626	200522	TS	Or48	H5	B2	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0627	200522	TS	Or46	H2	B1	-	-	-	-	-	-	+	+	-	-	-	-	-

Ref. No.	Animal	Isolation method	O-type/O-RFLP type	H	Lineage	*ipaH*	*stx*	*espP*	*escN*	*eae*	*cdtB*	*cnf*	*fimA*	*sfaD/E*	*neuB*	*iutA*	*ehxA*	*gafD*
MCI 0628	700217	RS	ONT (Or17)	H8	B1	-	C	G	-	-	-	-	+	-	-	-	-	-
MCI 0629	700217	RS	Or55	H+(NT)5	D	-	-	+/-[b]	-	-	-	-	+	-	-	-	-	-
MCI 0631	400354	RS	Or55	H+(NT)5	D	-	-	+/-[b]	-	-	-	-	+	-	-	-	-	-
MCI 0632	400354	RS	Or56	H2	B1	-	C	-	-	-	-	-	+	-	-	-	-	-
MCI 0635	301437	RS	O80	H+(NT)6	D	+/-	-	B	+	γ	-	-	+	-	-	+	106*	-
MCI 0637	301437	RS	Or50	H16NM	A	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0638	401473	RS	Or55	H+(NT)5	D	-	-	+/-[b]	-	-	-	-	+	-	-	-	-	-
MCI 0640	401473	RS	O118 (Or42)	H16NM	B1	-	-	-	-	-	-	-	+	-	-	-	-	-
MCI 0641	200522	RS	Or54	H25	B1	-	-	-	-	-	+	+	+	-	-	+	-	+
MCI 0642	200522	RS	Or55	H+(NT)5	D	-	-	+/-[b]	-	-	-	-	+	-	-	-	-	-
MCI 0644	200522	RS	-[e]	H2	B1	-	A	A	+	-	-	-	+	-	-	+	106*	-

Table 4.5: Molecular analysis of bovine *E. coli* isolates isolated during the course of this study: Isolates which yielded a band of similar molecular weight and intensity to the reference strains are noted with a '+'. Those yielding a band of lower intensity, suggesting either artifactual non-specific amplification or polymorphisms in the primer-binding site are denoted '+/-'. RFLP-patterns are included for *eae*, *stx*, *espP* and *ehxA*. No isolates were found to carry genes encoding *aat*, *bfpA*, F5, F41, *sfaD/E*, *kfiC*, *neuB* or *draA* and hence, these genes are omitted from the table.

Notes: "Lineage"= Phylogenetic group determined by method of Clermont *et al.* (2000). Isolation methods: F=faecal, RS=rectal swab, TS=Tissue section. ND=not yet determined, NT=Untypeable, NA=not amplified.

For *stx*, *espP*, *eae* and *ehxA*, RFLP patterns are also given. For *ehxA*: type 106* and 319* were assigned to isolates which exhibited *ehx*-RFLP patterns indistinguishable from MCI 0106 and MCI 0319 respectively.

a: an amplicon of the expected size was produced by the method of Aranda *et al.* (2004) but not by the method of Ramachandran *et al.* (2003). Therefore this isolate may be intimin positive, but it is untypeable by the methods described here.

b: an amplicon of the expected size was produced by the method of McNally *et al.* (2001) but not by the method of Brunder *et al.* (1999). Therefore this isolate may be *espP* positive, but it is untypeable by the methods described here.

c: *espP* RFLP-pattern did not match any of those given by Brunder *et al.* (1999)

d: *stx*2 positive by the method of Aranda *et al.* (2004) but the *stx* RFLP-pattern did not match any of those given by Bastian *et al.* (1998)

e: O-RFLP-PCR yielded no amplicon.

f: this isolate was found to be *stx* positive but the *stx* type remains to be determined.

4.2.3 Associations between *E. coli* subtypes and colonisation patterns

In order to predict associations between virulence factor genes among bovine GIT-resident *E. coli* and to give an overview of the genotypic nature of bovine *E. coli* isolates PCR-analysis data was combined from the two samples shown in Tables 4.2 and 4.4. In total, data from 132 *E. coli* isolates from 47 animals were included in this analysis, representing 67 O-RFLP types and 37 H-RFLP types. In total, 93 distinct O/H combinations were observed. Serologically untypeable isolates exhibiting O-RFLP pattern Or7 and a pattern corresponding to O45 (Or14) were the most commonly isolated O-RFLP types (recovered from 9 and 7 animals respectively).

Organisms which yielded no amplicon after *fliC* PCR were recovered from 13 animals, suggesting either that the strains encode no *fliC* gene or encode a variant *fliC* gene which is not amplified by the procedure described here. Ramos Moreno *et al.* (2006) noted that this protocol does not amplify *fliC* H17, H53 or H54. Eight *fliC* sequences that did not match reference types were identified, designated H+(NT1-NT8). Isolates giving a pattern that matched H21, H+(NT2) and H19NM were recovered from 10, 7 and 6 animals respectively.

The *fimA* gene was most commonly detected, occurring in 80.3% of strains. 32.6% of strains analysed were found to encode *fimA* and no other factor. Next most commonly detected were *cdtB* and *gafD*, each detected in 31.8% of isolates, then *iutA* and *cnf*, each detected in 30.3% of isolates, with all 4 of these genes present in 23.5% of *E. coli* analysed.

One or more genes encoding verocytotoxin were detected in 32 of the isolates analysed (24%). Of these strains, 16 carried genes with an RFLP pattern matching *stx*1, 6 strains exhibited a pattern matching *stx*2, 4 strains exhibited a pattern matching $stx2_{c/d}$ and 7 strains exhibited a pattern matching $stx2_e$ and 2 strains yielded amplicons which were not cut suggesting $stx2_g$. One strain (MCI 0441) gave a pattern which did not match reference patterns, possibly a previously undescribed variant *stx* gene and one strain was *stx* positive but not yet typed (MCI 0614). Two strains (MCI 0455 and MCI 0487) were found to carry three different *stx* variant genes. The carriage of three *stx* subtypes by bovine isolates has been noted previously (Bertin *et al.*, 2001). Twenty strains (15.2%) were found to encode *ehxA*, 11 of which were provisional type 319 and 9 of type 106.

18 strains (13.6%) were found to encode *espP*, of which 6 were of RFLP type C, 3 each of types B and G, 2 of type D, one of type A and 2 were unmatchable to reference patterns (MCI 0535, MCI 0656) with one yet to be determined (MCI 0522). RFLP-type C (likely to refer to *espP* type β) is one of the types found to be proteolytically inactive against pepsin A (Brockmeyer *et al.* 2007). Whether EspP (β) has other functions has not been determined, but as this was the most common type found among this panel of bovine *E. coli* isolates, it may have a function in colonisation. Conventional EspP (α) has been implicated in cellular adherence in the bovine host (Dziva *et al.*, 2007); however, proteolytic activity of the protease has not yet been shown to be necessary for EspP function as an adhesin.

13 strains (9.8%) were found to encode *escN* genes and 10 strains (7.6%) were found to encode an *eae* gene, of which 5 were type β, one each were type γ, ε and θ and 2 were untypeable as the PCR described by Ramachandran *et al.* (2003) failed to amplify genes from these strains.

Two strains were found to encode *papC* and only one strain (MCI 0521) was found to encode *neuB* and *sfaD/E*. No strains were found to encode *ipaH*, although 5 strains yielded an ambiguous faint band at the expected size. No strains were found to encode *aat*, *bfpA*, *draA*, *kfiC* or genes encoding F5 or F41 fimbrial subunits.

Virulence factor-encoding genes detected in more than 10 isolates were included in a *z*-test, using a null hypothesis that the proportion of strains encoding gene 1 which also encode gene 2 does not differ from the proportion of strains which encode gene 2 in the population as a whole. Results of the *E. coli* phylogenetic group determination by the method of Clermont *et al.* (2000) and the assignation of candidate rectal tropism (Table 4.4) were also included in the test for associations. The *z*-values were generated according to the binomial approximation of the normal distribution using the equation:

$$Z = \frac{(p - \pi)}{\sqrt{[\pi(1 - \pi)/n)]}}$$

where π refers to the proportion of strains encoding gene 1 of the total sample and *p* is given by the proportion of strains also encoding gene 2 of the total number of strains encoding gene 1. *Z* statistics were compared to a standard table of *Z* values, to give *P*-values indicating whether the null hypothesis should be accepted. *P*-values less than 0.05 were considered significant, although *P*-values below 0.01 were returned for certain associations. As well as detecting the association between virulence factors, this test was used to relate virulence factors to colonisation patterns (determined to be rectally tropic or not rectal tropic) Results of this test are given in Table 4.5.

The results of this analysis, presented in Table 4.5, indicate that there are positive associations between *stx*, *ehxA* and *espP*. Positive associations were also identified between *espP* and *escN*, *eae* and *escN* and *eae* and *ehxA*. Positive associations were also found between *cdtB*, *cnf*, *iutA* and *gafD*.Strains encoding *espP*, *escN*, *eae* or *ehxA* were more likely to encode *fimA* and less likely to encode *cdtB* or *gafD*. Strains encoding *escN*, *eae* or *ehxA* were also less likely to encode *cnf*. Strains encoding *cdtB*, *cnf*, *iutA* or *gafD* were significantly less likely to encode *fimA*.

Candidate rectal tropic strains were found to be more likely to encode any of *stx*, *espP*, *escN*, *fimA* and *ehxA* and were less likely to encode *cnf*, *iutA* or *gafD* ($P<0.01$). Non-rectal tropic strains were significantly more likely to encode *cnf*, *iutA* or *gafD* and were less likely to encode *stx* ($P<0.01$).

The assignation of phylogenetic groups revealed that the vast majority (72.7%) of *E. coli* isolates analysed are of Group B1. Isolates encoding *stx* or *espP* were positively associated with the B1 phylogenetic group. It was also clear that isolates which colonised cattle in significant numbers (those designated “RT:Yes” [rectal colonists] or “RT:No” [those colonising other sites]; Table 4.5) were almost exclusively of the B1 phylogenetic group and were significantly more likely to encode *stx*, *espP* or *ehxA*. Phylogenetic Group A made up 9.8% of the total number of isolates. The *cdtB*, *cnf*, *fimA*, *iutA* and *gafD* genes were detected less frequently among isolates of Group A. Group B2 accounted for 9.1% of isolates. Isolates of this phylogroup more often encoded *cdt*, *cnf*, *iutA* or *gafD*. The remaining 8.3% of isolates were of phylogenetic Group D. These isolates all encoded *fimA*, whereas *stx*, *cnf* and *gafD*

were found significantly less frequently in these strains. Of the 10 isolates of this Group D, only 2 were found to encode factors other than *fimA*.

	stx	*espP*	*escN*	*eae*	*cdtB*	*cnf*	*fimA*	*iutA*	*ehxA*	*gafD*	A	B1	B2	D	RT: Yes	RT: No
stx		4.11	-0.44	-0.53	-1.38	-1.16	-1.37	-0.73	3.18	-1.82	-0.44	3.25	-0.34	-1	0.96	-1.41
espP	7.8		2.17	1.75	-2.11	-1.88	2.22	-1.88	5.73	-2.11	-0.52	2.33	-0.43	-0.33	2.41	-0.1
escN	-1.17	3.07		7.37	-2.29	-4.17	3.11	-0.99	6.75	-4.43	0.67	0.92	-1.1	-0.08	2.77	-1.23
eae	-1.88	3.26	9.7		-3.04	-2.79	2.51	-0.04	4.35	-3.04	1.23	-0.6	-1.1	0.2	0.29	-2.2
cdtB	-1	-0.8	-0.62	-0.62		7.95	-5.38	6.64	-1.3	7.17	-1.19	-0.29	1.77	-1	-0.34	0.81
cnf	-0.89	-0.76	-1.19	-0.61	8.44		-4.61	7.18	-1.89	7.75	-1.19	-0.05	1.92	-0.7	-0.91	1.27
fimA	-0.21	0.18	0.18	0.11	-1.15	-0.92		-0.79	0.46	-1.15	-0.28	1.23	-0.64	0.25	0.22	0.06
iutA	-0.56	-0.76	-0.28	-0.01	7.05	7.18	-3.96		-0.02	6.7	-0.89	-1.15	1.92	-0.4	-0.91	1.27
ehxA	5.38	5.11	4.25	2.08	-3.04	-4.17	5.1	-0.04		-4.43	-0.59	3.8	-1.1	-0.4	3.87	-0.94
gafD	-1.31	-0.8	-1.19	-0.62	7.17	7.3	-5.38	6.32	-1.89		-0.9	0.24	1.49	-0.71	-0.91	1.41
A	-1.17	-0.73	0.67	0.93	-4.43	-4.17	-4.86	-3.11	-0.93	-3.36					-0.91	-1.23
B1	0.65	0.25	0.07	-0.03	-0.08	-0.01	1.59	-0.3	0.45	0.07					0.22	0.43
B2	-1	-0.66	-1.19	-0.91	7.17	7.3	-12.2	7.3	-1.89	6.01					0.09	-2.2
D	-3.2	-0.56	-0.09	0.18	-1.9	-2.92	5.1	-1.67	-0.76	-3.16					-0.91	-1.05
RT: Yes	3.4	4.5	3.65	0.29	-1.64	-4.17	5.1	-4.17	8.09	-4.43	-1.19	3.8	0.11	-1		
RT: No	-2.05	-0.07	-0.67	-0.91	1.62	2.41	0.6	2.41	-0.81	2.83	-0.67	3.13	-1.1	-0.48		

Table 4.6: Test for association between genotypes and colonisation characteristics among bovine *E. coli* isolates: *Z*-test was carried out as detailed in Section 4.2.3. Where $Z>1.96$, genes/characteristic vertical column are significantly more likely to occur in strains which also attain the gene/characteristic in the horizontal row ($P<0.05$). Where $Z<-1.96$, genes/characteristics given in the vertical column are significantly less likely to occur in strains which also attain the gene/characteristic in the horizontal row ($P<0.05$). Where $Z>2.58$ or where $Z<-2.58$, the significance of positive or negative association increases ($P<0.01$). Positive associations are highlighted in dark green ($P<0.01$) and light green ($P<0.05$). Negative associations are highlighted in red ($P<0.01$) and orange ($P<0.05$). Grey denotes that there was no statistically significant interaction between the two characteristics. White spaces denote characteristics that are mutually exclusive and invalid for comparison.

A/B1/B2/D – refers to the phylogenetic group, as determined by the methods of Clermont *et al.* (2000).

RT:Yes/RT:No – refers to strains identified as potentially rectal tropic (Table 4.4).

4.3 Discussion

The first objective of this analysis was to conduct genotypic characterisation of selected resident *E. coli* of the bovine GIT, in order to determine whether *E. coli* O157:H7 represents a typical or an atypical gastrointestinal colonist of cattle. PCR and microarray analysis revealed that *E. coli* of the bovine GIT represent a heterogeneous group, based on the carriage of virulence factors and genomic regions. CGI microarray confirmed that the core component of the *E. coli* genome, present in *E. coli* O157:H7 and *E. coli* K12 MG1655 is well conserved among bovine isolates, but that the O157- and K12- specific genomic regions (the O-islands and K-islands) are areas of heterogeneity. A large proportion of these genomic islands are comprised of bacteriophage or prophage-related elements (Perna *et al.*, 2001;Hayashi *et al.*, 2001b). It has been well documented that genomic diversification in *E. coli*, including the acquisition of factors which could lead to increased virulence, is largely dependent on the recombination events performed by bacteriophages, although the majority of transduced elements remain cryptic (Ogura *et al.*, 2006;Tobe *et al.*, 2006).

The role of bacteriophages in virulence factor gene transduction is further evidenced by the widespread prevalence of *stx* genes among bovine isolates, as *stx* genes are carried on temperate bacteriophages (Konowalchuk *et al.*, 1977;Scotland *et al.*, 1983). 24.2% of strains isolated in this study were found to encode *stx*. Strains encoding *stx* were isolated from 24 of the 47 animals (51.1%). This highlights the potential biohazard posed by faecal matter from healthy cattle. Varied estimates of the prevalence of STEC in cattle have been made: Previous studies have found STEC to be present in 0-71% of animals (Wilson *et al.*, 1992;Cerqueira *et al.*, 1999) and 0-100% of herds (Wilson *et al.*, 1992;Cobbold and Desmarchelier, 2000;Thran *et al.*, 2001a;Jenkins *et al.*, 2002). In weaned beef cattle, similar to those analysed in this study, estimates of the proportion of shedding animals range from 5.8% to 70% (Pradel *et al.*, 2000;Thran *et al.*, 2001b). Comparisons of the prevalence observed with this study with those identified in other studies is complicated by the fact that the methods used to identify the prevalence of STEC vary from study to study and most are based on faecal sampling techniques which have been shown to yield different populations of *E. coli* to those isolated directly from mucosal surfaces (Greenquist *et al.*, 2005). It must be noted that the major objective of this study was to link genotype with colonisation site, rather than survey the genotypes of *E. coli* present in cattle.

PCR-based detection of discriminative factors revealed that bovine resident *E. coli* broadly cluster into 3 main groups, carrying:-

1) *fimA* and none of the additional virulence factor-encoding genes tested for in the PCR-screen (matches genotypic pattern of non-pathogenic *E. coli*)
2) a combination of *cdt*, *cnf*, *iutA* and *gafD* (ExPEC related genes)
3) combinations of *stx*, *ehxA*, *escN* and *eae* and *fimA* (genes associated with EHEC and EPEC strains).

In assigning *E. coli* to these groups, it must be remembered that the virulence factors detected in this study represent only a subset of the virulence factors encoded by *E. coli* and that even the strains assigned to group 1 may carry virulence factors which are not detected in the gene-screening procedure described in this study and must still be considered potential pathogens before more extensive characterisation is carried out.

It is tempting to assume that isolates of group 3 are generally present in healthy cattle, but absent from healthy humans, as these include EHEC which are known human pathogens. However, this is not yet proven. Sampling and detection of EHEC-related virulence factors among *E. coli* of the human gastrointestinal tract has not been performed to a great extent, although it has been shown that *E. coli* O157:H7 can exist in the human GIT without overt clinical manifestations, especially in those who face increased likelihood of exposure to *E. coli* O157:H7, such as individuals involved with regular contact with cattle or environments contaminated by cattle (Wilson *et al.*, 1996;Silvestro *et al.*, 2004).

Strains encoding *cdtB*, *cnf* and *gafD* (F17 fimbriae) were found to constitute a large proportion of the *E. coli* population detected in animals analysed in this study. Strains encoding these factors have been linked to cases of enteric disease in calves (Shimizu *et al.*, 1987;Lintermans *et al.*, 1988;Orden *et al.*, 1999). The prevalence of such strains among the natural microbiota of the healthy animal has been noted by others (Blanco *et al.*, 1998a;Blanco *et al.*, 1998b;Güler *et al.*, 2008) strongly suggests that these strains function as opportunistic pathogens, relying on a compromised host for pathogenesis and are non-pathogenic under normal circumstances.

The panel of *E. coli* described here were devoid of *aat* genes, which corroborates the finding that EAEC are generally not detected in ruminants (Cassar *et al.*, 2003). The *bfpA* gene was not detected in any isolates suggesting that "typical" EPEC are not regularly found in cattle; however, atypical EPEC-like strains (those encoding *eae* but not *bfpA* or *stx*) represented 6.8% of the strains isolated. Atypical EPEC are more closely related to EHEC than to conventional EPEC strains such as EPEC O127:H6 (Trabulsi *et al.*, 2002). Strains encoding F5 (K99) and F41 fimbriae, common among bovine ETEC pathogens (Section 4.2.1.3) were not found. This could suggest that these strains persist in a reservoir other than the bovine gut and are unlikely colonise asymptomatically.

The genes put forward as mediators of persistence of *E. coli* strains in humans (Nowrouzian *et al.*, 2001) related to the formation of P and S fimbriae, along with capsular antigens K1 and K5 were rarely detected among the isolates analysed. This could suggest that mechanisms of *E. coli* persistence in ruminants differ from persistence in the human GIT, although characterisation of the capsular antigens of strains analysed here was not performed during this study. It may be that K1 and K5 capsular antigens are common only among human *E. coli* isolates, while other as yet undefined capsular antigen-types may have a role in colonisation of the bovine GIT, although genes likely to encode K1 and K5 capsular antigens have been detected in the genomes of *E. coli* related to cases of bovine mastitis and therefore are likely to have originated from bovine faeces (Kaipainen *et al.*, 2002). The aerobactin-receptor gene (*iutA*) was found to be prevalent among bovine *E. coli* isolates, which might suggest that the mechanisms of iron acquisition could also be important in colonisation of cattle. In each case, detection of an individual virulence factor does not directly implicate that factor in colonisation of the bovine gastrointestinal tract, but the relation of these factors to colonisation patterns may suggest that the factors may be worthy of further investigation to determine the role in colonistation (if any) that these factors fulfil. The genes detected in this study may also be markers for other as yet undefined factors which define the colonisation pattern. Further genomic and functional analysis of these strains may reveal the true factors which confer the colonisation patterns observed.

It must be noted that gene detection by PCR is fallible. While attempts were made to conduct analysis using previously validated PCR primer sets and conditions, differing specificities of

PCR reactions are unavoidable. In general, probe-based methods are more reliable for gene detection than PCR-based methods. Both false-negative and false-positive results are more likely with PCR. False-negative results may be obtained with as little as a single nucleotide change at a primer-binding site while false-positive results can be obtained due to cross contamination or by non-specific priming and amplification of irrelevant genomic loci. PCR does however provide an accessible, low cost, high throughput method of gene detection, requiring little downstream analysis or sophisticated equipment. Gene detection by microarray and nanoarray partially corroborated PCR results, but absolute genotyping of bovine isolates would only be possible by more extensive methodology, such as whole genome sequencing.

The second major objective of this study was to assess the colonisation patterns of bovine resident *E. coli*. The data obtained suggest that colonisation is non-uniform. *E. coli* was found in greater numbers at the terminal rectum and proximal colon. As mentioned previously, *E. coli* O157:H7 colocalises to lymphoid follicle-dense regions in both human and bovine infections, namely the recto-anal junction in cattle and the ileal Peyer's patches in humans. Data presented here appears to suggest that the terminal ileum is generally less densely colonised by *E. coli* in cattle than other sites, although this region is densely populated by lymphoid follicles (as is the terminal rectum). Thus, if *E. coli* shows a general preference for lymphoid follicle-dense regions, other factors could act to prevent *E. coli* from establishing at the ileum. The ileum may present an unfavourable physiochemical environment for the persistence of *E. coli* at this site. It is possible that *E. coli* is not tolerated by the host immune system at the ileum, especially as the level of cells associated with host immunity are found in great numbers at this site. Ileal colonisation may be a function associated exclusively with virulence: it is clear that ileal colonisation in humans is important for the pathogenesis of *E. coli* O157:H7 so prevention of ileal colonisation could be an important mechanism by which cattle generally resist EHEC pathogenesis. The gastrointestinal location of *E. coli* O157:H7 in humans carrying the organism asymptomatically remains to be determined, although it is feasible that the organism is carried at sites other than the ileum in these individuals.

The major limitation of this analysis is that sampling of sites in the upper regions of the bovine gastrointestinal tract is only possible to perform at a single timepoint (post-mortem) without resorting to invasive sampling procedures. Initial colonisation of sites in the upper gastrointestinal sites by *E. coli* O157:H7 has been noted (Cray, Jr. and Moon, 1995;Brown *et*

al., 1997;Dean-Nystrom *et al.*, 1998a;Laven *et al.*, 2003), while persistence has been related to colonisation at the terminal rectum (Naylor *et al.*, 2003;Low *et al.*, 2005;Lim *et al.*, 2007) (as described in Section 1.3.3). In all cases, it is impossible to determine whether the *E. coli* isolated during this study represent persistent colonists or were carried transiently. However, calves that were sampled by rectal swabbing 3 weeks prior to euthanasia were found not to contain the strains of *E. coli* which were found to be abundant at post mortem, suggesting that strain-turnover of *E. coli* in the bovine GIT is a fairly rapid occurrence; however, the method of rectal swabbing was not consistent with the sampling of rectal tissue sections, in that PBS washing was not performed. It is possible that the strains isolated by rectal swab represent transient faecal bacteria and true resident isolates may have been missed in this initial isolation. Others have found that resident *E. coli* strains of the human GIT have persisted for more than 3 years (Wold *et al.*, 1992;Nowrouzian *et al.*, 2003). As yet analysis of persistence of *E. coli* strains in cattle has focussed on *E. coli* O157:H7 (Akiba *et al.*, 2000;Lejeune *et al.*, 2004).

In a few cases, colonisation patterns of *E. coli* isolates were apparently conserved when isolates that were indistinguishable by molecular analysis were found in multiple animals. These included the organisms designated *E. coli* Or37:HNT1 represented by isolates MCI 0517, MCI 0528 and MCI 0534 (*stx*2+, *ehxA*: type 319, *fimA*+) which were found only in the rectum in three animals. Similarly, isolates designated *E. coli* O43(Or41):H25 (*escN*+, *cdtB*+, *ehxA*: type 106) represented by isolates MCI 0544 and MCI 0594, were recovered from two animals, restricted to the rectum in each case. In the youngest animal analysed, an organism designated *E. coli* O26:HNT1 MCI 0483 (*escN*+, *eae*β, *espP*: type D, *fimA*+, *ehxA*: type 106) was found at 10000-fold density at the rectum than at the proximal colon or the ileum. This observation is in keeping with the findings of Shaw *et al.* (2004) who noted that shedding of *E. coli* O26 was especially associated with very young calves.

The strains described in the paragraph above represent the most promising candidate rectal tropic *E. coli* strains recovered during the study, which may be worthy of further investigation. Although rectal colonisation is only predicted for these organisms and not yet confirmed by rechallenge and reisolation, it is interesting to note that prevalence at the rectum above other sites correlated positively with the carriage of *stx*, *eae*, *escN*, *espP* and *ehxA* genes. Carriage of *stx* or *eae* was found to negatively associate with strains showing no apparent site-preference.

These observations contrast with finding that isolates encoding ExPEC-related virulence factors and those encoding no detected virulence factors except for *fimA* were found to be evenly distributed between the sites analysed. The most obvious example of this was ExPEC-related *E. coli* ONT (Or7):HNT2 (*cdtB*+, *cnf*+, *fimA*+, *iutA*+, *gafD*+) which was recovered from five animals and was not found to associate with any site in preference to others. To refine knowledge regarding the colonisation pattern of each organism oral challenges of calves may be a suitable method. Such methodology may also give important information on the colonisation behaviour of strains to be assessed for utility as a probiotic organism (as discussed further in Chapter 5).

It is possible that strains of EHEC-related genotypes are highly adherent and that they encode factors which aid colonisation and persistence at the terminal rectum. EHEC factors involved in the pathogenic process in humans could be the same factors required for colonisation of the bovine terminal rectum, as has been suggested for intimin, Shiga-like toxin and the EspP protease (Robinson *et al.*, 2006;Sheng *et al.*, 2006b;Dziva *et al.*, 2007). The trait could depend on other, as yet uncharacterised factors associated with EHEC-related genotypes. It is equally plausible that strains of EHEC-related genotypes lack factors allowing them to colonise and persist at sites other than the terminal rectum in the bovine host, while strains of other genotypes are able to colonise these regions effectively or may be tolerated by the host. *E. coli* O157:H7 has been observed to form T3SS-dependent A/E-lesions at the bovine terminal rectum (Naylor *et al.*, 2005). It could also be that the host responds more aggressively to A/E-lesion-forming or shiga-toxigenic bacteria at sites in the upper gastrointestinal tract, while such strains are tolerated at the bovine terminal rectum. To date, no extensive analysis has been conducted of the persistence of non-EHEC- related *E. coli* in ruminants.

To summarise, data presented in this chapter highlights the diversity and potential pathogenicity of *E. coli* of the bovine GIT. There is some evidence to suggest that *E. coli* generally colonises the terminal rectum and proximal colon to a greater extent than the proximal rectum, distal colon or ileum. EHEC-related genotypes were most strongly associated with colonisation of the terminal rectum above other sites in the bovine gastrointestinal tract, while strains of ExPEC-related or those lacking established virulence factors generally exhibited a more even distribution throughout the gastrointestinal tract.

Chapter 5: Evaluation of bovine resident *E. coli* as potential probiotic agents

5.1 Introduction

As mentioned previously, the bovine gastrointestinal tract (specifically the bovine terminal rectum) provides a reservoir for *E. coli* O157:H7. It is envisaged that effective control of EHEC colonisation of cattle could prevent or reduce transmission to man and hence reduce the incidence of disease in humans. Efforts have been made to reduce prevalence of EHEC in finished food products. Improved detection methods have been developed and the adoption of these methods may prevent contaminated articles reaching the consumer (Bennett *et al.*, 1996). Pre-harvest control would be ideal, as human EHEC infections have also been related to contact with farm animals or environments contaminated with animal faeces. Numerous strategies have been put forward to reduce the carriage of *E. coli* O157:H7 by cattle and are currently being evaluated for that purpose (discussed in detail in Section 1.3.4).

The application of bacteriocin-producing *E. coli* has recently been proposed as an option for reduction of EHEC prevalence in cattle (Zhao *et al.*, 1998;Tkalcic *et al.*, 2003;Zhao *et al.*, 2003;Schamberger *et al.*, 2004). Bacteriocins are molecules produced by one bacterium, which may inhibit another susceptible bacterium. Bacteriocins include medium to large proteins (colicins) and small peptides (microcins).

More than 25 different *E. coli* colicins have been characterised to date, with molecular masses ranging from 29-75kDa (Cursino *et al.*, 2002). Colicins are normally encoded on plasmids which also encode proteins conveying immunity to the colicin encoded (Hardy *et al.*, 1973). Expression of colicins is induced by stressors such as a lack of nutrients and bacterial detection of DNA damaging agents triggering the SOS response system. Most colicins are not normally secreted and are generally released on lysis of a colicin producing cell, often facilitated by a lysis protein which co-expressed with the functional colicin. Colicins may target specific receptors on sensitive strains and active uptake by Ton or Tol translocation systems has been observed (Cascales *et al.*, 2007).

Microcins can be distinguished from colicins due to their low molecular weight (<10kDa) (Asensio and Pérez-Díaz 1976). Microcin production and immunity genes may be chromosomally or plasmid-encoded (Duquesne *et al.*, 2007). The synthesis of the majority of microcins occurs in the stationary growth phase and has not been found to be induced by

agents that trigger the SOS response. Microcin release is not lethal to the producer as specific export systems are utilised (Hwang *et al.*, 1997;Delgado *et al.*, 1999;Pavlova and Severinov, 2006).

It was envisaged that strains acquired from the bovine host may be suitable candidates for investigation as potential probiotic organisms. These strains are likely to colonise the bovine GIT effectively without pathogenesis as all were isolated from healthy animals. As competition at the mucosal surface is manifested in a number of different ways; the simplest form being "niche-occupation" by growth, competition for nutrients and binding surfaces, it was hypothesised that *E. coli* isolated from the bovine host (particularly those colonising the bovine terminal rectum) may have increased persistence and occupation of the niche colonised by *E. coli* O157:H7 (i.e. the bovine terminal rectal mucosal epithelium). Bacterial isolates studied include a group of strains provided by Stuart Naylor (MCI 0308-MCI 0412) from animals in which *E. coli* O157:H7 (MCI 0010) was found to colonise poorly after oral challenge (Stuart Naylor, Personal Communication). It was deduced that the *E. coli* present in these animals may have prevented EHEC colonisation, although there are many other factors which could have acted against establishment of EHEC colonisation in this instance.

Screening for bacteriocin-encoding genes by PCR was performed to predict the carriage of bacteriocins likely to be effective against *E. coli* O157:H7. Colicin E7 and microcins H47, J25 and V and have previously been shown to inhibit the growth of *E. coli* O157:H7 (Sable *et al.*, 2000;Schamberger and Diez-Gonzalez, 2004;Abercrombie *et al.*, 2006) and hence were targeted in the PCR-based gene-screening procedure. Colicin B and E2 were also screened for as these have been identified as common colicins of *E. coli* isolates (Schamberger and Diez-Gonzalez, 2004). Analysis also included *in-vitro* competition assays, to determine the potential inhibitory activity of each potential organism. The data presented in Chapter 4 aids the selection of an organism likely to effectively colonise the bovine GIT and the pathotyping information was used as a predictor of the potential biosafety of each prospective strain.

Note: The work presented in this chapter represents the collaborative efforts of the author and Joanna Laing (BSc, University of Edinburgh). The author and Joanna invested equal intellectual and practical input into the production of the results presented herein.

5.2 Results

5.2.1 Detection of bacteriocin-encoding genes by PCR

Detection of six bacteriocin genes was performed by PCR using previously described oligonucleotide primers (Gordon and O'Brien, 2006). Eighty-eight bovine *E. coli* isolates were screened for genes encoding colicins B, E2 and E7 and microcins H47, V, J25 by PCR. Results are summarised in Table 5.1 and supporting images are included in Appendix II. Ten strains (11.4%) possessed the genes for a colicin or a microcin. The most commonly detected genes were those encoding colicin E2 and microcin H47, each detected in 6 strains (6.8%). Colicin B was detected in 3 strains (3.4%). Microcin V, J25 or colicin E7 genes were not confidently detected, although no positive controls were available at the start of this study. PCR for microcin V yielded a faint band of the expected size from MCI 0345, which could suggest carriage of this gene, although sequencing would be required to verify this observation. Of the 10 bacteriocin-encoding strains, 5 were positive for two bacteriocins; microcin H47 in combination with either colicin E2 (3 strains) or Colicin B (2 strains).

The fragments amplified by the colicin E2 and microcin H47 primers from MCI0410 and MCI0319 respectively were sequenced to confirm their identity. In both cases, sequences were obtained that were highly homologous with the target gene. The sequence generated from the colicin E2 PCR product suggested that more then one gene may have been amplified in the PCR reaction, as “double sequence” was observed. This could suggest that multiple colicin E2 homologues may be encoded in the genome of MCI0410.

Bovine *E. coli* isolate	*mccH47*	*mccV*	*colB*	*colE2*
MCI 0104	-	-	-	-
MCI 0105	-	-	-	-
MCI 0106	-	-	-	-
MCI 0108	-	-	-	-
MCI 0109	-	-	-	-
MCI 0110	-	-	-	-
MCI 0114	-	-	-	-
MCI 0120	-	-	-	-
MCI 0121	-	-	-	-
MCI 0122	-	-	-	-
MCI 0160	-	-	-	-
MCI 0162	-	-	-	-
MCI 0164	-	-	-	-
MCI 0165	-	-	-	-
MCI 0167	-	-	-	-
MCI 0168	-	-	-	-
MCI 0169	-	-	-	-
MCI 0170	-	-	-	-
MCI 0171	-	-	-	-
MCI 0172	-	-	-	-
MCI 0173	-	-	-	-
MCI 0174	-	-	-	-
MCI 0176	-	-	-	-
MCI 0177	-	-	-	-
MCI 0179	-	-	-	-
MCI 0181	-	-	-	-
MCI 0182	-	-	-	-
MCI 0308	-	-	-	-
MCI 0319	+	-	-	+
MCI 0327	+	-	-	+
MCI 0344	-	-	-	-
MCI 0345	-	+/-	-	-
MCI 0354	-	-	-	-
MCI 0356	-	-	-	-
MCI 0362	-	-	-	-
MCI 0368	-	-	-	-
MCI 0369	-	-	-	-
MCI 0377	-	-	-	+
MCI 0378	-	-	-	-
MCI 0379	-	-	-	-
MCI 0382	-	-	-	-
MCI 0386	+	-	-	+
MCI 0392	+	-	-	-
MCI 0398	-	-	-	-
MCI 0404	-	-	+	+
MCI 0410	-	-	+	+
MCI 0430	-	-	-	-
MCI 0435	-	-	-	-
MCI 0437	-	-	-	-
MCI 0439	-	-	-	-
MCI 0441	-	-	-	-

Bovine *E. coli* isolate	MccH47	MccV	ColB	ColE2
MCI 0454	-	-	-	-
MCI 0455	-	-	-	-
MCI 0465	-	-	-	-
MCI 0483	-	-	-	-
MCI 0487	-	-	-	-
MCI 0490	-	-	-	-
MCI 0496	-	-	-	-
MCI 0501	-	-	-	-
MCI 0512	-	-	-	-
MCI 0517	-	-	-	-
MCI 0518	-	-	-	-
MCI 0523	-	-	-	-
MCI 0528	-	-	-	-
MCI 0529	-	-	-	-
MCI 0534	-	-	-	-
MCI 0535	-	-	-	-
MCI 0541	-	-	-	-
MCI 0544	+	-	-	-
MCI 0546	-	-	-	-
MCI 0549	-	-	-	-
MCI 0551	-	-	-	-
MCI 0557	-	-	-	-
MCI 0561	-	-	-	-
MCI 0564	-	-	-	-
MCI 0568	-	-	-	-
MCI 0575	-	-	-	-
MCI 0581	-	-	-	-
MCI 0586	-	-	-	-
MCI 0594	+	-	-	-
MCI 0595	-	-	-	-
MCI 0597	-	-	+	-
MCI 0602	-	-	-	-
MCI 0606	-	-	-	-
MCI 0608	-	-	-	-
MCI 0611	-	-	-	-
MCI 0615	-	-	-	-
MCI 0621	-	-	-	-
Total	**6**	**1 (+/-)**	**3**	**6**

Table 5.1: PCR-based screening of bovine *E. coli* isolates for bacteriocin-encoding genes: No strains were found to be positive for MccJ25 or ColE7 and hence these genes were omitted from the table. The band observed from MCI 0345 for MccV was faint and hence considered ambiguous (+/-). Mcc: microcin, Col: colicin, +: detected, -: not detected.

5.2.2 *In vitro* inhibition assays

5.2.2.1 Lawn-inhibition assays

Two methods were evaluated to obtain bacterial products for testing against potentially sensitive bacteria (as set out in Section 2.7). Bacterial supernatants were recovered by centrifugation and 10µl spots of this preparation were overlaid onto bacterial lawns. A second method employing filter-paper discs soaked in chloroform and laid onto bacterial colonies was performed in order to recover intracellular bacterial products as certain bacteriocins are non-secreted (as explained in Section 5.1). For the 5 strains tested initially, the supernatant method proved more sensitive as the lysate disc and was adopted as it proved less laborious. Results of the initial assessment of these methods are presented in Figure 5.1.

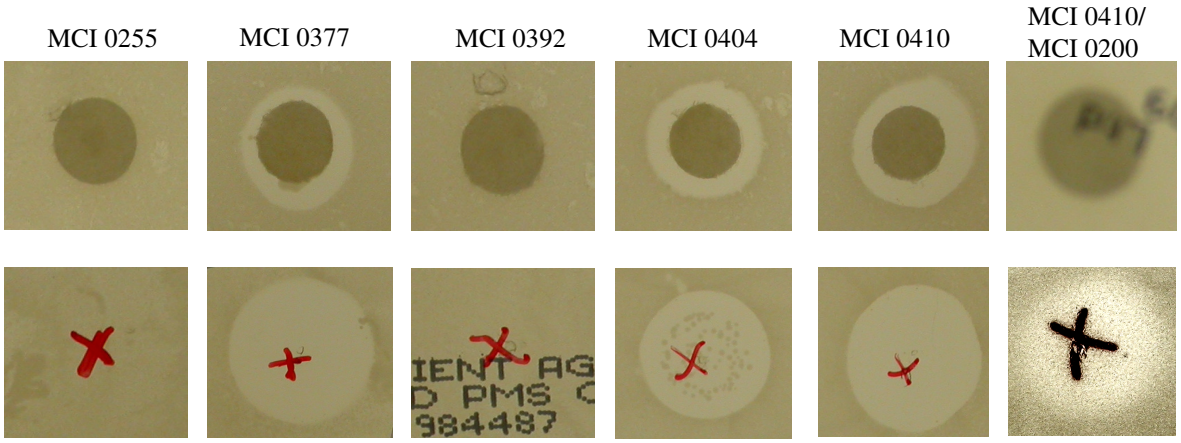


Figure 5.1: Assessment of methods for bacterial inhibitory activity detection: The upper images show the lysate-disc method of inhibition assessment, while the lower panels show the results of the supernatant spot-on-lawn method. Products from MCI 0255 were used as negative controls in each case. The rightmost panel shows the greater sensitivity of the spot on lawn method, as a zone of inhibition is observed for unconcentrated MCI 0410 supernatant on a MCI 0200 lawn, while no such zone of inhibition was observed using the filter-paper disc method. Within the area of inhibition caused by MCI 0404 breakthrough colonies were observed, which could suggest that the test strain may have acquired resistance to the inhibitory activity of MCI 0404 supernatant.

In total, nineteen strains were identified from which supernatant or bacterial lysate inhibited lawns of laboratory strain MCI 0255 (listed in Table 5.2). For these strains mitomycin C-induced culture was prepared and either culture supernatant or whole bacterial culture was centrifuged into chloroform to produce a supernatant/lysate mixture (Section 2.7.3, Materials & Methods). These products were concentrated 60-fold using an Amicon 3kDa-cutoff filter and tested against the panel of *E. coli* strains listed in Table 5.2. In all cases, supernatant and lysate prepared in the same manner from MCI 0255 was used as a negative control.

No single isolate was found to be able to cause clear zones of inhibition on all of the O157:H7 strains tested, which suggests that sensitivity to inhibitory compounds is not uniform among *E. coli* O157:H7 subtypes. This could indicate differing sensitivities to inhibitory substances among *E. coli* O157:H7 strains. Three potentially non-pathogenic bovine *E. coli* isolates (MCI 0105, MCI 0308 and MCI 0430) were also tested for sensitivity to bacterial products, to determine whether inhibitory *E. coli* strains would affect the natural microflora of the bovine GIT.

Initially, the best candidate for further inhibition studies proved to be MCI0410 as it was the only organism from which unconcentrated supernatant produced a visible zone of inhibition on a lawn of *E. coli* O157:H7 (MCI 0200) and concentrated supernatant inhibited four of the six *E. coli* O157:H7 (Δstx) strains. However, MCI 0410 supernatant inhibited MCI 0105 and MCI 0308, which are likely to be non-pathogenic members of the bovine gastrointestinal flora. Strains MCI 0483 and MCI 0512 exhibited greater selectivity against *E. coli* O157 strains tested. MCI 0483 and MCI 0512 were isolated long after work commenced using MCI 0410. The analysis which follows pertaining to MCI 0410 is yet to be performed for MCI 0483 or MCI 0512.

		Bacterial lawn									
		K12 lab strain	Bovine resident *E. coli* isolates			*E. coli* O157:H7 (Δstx) isolates					
		255	105	308	430	10	24	78	191	200	279
	255L	-	-	-	-	-	-	-	-	-	-
	255S	-	-	-	-	-	-	-	-	-	-
	165S	+	-	-	-	-	-	-	+/-	-	-
	170S	+	-	-	-	-	-	-	+/-	-	-
	171S	+	-	-	-	-	-	-	-	-	+/-
	172L	+	-	-	-	-	-	-	-	-	-
	172S	+	-	-	-	-	-	-	+/-	-	-
	180S	+	-	-	-	-	+/-	-	-	-	+/-
	379S	+	-	-	-	+/-	+	-	-	-	-
	398S	+	+/-	-	-	-	+	-	-	-	-
	404S	+	+	+	-	+	-	-	-	-	-
	410S	+	+/-	+	-	+	+	-	+	+/-	-
	436S	+	-	-	-	-	-	-	-	-	-
	437S	+	+/-	-	-	-	+/-	-	-	-	-
	483L	+	-	-	+/-	+	+	+	+/-	+/-	+
	512L	+/-	-	+/-	-	+/-	-	+/-	+/-	+/-	+/-
	523S	+	-	-	-	-	-	-	-	-	-
	544S	+	-	-	-	-	-	-	-	-	-
	549S	+	-	-	-	-	-	-	-	-	-
	557S	+	-	-	-	-	-	-	-	-	-
	575S	+	+/-	-	-	-	-	-	-	-	-
	615S	+/-	-	-	+	-	-	-	-	-	-

Table 5.2: Activity of bacterial products on a panel of *E. coli* isolates: Strains tested for sensitivity are denoted by their MCI reference number, which corresponds to Table 2.1. Bacterial products are denoted by the MCI reference number and "S" or "L" to indicate whether bacterial supernatant or lysate was used as the test substance. Clear inhibition, indicating that the substance was bactericidal is indicated with a "+" sign; substances giving a turbid zone of inhibition, indicating that the substance was bacteriostatic, are denoted "+/-".

PCR-screening results suggested that MCI 0410 carries at least one copy of a colicin E2-like gene. Colicin E2 has a pI value of 7.63 (Herschman and Helsinki, 1967) and therefore, in order to attempt to separate inhibitory substances (possibly including a colicin E2-like protein) while retaining their activity, acidic polyacrylamide gel electrophoresis was employed for the analysis of concentrated supernatant from MCI 0410 under non-denaturing conditions. The lane containing MCI 0410 supernatant was cut into 2-3mm sections and placed on lawns of MCI 0255 and MCI 0200. None of the excised bands caused inhibition of MCI 0200 while all excised bands (including the the negative control, taken from a clean area of the gel) inhibited MCI 0255. This suggests that the acid contained in the gel is sufficient to inhibit MCI 0255 and would mask any inhibitory action of proteins contained within the gel slices. Therefore the procedure was repeated using SDS-PAGE. This method would normally be expected to denature the proteins. However, certain proteins may retain their activity after SDS-PAGE (Bischoff *et al.*, 1998). MCI 0410 supernatant produced an intense band at approximately 62kDa after SDS-PAGE as shown in Figure 5.3. Only gel sections taken from this large band produced a clear zone of inhibition on a lawn of MCI 0255 while other bands and the negative control did not. However, this band was not observed to inhibit a lawn of MCI 0200. No comparatively intense bands were seen in the SDS-PAGE pattern of MCI 0165, 0170 or 0172 and no bands from these gels showed inhibition of MCI 0255 lawns (not shown).

The peptide mass fingerprint of the 62kDa band was determined by MALDI-TOF mass spectrometry and searched against the MASCOT database. The most significant hit was colicin E2 (Mowse score =180, $P<0.05$, Figure 5.4).

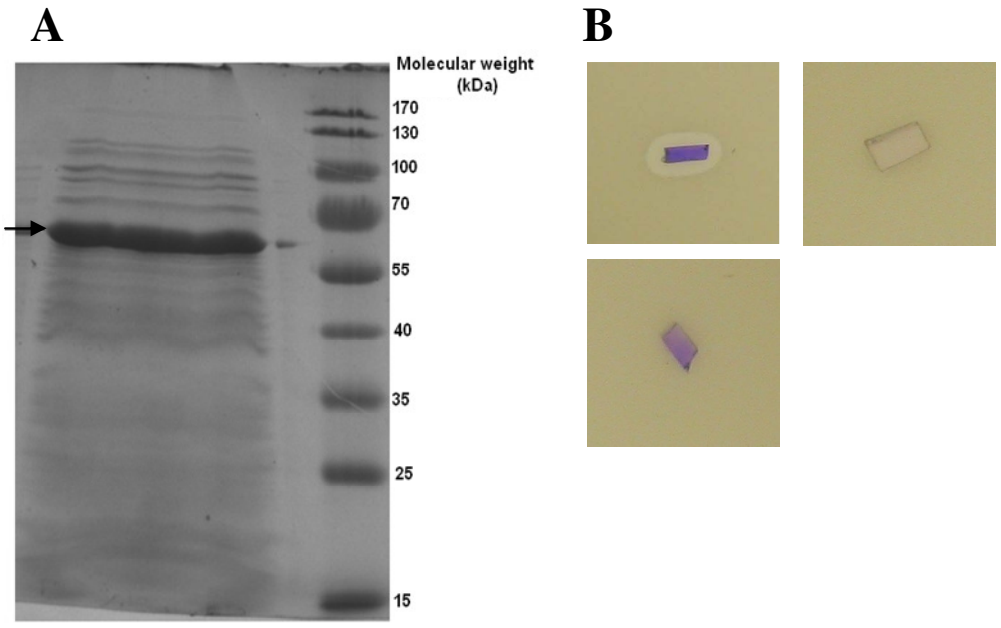


Figure 5.2: Analysis of MCI 0410-secreted proteins: (A) Banding pattern of the secreted products of MCI 0410 when separated by SDS-PAGE. The band denoted with the arrow was cut out of the gel and placed on soft agar lawns of MCI 0200 and MCI 0255. **(B)** Inhibition of MCI 0255 bacterial lawn was observed (top left) whereas the negative control using a piece of clean SDS-PAGE gel caused no inhibition (top right). Inhibition of MCI 0200 was not detected (bottom left).

Match to: **CEA2_ECOLX** Score: **180** Expect: **3.3e-13**
Colicin-E2 - *Escherichia coli*

Nominal mass (M_r): **61592;** Calculated pI value: **9.02**
NCBI BLAST search of CEA2_ECOLX against nr

Taxonomy: *Escherichia coli*

Number of mass values searched: **92**
Number of mass values matched: **29**
Sequence Coverage: **42%**

Matched peptides shown in **Bold Red**

```
     1 MSGGDGRGHN TGAHSTSGNI NGGPTGLGVG GGASDGSGWS SENNPWGGGS
    51 GSGIHWGGGS GHGNGGGNGN SGGGSGTGGN LSAVAAPVAF GFPALSTPGA
   101 GGLAVSISAG ALSAAIADIM AALKGPFKFG LWGVALYGVL PSQIAKDDPN
   151 MMSKIVTSLP ADDITESPVS SLPLDKATVN VNVRVVDDVK DERQNISVVS
   201 GVPMSVPVVD AKPTERPGVF TASIPGAPVL NISVNNSTPE VQTLSPGVTN
   251 NTDKDVRPAG FTQGGNTRDA VIRFPKDSGH NAVYVSVSDV LSPDQVKQRQ
   301 DEENRRQQEW DATHPVEAAE RNYERARAEL NQANEDVARN QERQAKAVQV
   351 YNSRKSELDA ANKTLADAIA EIKQFNRFAH DPMAGGHRMW QMAGLKAQRA
   401 QTDVNNKQAA FDAAAKEKSD ADAALSAAQE RRKQKENKEK DAKDKLDKES
   451 KRNKPGKATG KGKPVGDKWL DDAGKDSGAP IPDRIADKLR DKEFKNFDDF
   501 RKKFWEEVSK DPDLSKQFKG SNKTNIQKGK APFARKKDQV GGRERFELHH
   551 DKPISQDGGV YDMNNIRVTT PKRHIDIHRG K
```

Figure 5.3: Results of MALDI-TOF analysis of the band resulting from SDS-PAGE of MCI 0410 secreted protein: The most significant hit was against *E. coli* colicin type E2, giving 42% coverage and a MOWSE score of 180.

5.2.3 *In vitro* competitive assays

The media employed to distinguish *E. coli* O157:H7 from the bovine isolates used in these assays were Sorbitol-McConkey's (SMAC) agar and Tryptone-Bile salts-X-glucuronide (TBX). The *E. coli* O157:H7 isolate employed in this procedure (MCI 0200) is non-sorbitol-fermenting and glucuronidase negative and therefore produces brown colonies on SMAC and white colonies on TBX. The vast majority of *E. coli* other than conventional *E. coli* O157:H7 isolates are sorbitol-fermenting and glucuronidase positive, producing pink colonies on SMAC and blue colonies on TBX. Hence, *E. coli* O157 incubated with a second *E. coli* isolate may be distinguished and enumerated from a mixed culture using these indicator-media. For each triplicate plating exercise, at least one plate of each medium-type was employed, in order to confirm that the organism had not gained the ability to ferment sorbitol or to produce active glucuronidase during the course of the assay. Acquisition of both characteristics during the assay is unlikely.

5.2.3.1 Competitive co-culture

In all assays performed, there was a significant difference ($P<0.05$) between the number of viable MCI 0200 when grown on its own ($1.05x10^9 \pm 1.3x10^8$ cfu/ml; $n=6$ after 4hrs) compared to growth in the presence of a second *E. coli* isolate. However, there was no significant difference ($P>0.05$) in the number of MCI 0200 when grown in the presence of any bovine isolate compared to growth in the presence of a laboratory strain (MCI 0255) (see Figure 5.5). When coincubated with MCI 0255, MCI 0200 grew to a density of $4.11x10^8$ cfu/ml ($\pm 7.39x10^7$cfu/ml; $n=6$) within four hours. Similar growth levels were observed when grown with MCI 0410 ($3.09x10^8 \pm 1x10^8$cfu/ml; $n=9$), MCI 0170 ($4.66x10^8 \pm 1.1x10^8$cfu/ml; $n=6$) or MCI 0172 ($3.66x10^8 \pm 4.19x10^7$cfu/ml, $n=6$).

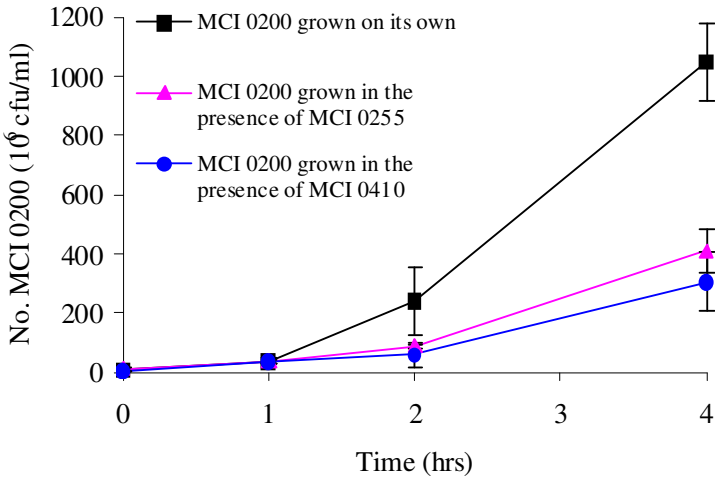


Figure 5.4: Competitive co-culture: Growth of *E. coli* O157:H7 (MCI 0200) with a second strain significantly decreases the growth of MCI 0200. However, there is no significant difference between the growth of *E. coli* MCI 0200 with MCI 0410 compared with MCI 0200 grown with MCI 0410.

5.2.3.2 Growth of *E. coli* O157:H7 in MCI0410 supernatant

MCI 0200 grew to a density of $1.1x10^9 \pm 7.8x10^7$ cfu/ml within 4 hours when grown in LB without another strain. When grown in a 1:1 mixture of sterile LB medium:MCI 0255 culture supernatant, density of MCI 0200 growth was significantly reduced at the 4 hour timepoint ($3.91x10^7$cfu/ml, $\pm 1.13x10^7$cfu/ml; n=12). When grown in the presence of MCI 0410 supernatant, numbers of MCI 0200 declined slightly in the first hour followed by a gradual incline to $5.9x10^6$cfu/ml ($\pm 4.42x10^6$cfu/ml; n=12) by 4hrs. Although MCI 0200 seems to be suppressed to a greater extent by MCI0410 supernatant than MC0255 supernatant, the difference is not statistically significant ($p>0.05$).

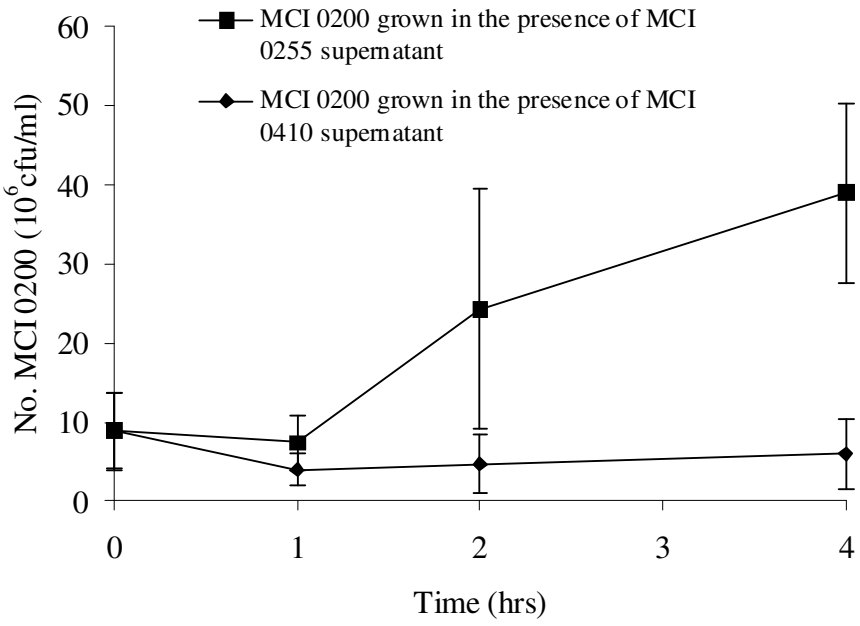


Figure 5.5: Growth of MCI 0200 in 50% MCI 0410 supernatant in LB: Although MCI 0410 supernatant appeared to reduce the number of MCI 0200 more so than the supernatant of MCI 0255, the difference was not significant (P>0.05). Data shown is the average of two separate experiments (± SEM n=6 for each experiment).

5.2.3.3 Competitive adherence to EBL cells

E. coli O157:H7 (MCI0200) was coincubated with a panel of bovine *E. coli* isolates (MCI 0165, 0170, 0172 and 0410) on EBL cell monolayers (as described in Section 2.7.5.3) to predict their ability to reduce the numbers of *E. coli* O157:H7 binding to bovine epithelial cells (shown in Figure 5.7).

When added to EBL cells without a competitor, the number of viable adherent MCI 0200 was $1.1x10^5 \pm 1.3x10^4$ cfu/cm^2. When coincubated with MCI 0165, 0170 or 0172 there was no significant difference in the number of adherent MCI 0200 compared with MCI 0200 incubated without a second strain (P>0.05). When coincubated with MCI 0410, however, there was a significant difference (P<0.05) in that no adherent MCI 0200 colonies were detected. It must be noted that overnight cultures of MCI 0255 failed to grow in MEM-HEPES (the medium chosen for growth of *E. coli* isolates for addition to cell monolayers), therefore

the assay lacked the laboratory strain (a likely non-competitive *E. coli* strain) as a negative control; however, MCI0410 performed better than any of the other bovine isolates tested for inhibition of MCI0200 ($P<0.05$).

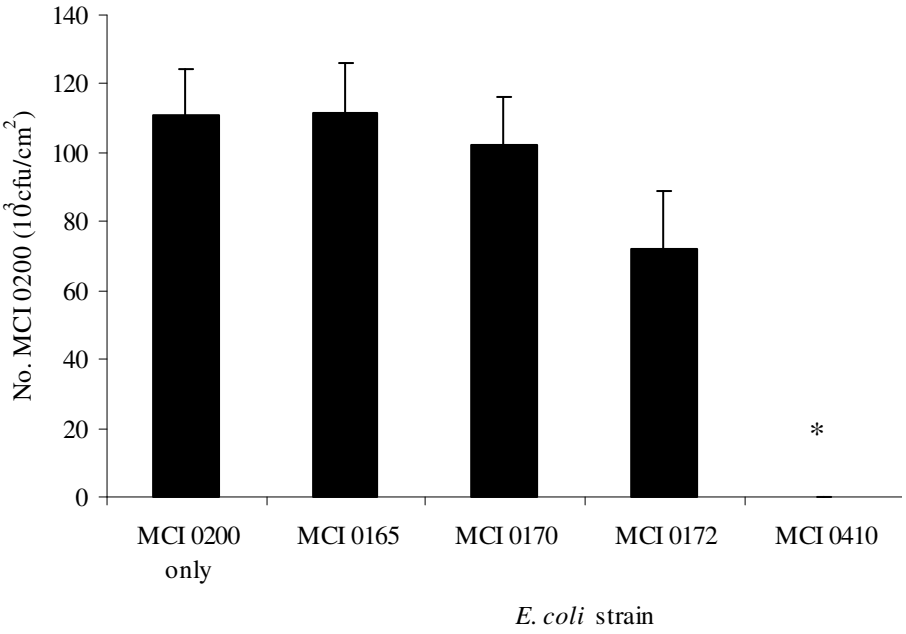


Figure 5.6: Competitive adherence between MCI 0200 and bovine isolates: The number of viable MCI 0200 from the lysate was counted on SMAC after one hour of challenge and represented as colony forming units (CFU) per cm^2 (+1 SEM). When coincubated with MCI 0165, MCI 0170 or MCI 0172, there was no significant difference in the number of adherent MCI 0200 CFU compared with control (MCI 0200 incubated with cells only). When co-incubated with MCI 0410 complete inhibition was observed, in that no colonies of MCI 0200 were recovered from the cell monolayer.

5.3 Discussion

Data presented above represents the initial *in vitro* assessment of *E. coli* strains for applicability as probiotic agents. *E. coli* MCI 0410 appeared to be a good initial candidate as supernatants from this organism caused zones of inhibition on lawns of *E. coli* O157:H7 and also that out-competed a strain of *E. coli* O157:H7 in adherence to monolayers of a bovine epithelial cell line. However, PCR-screening data presented in Chapter 4, revealed that this strain carries genes encoding EHEC virulence factors, including *espP* (β) and a subtype of *stx*2. As verotoxin positive *E. coli* present a significant health risk, the strain would provide limited worth as a probiotic organism in its current form. It is conceivable that virulence gene-knockout organisms may be created and evaluated for probiotic potential. Alternatively, genes encoding competitive factors could be cloned into *E. coli* isolates with less pathogenic potential.

Strains isolated later in the study may be better candidates for the development of a probiotic organism. MCI 0483 (*E. coli* O26:H+(NT1), *escN*+, *eae*β+, *espP*+, *ehxA*+) performed most impressively against the panel of *E. coli* O157:H7 strains and did not cause clear inhibition of any of the *E. coli* isolates predicted to be non-pathogenic except for the laboratory strain MCI 0255. However, *E. coli* O26 isolates are recognised human pathogens and verotoxin producing *E. coli* O26 isolates are common (Goldwater and Bettelheim, 1998). It has been documented that *E. coli* O26 is prevalent in young calves but isolated less often in older cattle (Shaw *et al.*, 2004). As MCI 0483 was isolated from a young calf, this organism may be less effective in the colonisation of older animals. Products from strain MCI 0512 (*E. coli* O18ac:H49 *cdt*+, *fimA*+) caused partial inhibition of most of the *E. coli* O157:H7 strains tested. Although the zone of inhibition observed appeared more turbid than that caused by MCI 0410 or MCI 0483 at the concentrations tested, suggesting that the inhibition caused may be easier to for *E. coli* O157:H7 to overcome, this strain may be a good candidate for further investigation as its lack of virulence factor-encoding genes detected could suggest that the organism has a lower probability of pathogenesis than the other isolates obtained.

Data presented in this chapter highlights the competitive fitness of potentially pathogenic *E. coli* strains. Competitive factors such as colicins and microcins may increase environmental persistence of pathogenic bacteria and facilitate transmission to humans. This means that

caution should be exercised in designing probiotic strategies for the control of *E. coli* O157:H7 in cattle. Although numerous studies support the efficacy of competitive *E. coli* for use as a probiotic organism (Zhao *et al.*, 1998;Tkalcic *et al.*, 2003;Zhao *et al.*, 2003;Schamberger *et al.*, 2004), none appear to have considered the interaction of a probiotic organism with the indigenous microflora after release into the cattle population. It has been widely documented that *E. coli* have the ability to acquire genomic regions, especially by the action of bacteriophages (Dobrindt and Hacker, 2001;Pallen and Wren, 2007). This is manifested most devastatingly in the acquisition of *stx*-encoding genes, which are carried on lysogenic lambdoid bacteriophages (Smith *et al.*, 1983;O'Brien *et al.*, 1984). If a hyper-competitive *E. coli* strain were to be released into the environment, it must be proven to resist acquisition of the *stx*-encoding bacteriophage and other virulence factors. Another potential problem with the administration of a competitive microorganism to cattle is that genes conferring the competitive advantage may be transferred to a pathogenic microorganism. Either of these occurrences could lead to the emergence of a hyper-competitive organism with potential pathogenicity. The insertion of bacteriophages similar to the *stx*-encoding bacteriophages lacking the *stx* genes into normal *stx*-insertion sites (such as at the *yeh*V or *wrb*A sites) may prevent insertion of further *stx*-encoding bacteriophages (Serra-Moreno *et al.*, 2007); however, insertion of *stx*-phages has been found to occur at other genomic loci (Besser *et al.*, 2007) and it is not unlikely that undescribed variant forms of *stx*-encoding phages may insert at unknown genomic sites. Ensuring that the probiotic strain lacks the *yae*T protein, which is exploited as a receptor by *stx*-encoding bacteriophages may reduce the likelihood of *stx*-gene acquisition (Smith *et al.*, 2007). The diversity of *stx*-encoding bacteriophages (Shaikh and Tarr, 2003;Muniesa *et al.*, 2004;Besser *et al.*, 2007) coupled with the limited understanding of the full range of the modes of action of virulence factor-encoding bacteriophages means that it would be a major undertaking to produce a strain resistant to all types of horizontal gene transfer. With public resistance to the application of genetically modified organisms to food products, it may prove more simple to screen further isolates for suitability until a naturally occurring strain is identified which has efficacy against EHEC, lacks virulence properties and is resistant to acquisition of virulence factors or transduction of competitive factors to other organisms.

Data presented above suggests that the sensitivity of *E. coli* O157:H7 to inhibitory compounds produced by *E. coli* isolates differ between strains of *E. coli* O157:H7. Activity of inhibitory

substances against potential non-pathogenic bovine *E. coli* isolates was also detected for certain *E. coli* isolates, including MCI 0410. Further effects of the disruption of gastrointestinal microflora are not yet established; however, as the gastrointestinal microbiota is important for the health of the animal (Section 1.5.1) the predicted disruption to this population should be considered in the design of probiotic strategies.

In determining the probiotic potential of *E. coli* MCI 0410, it was noted that competition seemed to be most significantly increased when the organism was co-incubated with bovine epithelial cells, which could highlight the role of host cells in the activation of the competitive nature of *E. coli*. However, incubation with cultured cells was carried out in entirely different medium to competitive growth in liquid culture and the effects of components of the bacterial growth medium would need to be examined in order to validate this observation.

In summary, data presented above provide an indication that strains isolated during this study could have inhibitory activity against *E. coli* O157:H7. However, virulence factor detection shows that some of these isolates are potentially pathogenic meaning that certain isolates would not be directly applicable in their current form. Nevertheless, certain isolates identified in this study may be worthy of further investigation with a view to developing probiotic control strategies against *E. coli* O157:H7.

Chapter 6: Pan-genomic characterisation of disease associated human EHEC isolates

6.1 Introduction

As described in Chapter 1, EHEC infection of humans is often related to contact with bovine faecal material, especially through contamination of food products or direct contact with bovine faeces (Mead *et al.*, 1997;Locking *et al.*, 2001;Strachan *et al.*, 2006). Scotland has frequently been the setting for outbreaks of human EHEC-infection and *E. coli* O157 infection has been more frequent in Scotland compared with the rest of the UK since 1988, as shown in Figure 1.1 (Lynn *et al.*, 2005). Incidence of *E. coli* O157 infection in Scotland peaked in 1996, when a large outbreak of *E. coli* O157:H7 occurred, related to contamination of cooked meat products with material from raw meat in a Wishaw butchery (The Pennington Group, 1998).

At the beginning of this study, genome sequences for two strains of *E. coli* O157:H7 had been published. The first was strain EDL933, isolated from one of the earliest recognised outbreaks of human *E. coli* O157:H7 infection, which was linked to a fast food chain in Oregon. During this outbreak at least 47 people became infected (Riley *et al.*, 1983;Perna *et al.*, 2001). The second published genome was that of strain RIMD 0509952, which was isolated from an outbreak in Sakai, Japan, during which over 6000 schoolchildren were affected by EHEC disease (Watanabe *et al.*, 1996;Hayashi *et al.*, 2001b). Since then a large-scale sequencing project of *E. coli* O157:H7 genomes has begun at the J. Craig Venter Institute (http://msc.jcvi.org/o157h7/index.shtml accessed 10/08/2008).

The objectives of work presented in this Chapter were to predict the diversity of EHEC genomes and establish whether the strains of *E. coli* O157 related to human disease in Scotland differ from reference strains on the genomic level.

Targeted analysis of individual virulence factor genes was initially carried out in order to predict the distribution of alleles of these genes among EHEC isolates. Genes encoding Shiga-like toxin and the EspP protease were chosen, firstly as they each have proposed roles in the progression and severity of human infection (O'Brien and Holmes, 1987;Brunder *et al.*, 1997) and secondly that these genes were the most common EHEC-virulence factor-encoding genes detected among bovine *E. coli* isolates (Chapter 4). This analysis may facilitate preliminary comparisons of the bovine "predicted-EHEC" population with those directly implicated in cases of human infection.

A pan-genomic approach was adopted, including whole genome microarray to predict areas of genome-diversity in pathogenic *E. coli* O157 strains. Isolates related to significant recent outbreaks in Scotland were selected for identification of genomic portions present in Scottish isolates which are absent in genome-sequenced strains, using a technique termed suppressive subtractive hybridisation (SSH).

Suppressive subtractive hybridisation (SSH) is a method whereby the sequences present in one population of DNA (the "tester") but absent in another (the "driver") may be selectively amplified. In this instance, genomic DNA from a genome-sequenced strain of *E. coli* O157:H7 (EDL933, MCI 0045) was used as the driver and genomic DNA from three strains related to outbreaks in Scotland were individually used as tester DNA populations. The first tester strain was an isolate related to the Wishaw outbreak of 1996 (MCI 0489). This strain was included to represent Scottish *E. coli* O157 which were prevalent in the mid 1990s, when incidence of *E. coli* O157 infection was at its peak (Lynn *et al.*, 2005). *E. coli* O157 MCI 0453 was selected as a representative of *E. coli* O157 strains most frequently related to incidence of human infection in Scotland recently. MCI 0453 is of phage type 21/28, which accounts for 44% of identified cases of *E. coli* O157 infection in Scotland in 2007 (Locking *et al.*, 2006). This particular strain caused HUS in two children in the same household and infected their mother. MCI 0507 was chosen as a representative of sorbitol-fermenting *E. coli* O157, which is an emerging group of human pathogens. This isolate was related to an outbreak in a Fife Nursery in 2006, during which there were 14 confirmed cases of EHEC infection (Health Protection Scotland, 2007).

SSH-analysis was carried out to predict whether Scottish *E. coli* O157 isolates carry additional virulence factors. Strains encoding novel virulence factors could theoretically contribute to the increased prevalence of *E. coli* O157 infection in Scotland, although other factors such as increased likelihood of contact with cattle due to the rural environment and increased cattle population present in Scotland (Strachan *et al.*, 2006), coupled with the increased observed prevalence of *E. coli* O157:H7 in Scottish cattle herds (Synge *et al.*, 2003) are likely to be important in the increased incidence of human EHEC infection in Scotland.

6.2 EHEC strains used in this analysis

Bacterial isolates used in this study are summarised in Table 6.1. These include 48 isolates provided by Lesley Allison at the Scottish *E. coli* Reference Laboratory (SERL, Western General Hospital, Edinburgh) which are related to human disease in Scotland and 10 isolates from other sources.

Ref No	Strain	Source	Phage Type	Intimin	Verotoxin	Details
MCI 0033	*E. coli* O157:H7 319	Human	2	+	2+, 2c+	Redhouse dairy outbreak (1994)
MCI 0047	*E. coli* O157:H7 (MPRL 6303)	Bovine	32	ND	2+	unknown
MCI 0048	*E. coli* O157:H7 (MPRL 6043)	Bovine	2	ND	2+	unknown
MCI 0049	*E. coli* O157:H7 (MPRL 6409)	Bovine	49	ND	2+	Northern Ireland
MCI 0050	*E. coli* O26 (MPRL 4269)	Human	ND	ND	-	unknown
MCI 0051	*E. coli* O26 (MPRL 4270)	Human	ND	-	1+	Monkland s Outbreak (HUS)
MCI 0064	*E. coli* O157:H7 (NCTC12900)	Human	ND	+	-	Public Health Laboratory, Austria, (1992)
MCI 0066	*E. coli* O157:H7 (WallaWalla1)	Human	25 [g]	+	2+	Washington State Outbreak, (1986)
MCI 0218	*E. coli* O157:H7 (84-289)	Human	5 [g]	+	2+	Ottawa (1987, Nursing home, HUS)
MCI 0230	*E. coli* O157 (SERL1)	Bovine	21/28	+	2+	Outbreak Y-1 (2004, Dumfries & Galloway)
MCI 0233	*E. coli* O157 (SERL4)	Bovine	21/28	+	2+	Outbreak Y-2 (2004)
MCI 0234	*E. coli* O157 (SERL5)	Human	21/28	+	2+	Outbreak Y-2 (2004, Dumfries & Galloway, 13 year old)
MCI 0235	*E. coli* O157 (SERL6)	Human	21/28	+	2+	Outbreak Y-2 (2004, Foreign patient[d], 3 year old, HUS)
MCI 0236	*E. coli* O157 (SERL7)	Human	8	+	1+, 2+	Outbreak Y-3 (2000, Lothian, 13 year old)
MCI 0238	*E. coli* O157 (SERL9)	Human	21/28	+	2+	Sporadic case (2004, Tayside, 62 year old, HUS)
MCI 0239	*E. coli* O157 (SERL10)	Human	14	+	1+, 2+	Sporadic case (2004, Lothian, 40 year old, BD)
MCI 0240	*E. coli* O157 (SERL11)	Human	33	+	1+, 2+	Sporadic case (2004, Ayrshire & Arran, 63 year old, BD)
MCI 0241	*E. coli* O157 (SERL12)	Human	31	+	2+	Sporadic case[b] (2004, Lothian, 4 year old)

MCI 0242	*E. coli* O157 (SERL13)	Human	21/28	+	2+	Outbreak Y-4, (2001, Greater Glasgow & Clyde, 11 year old, HUS)
MCI 0245	*E. coli* O26 (SERL16)	Human	ND	+	1+	Sporadic case (2002)
MCI 0246	*E. coli* O162 (SERL17)	Human	ND	-	1+	Sporadic case (2002)
MCI 0247	*E. coli* O113 (SERL18)	Human	ND	-	2+	Sporadic case (2003)
MCI 0248	*E. coli* O177 (SERL19)	Human	ND	+	2+	Sporadic case (2003)
MCI 0249	*E. coli* O118 (SERL20)	Human	ND	-	1+, 2+	Sporadic case (2004)
MCI 0280	*E. coli* O157:H7 (TT12A)	Human	ND	+	1+, 2+	Sporadic case (1992, Japan, HC)
MCI 0444	*E. coli* O157[a] SERL H1410	Human	RDNC	+	2+	Sporadic case (2002)
MCI 0445	*E. coli* O157[a] SERL H 2687	Human	RDNC	+	2+	Sporadic case (2003)
MCI 0446	*E. coli* O157 SERL H 7370	Human	8	+	1+, 2+	Sporadic case (2003)[c]
MCI 0447	*E. coli* O157 SERL H 7436	Human	8	+	1+, 2+	Sporadic case (2003)[c]
MCI 0448	*E. coli* O157 SERL H7483	Human	8	+	1+, 2+	Sporadic case (2003)[c]
MCI 0449	*E. coli* O157 SERL H 10080	Human	21/28	+	2+	Sporadic case (2006)[e]
MCI 0450	*E. coli* O157 SERL H 10744	Ovine	21/28	+	2+	Recent outbreak (2006)
MCI 0451	*E. coli* O157 SERL H 10645	Human	21/28	+	2+	Recent outbreak (2006)
MCI 0452	*E. coli* O157 SERL H 10746	Soil	21/28	+	2+	Recent outbreak (2006)
MCI 0453	*E. coli* O157 SERL H 10873	Human	21/28	+	2+	Familial outbreak (2006, HUS)
MCI 0489	*E. coli* O157 SERL H 1477	Human	2	+	2+	Wishaw Outbreak (1996, 65 year old, fatal)
MCI 0507	*E. coli* O157[a] SERL H8478	Human	RDNC			Nursery Outbreak (2006, Fife)
MCI 0665	*E. coli* O103 SERL XH2644G	Human	ND	-	1+	Sporadic case (2005)
MCI 0666	*E. coli* ONT SERL XH6233T	Human	ND	-	2+	Sporadic case (2005)
MCI 0667	*E. coli* O145 SERL XH6741H	Human	ND	+	2+	Sporadic case (2006)
MCI 0668	*E. coli* O26 SERL XH7788D	Human	ND	+	1+, 2+	Sporadic case (2006)
MCI 0669	*E. coli* O179 SERL XH8074K	Human	ND	-	2+	Sporadic case (2006)
MCI 0670	*E. coli* O111 SERL XH8352A	Human	ND	+	1+, 2+	Sporadic case (2006)
MCI 0671	*E. coli* O26 SERL XH9606R	Human	ND	+	1+, 2+	Sporadic case (2006)
MCI 0672	*E. coli* O113 SERL XH9674W	Human	ND	+	1+, 2+	Sporadic case (2006)

MCI 0673	*E. coli* O26 SERL XH10413N	Human	ND	+	1+	Sporadic case (2006)
MCI 0674	*E. coli* O26 SERL XH10544D	Human	ND	+	1+	Sporadic case (2006)
MCI 0675	*E. coli* ONT SERL XH10638L	Human	ND	+	1+	Sporadic case (2006)
MCI 0676	*E. coli* ONT SERL XH10743A	[f]	ND	+	1+, 2+	Sporadic case (2006)
MCI 0677	*E. coli* ONT SERL XH10745C	[f]	ND	+	1+	Sporadic case (2006)
MCI 0678	*E. coli* ONT SERL XH10917E	Human	ND	-	2+	Sporadic case (2006)
MCI 0679	*E. coli* ONT SERL XH11142L	Human	ND	+	1+	Sporadic case (2007)
MCI 0680	*E. coli* O103 SERL XH11266N	Human	ND	+	1+	Sporadic case (2007)
MCI 0681	*E. coli* O26 SERL XH11904R	Human	ND	+	1+	Sporadic case (2007)
MCI 0682	*E. coli* O26 SERL XH11946B	Human	ND	+	1+	Sporadic case (2007)
MCI 0683	*E. coli* O26 SERL XH12037J	Human	ND	+	1+	Sporadic case (2007)
MCI 0684	*E. coli* O103 SERL XH12243P	Human	ND	+	1+	Sporadic case (2007)
MCI 0695	*E. coli* O76 SERL XH12464R	Human	ND	-	1+	Sporadic case (2007)

a: Sorbitol-fermenting *E. coli* O157 isolates

b: Patient had visited the farm that had been related to outbreak Y-1

c: indistinguishable by PFGE (L. Allison, Pers. Comm.)

d: The foreign patient acquired the infection in Scotland (L. Allison, Pers. Comm.)

e: Recurrent clone (L. Allison, Pers. Comm.)

f: Veterinary isolates

g: determined by Ratnam *et al.* (1988)

Table 6.1: Bacterial isolates used in this study: Where known, details including phage type (for O157 isolates), verotoxin type and case information are included (HUS: haemolytic uraemic syndrome, BD: bloody diarrhoea, HC: haemorrhagic colitis. RDNC: phages reacted but did not conform to any known pattern, ND: not determined/unknown).

6.3 Results

6.3.1 Virulence factor-typing by PCR-RFLP of disease associated EHEC isolates

Previously published methods of PCR-RFLP-typing of *stx* and *espP* genes described in Section 2.6.3 (Bastian *et al.*, 1998;Brunder *et al.*, 1999) were adopted to obtain the allelic profile for these genes among the isolates included in this study (listed in Table 6.1). Resultant patterns and deduced *stx* and *espP* types are shown in Figures 6.1 and 6.2 and summarised in Table 6.2.

Of the 26 non-sorbitol-fermenting *E. coli* O157 isolates included in the analysis, 24 were of *espP* pattern A. Pattern A corresponds to the *espP* type α allele which is commonly encoded by *E. coli* O157:H7 (Brockmeyer *et al.*, 2007). All three sorbitol-fermenting *E. coli* O157 isolates yielded no amplicon. Nine of the ten *E. coli* O26 isolates yielded *espP* pattern B, which relates to the EspP type α gene which is commonly encoded by *E. coli* O26 (Brockmeyer *et al.*, 2007). No amplicon was generated from one *E. coli* O157 isolate (MCI 0239) and another (MCI 0064) yielded *espP* type which could not be related to expected patterns given in Figure 4.6, which suggest that this isolate encodes a previously undescribed variant of EspP. Of the 18 representatives of other EHEC serotypes, *espP* amplicons were generated from 5 strains, including *E. coli* O103 (MCI 0665), *E. coli* O145 (MCI 0667), *E. coli* O177 (MCI 0248) and *E. coli* ONT (MCI 0679) which exhibited pattern B (*espP*α_{O26}) and *E. coli* O179 (MCI 0669) which yielded pattern C, which corresponds to proteolytically inactive EspP type β. These observations reflect the prevalence and types of *espP* genes detected in EHEC isolates given in Brockmeyer *et al.* (2007) in which *espP* genes were detected in the genomes of 62.5% of STEC isolates. Presence or absence of the *espP* gene and the subtypes carried was found to be serotype-specific.

Although the *stx* genes were detected in a greater number of the EHEC isolates analysed herein, greater levels of heterogeneity were detected in the *stx* alleles than was identified in *espP* alleles. Of the 26 non-sorbitol-fermenting *E. coli* isolates, 19 yielded a pattern corresponding to *stx*$2_{c/d}$. Of *stx*$2_{c/d}$ -encoding *E. coli* O157 strains, thirteen also appear to encode a copy of the conventional *stx*2 gene, including all three of the phage type 2 isolates

and 7 of the 10 phage type 21/28 isolates as stx2$_c$. A further five *stx*2$_{c/d}$ -encoding isolates also carry a gene for *stx*1, including all four of the strains of phage type 8. One strain was observed to encode only *stx*2$_{c/d}$ (MCI 0241). All three sorbitol-fermenting *E. coli* O157 strains were found to encode *stx*2. Of the ten *E. coli* O26 isolates analysed, 9 were found to carry *stx*1. Of those, 2 were also found to carry *stx*2.

Compared against the *espP* and *stx* gene subtypes identified in bovine isolates, the most striking difference is the absence of the *stx*2/*stx*2$_{c/d}$ (pattern A + B) genotype among the bovine isolates whereas this is one of the most common patterns observed among the human EHEC isolates. Certain bovine isolates exhibited a pattern suggesting that three different *stx*-encoding alleles were carried (*stx*1/*stx*2/*stx*2$_e$) which has previously been observed among bovine STEC (Bertin *et al.*, 2001).

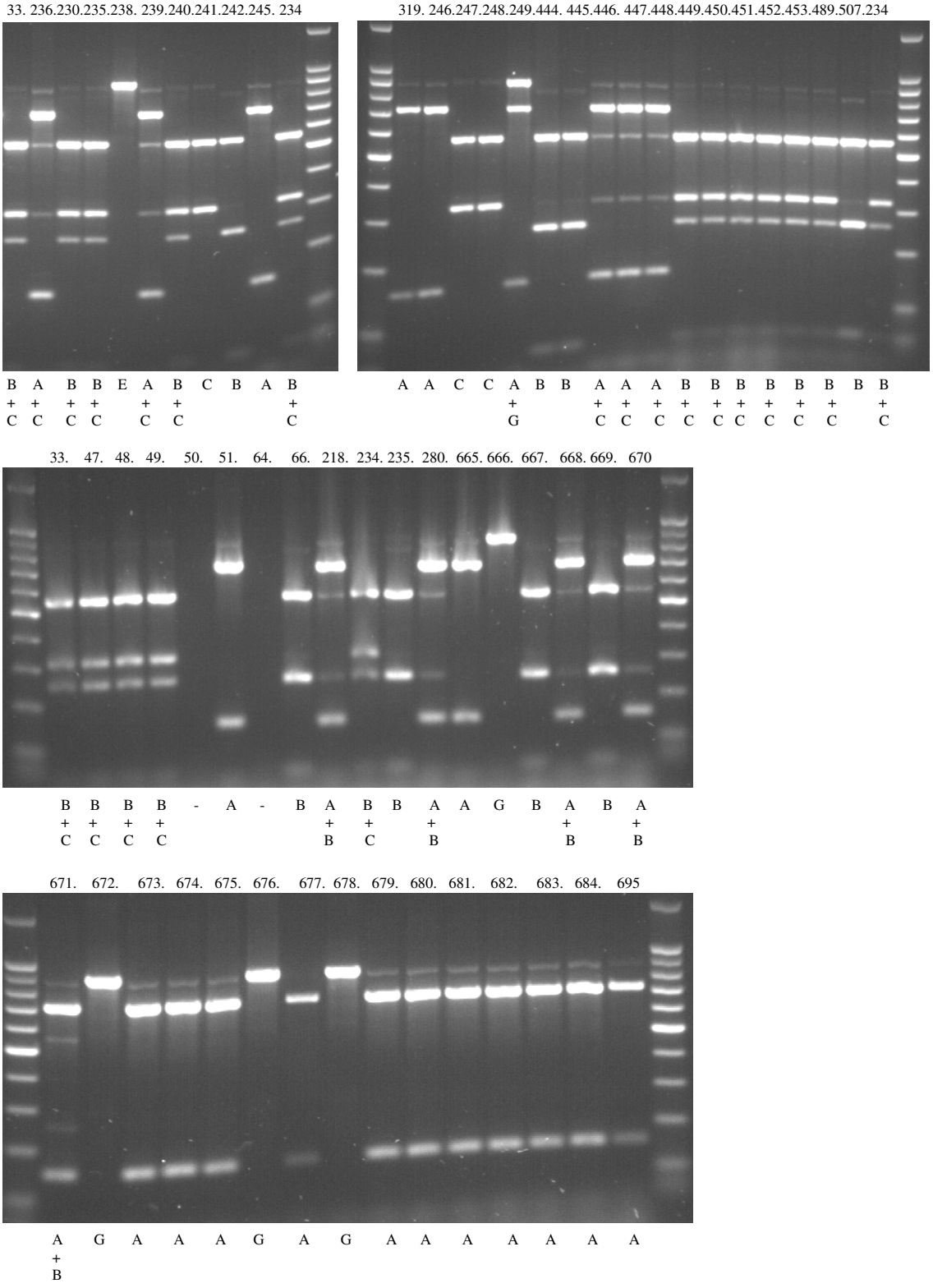
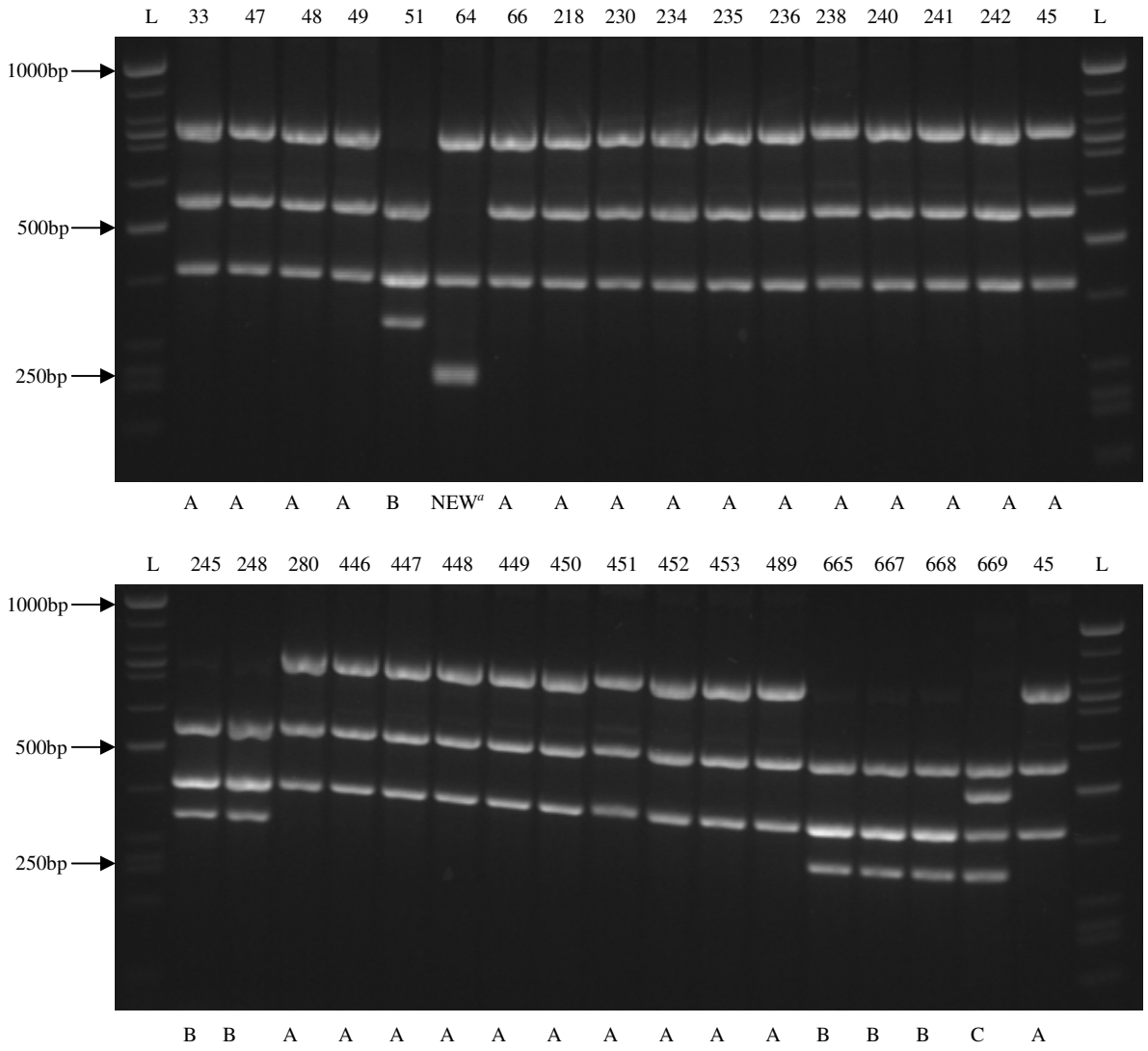


Figure 6.1: stx-RFLP patterns visualised by agarose gel elecrophoresis: Numbers above each lane correspond to MCI reference numbers given in Table 6.1. RFLP-pattern assignations are given beneath each lane, corresponding to Figure 4.7. MCI 0319 was used as a reference pattern for *stx*1 (see Chapter 4).



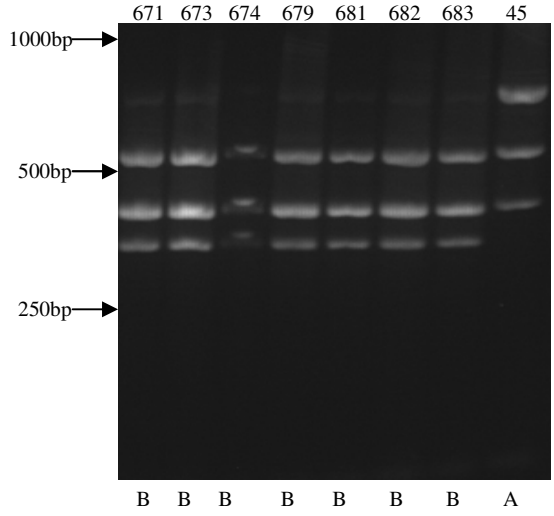


Figure 6.2: Determination of espP RFLP-types: Strain (corresponding to MCI-Reference number) is given above gel lane. MCI 0045 was included on each gel as a reference pattern for *esp*P pattern A. Patterns were matched to those listed in Figure 4.6 and pattern assignations are given beneath each lane. Strains not shown were those which yielded no amplicon after PCR.

		stx-RFLP pattern													*espP*-RFLP pattern				
Serotype	**Number analysed**	**-**	**A** ***stx*1**	**B** ***stx*2**	**C** ***stx*2$_{c/d}$**	**D** ***stx*2$_{e}$**	**G** ***stx*2$_{g}$**	**A + B**	**A + C**	**A + D**	**A + D + B**	**B + C**	**A + G**	**NEW***	**A** ***espP*α$_{O157}$**	**B** ***espP*α$_{O26}$**	**C** ***espP*β**	**NEW***	**-**
O157 (nSF)	26	1	0	3	1	0	1	2	5	0	0	13	0	0	24	0	0	1	1
O157 (SF)	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
O26	10	1	7	0	0	0	0	2	0	0	0	0	0	0	0	9	0	0	1
O103	3	0	3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2
O113	2	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2
O111	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
O118	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
O145	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
O162	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
O177	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
O179	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
O76	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
ONT	6	0	3	0	0	0	3	0	0	0	0	0	0	0	0	1	0	0	5
TOTAL (EHEC)	**57**	**2**	**15**	**7**	**4**	**0**	**5**	**5**	**5**	**0**	**0**	**14**	**1**	**0**	**24**	**13**	**1**	**1**	**18**
Bovine isolates**	**132**	**101**	**13**	**4**	**4**	**4**	**2**	**0**	**0**	**1**	**2**	**0**	**0**	**1**	**1**	**3**	**7**	**1**	**120**

Table 6.2: Summary of *stx* and *espP* RFLP-types: Patterns observed after *espP* and *stx* RFLP were matched to those given in Figures 4.6 and 4.7. Numbers of isolates bearing each RFLP-pattern for each serotype were compiled in the table above, with "-" referring to isolates yielding no amplified fragment from the PCR for each factors. SF: sorbitol fermenting, nSF: non sorbitol fermenting.

* Pattern does not conform to any predicted genotype.

**Assorted serotypes, as described in Chapter 4.

6.3.2 Analysis of *E. coli* O157 isolates by genomic microarray

Eleven isolates were selected for analysis by genomic microarray, using the method set out in Section 2.6.6. The isolates represented four outbreaks and four sporadic cases of disease, the available details of which are given in Table 6.1. From one of the outbreaks two epidemiologically-linked isolates (a bovine isolate linked to the cases of human infection and a corresponding human isolate from the same outbreak, namely MCI 0233 and MCI 0234 respectively) were included to predict whether the epidemiological investigation carried out by the SERL had been successful in identifying two strains with conserved genomic characteristics. MCI 0066 was included in this analysis as a representative American *E. coli* O157:H7 strain, isolated during a foodborne EHEC outbreak in Washington State in 1986 (Ratnam *et al.*, 1988)

Although the vast majority of oligonucleotides representing the genomes of *E. coli* O157:H7 EDL933 and *E. coli* O157:H7 Sakai were found to be present in the genomes of the Scottish *E. coli* O157 isolates, the results of the genomic microarray analysis identified several genomic regions encoded by reference *E. coli* O157:H7 isolates which were absent or divergent among the Scottish *E. coli* O157 isolates. The most notable absences are summarised in Figures 6.4 and 6.5. Figure 6.4 illustrates the diversity among *stx*-encoding bacteriophages carried in the genomes of *E. coli* O157 strains. Regions of the *stx*1 phage were found to be absent in the two strains which were found to encode *stx*1 (MCI 0236 and MCI0239). However, regions of this phage were apparently present in strains without *stx*1, suggesting that there may be regions homologous to the *stx*1 phage present elsewhere on the genome, possibly among variant *stx*2-encoding bacteriophages. The two strains which were found to encode a conventional $stx2_{EDL933}$ gene and were also found to be positive for the majority (but not all) of the oligonucleotides representing the *stx*2 phage present in the genome of the reference *E. coli* O157:H7 strains. Strains MCI 0236 and MCI 0241 which encoded *stx*1 along with variant $stx2_{c/d}$ were found to be negative for the entire *stx*2-encoding bacteriophage except for oligonucleotides representing the *stx*2 gene itself. Copies of the conventional $stx2_{EDL933}$ gene were not detected in these strains by PCR-RFLP analysis (presented in Section 6.3.1).

Diversity was also exhibited within certain O-islands; the largest of these diverse regions are highlighted in Figure 6.5. Each of the isolates analysed was negative for a large portion of oligonucleotides representing genes carried on O-island 51, which are carried by a cryptic prophage element designated CP-933-C (Perna *et al.*, 2001). MCI 0233 and MCI 0234 (bovine and human isolates respectively, epidemiologically linked to the same outbreak) were each negative for O-island 76 in its entirety. O-island 76 is also comprised of a cryptic prophage element, CP933-T. MCI 0066 and MCI 0241 were both negative for the majority of O-island 172, which covers a putative insertion element. Further diversity was restricted to small genomic regions and individual genes– also encoded on O-islands. All of the Scottish isolates analysed were negative for oligonucleotides representing genes z1797 and z1799 carried on O-island 50 (both encoding proteins involved in bacteriophage function). MCI 0236 and MCI 0239 alone were positive for oligonucleotides representing genes z2200 and z2201 (putative fimbrial component-encoding genes carried on O-island 61) and oligonucleotides representing genes z5087, z5089 and z5094, which encode putative prophage-related proteins, carried on O-island 148 (the LEE). All strains were found to be positive for the vast majority of pO157-encoded genes, except for oligonucleotides representing genes L7071 and L7072 (both hypothetical proteins).

All strains were found to be negative for the majority of K-island-encoded genes, except that all strains were found to be positive for oligonucleotides representing genes b2361 and b2362 which are both hypothetical proteins encoded on K-island 121. The vast majority of diverse regions described here are bacteriophage or prophage-related. This observation supports the notion that bacteriophages are the major agents of genome diversification among *E. coli* O157 strains, as proposed by others (Bielaszewska *et al.*, 2007a).

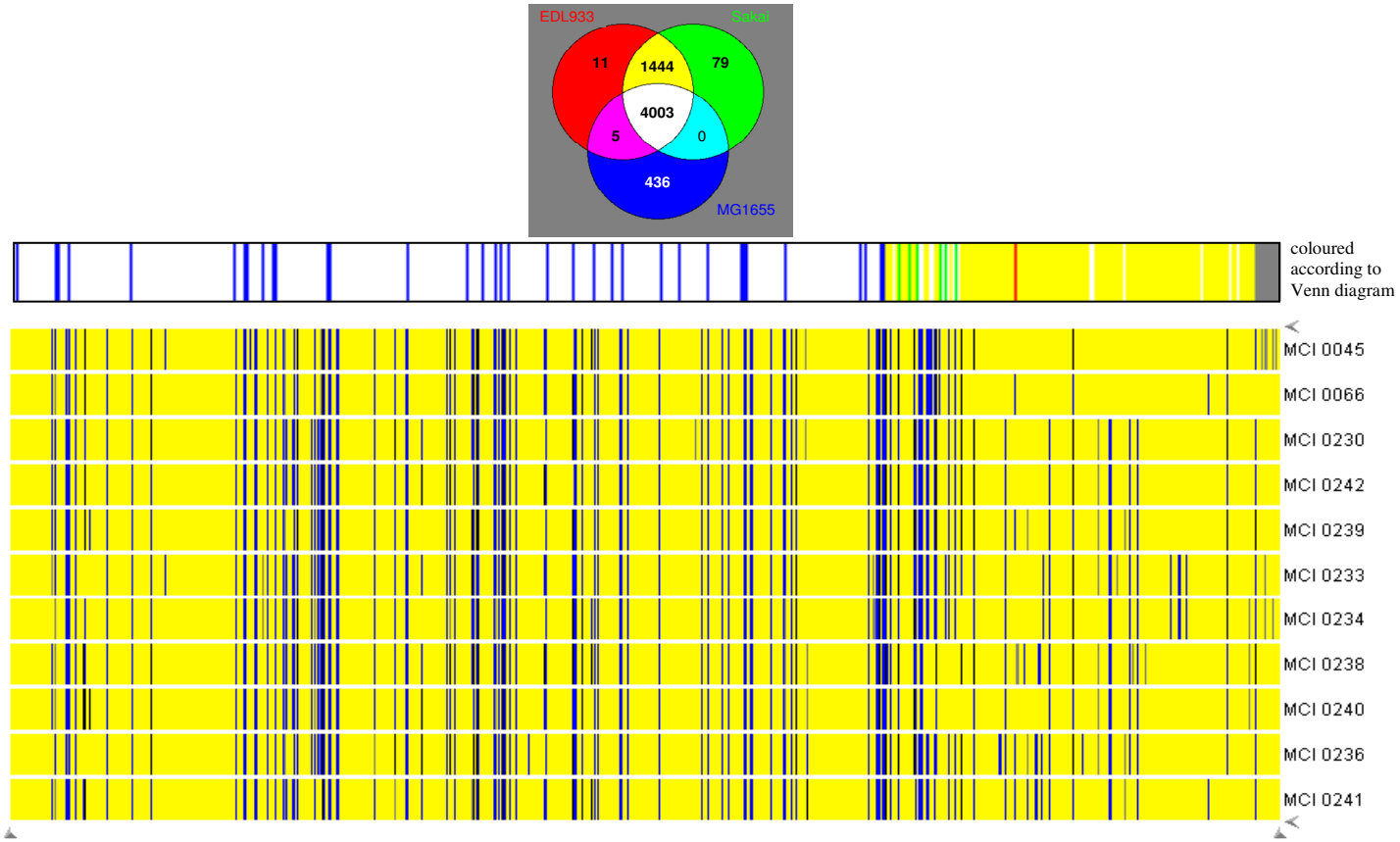


Figure 6.3: Results of whole genome microarray analysis of *E. coli* O157 isolates: The Venn diagram above shows the numbers of oligonucleotides specific to each of the three control strains represented on the array and those common to two or all three of the control strain genomes. Each oligonucleotide is represented by a vertical bar, the topmost bar coloured according to the Venn diagram (the grey area at the rightmost end of the bar corresponds to the genes encoded on pO157) and subsequent bars coloured by presence or absence in each strain analysed (yellow: presence, blue: absence, grey: ambiguous). It can be seen from this representation that the Scottish *E. coli* O157 strains included in this analysis exhibit a similar pattern of gene conservation.

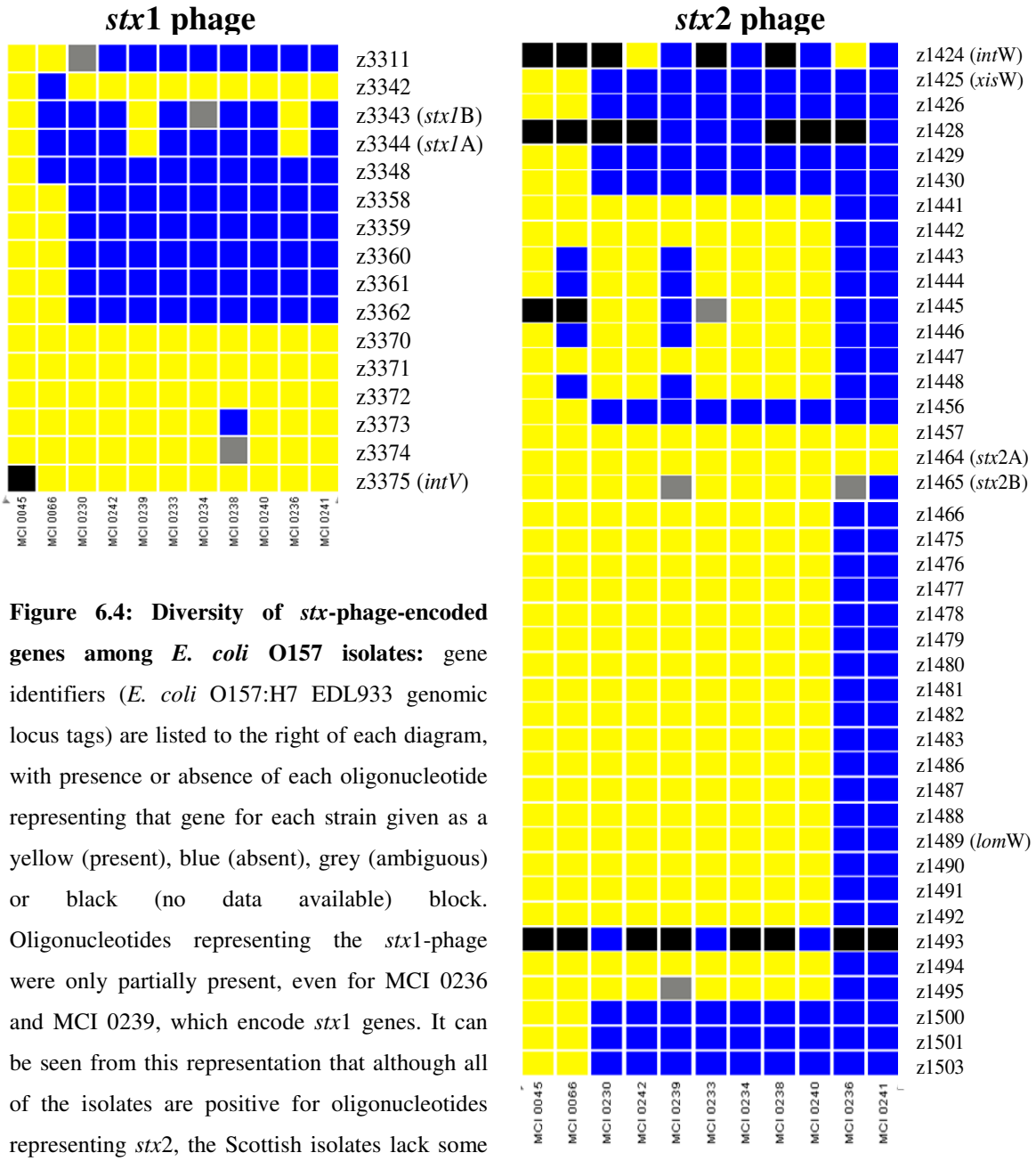


Figure 6.4: Diversity of *stx*-phage-encoded genes among *E. coli* O157 isolates: gene identifiers (*E. coli* O157:H7 EDL933 genomic locus tags) are listed to the right of each diagram, with presence or absence of each oligonucleotide representing that gene for each strain given as a yellow (present), blue (absent), grey (ambiguous) or black (no data available) block. Oligonucleotides representing the *stx*1-phage were only partially present, even for MCI 0236 and MCI 0239, which encode *stx*1 genes. It can be seen from this representation that although all of the isolates are positive for oligonucleotides representing *stx*2, the Scottish isolates lack some part of the *stx*2-phage encoded genes, especially in the case of MCI 0241 and MCI 0236.

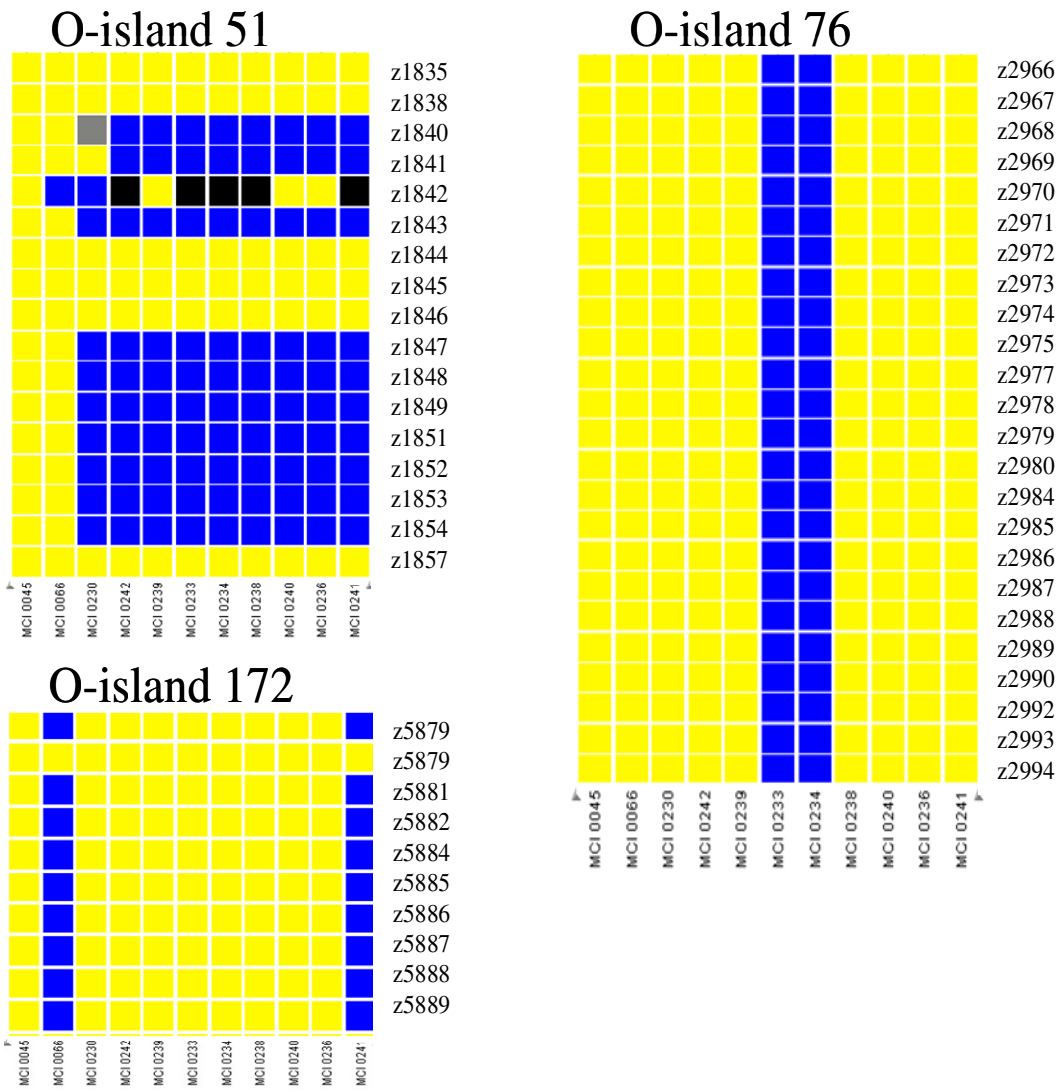


Figure 6.5: Diversity of carriage of O-island-encoded genes among *E. coli* O157 isolates: gene identifiers (*E. coli* O157:H7 EDL933 genomic locus tags) are listed to the right of each diagram, with presence or absence of each oligonucleotide representing that gene for each strain given as a yellow (present), blue (absent), grey (ambiguous) or black (no data available) block. It can be seen that all of the Scottish O157 isolates lack a major part of O-island 51. MCI 0233 and MCI 0234 are negative for O-island 76 in its entirety and MCI 0066 and MCI 0241 lack the majority of oligonucleotides representing O-island 172.

6.3.3 Genomic subtractive hybridisation of Scottish outbreak-related *E. coli* O157 isolates

Subtractive hybridisation was carried out according to Section 2.6.8 (Materials & Methods) resulting in three clone libraries corresponding to each of the tester strains (MCI 0453, MCI 0489 and MCI 0507). Two hundred and eighty-eight colonies from each library were screened by Southern hybridisation (Section 2.6.8.1) using genomic DNA from the driver (MCI 0045) as a probe (not shown). The TotalLabArray program was used to assign a value for pixel density to each resulting spot. For the clones giving the lowest intensity spots (likely to represent sequences that are absent in the EDL933 genome) plasmid DNA was purified (Section 2.6.1.4) and submitted for sequencing at MWG Biotech. Sequence data was edited to remove vector sequence and analysed by BLASTN. Those yielding no hit were analysed by BLASTX, to give predicted translated protein hits for each sequence. Sequencing was carried out in small batches until the same sequences were recovered multiple times, indicating that most of the unique sequences were likely to have been recovered. In total, 202 plasmids were chosen for sequencing from the three subtracted libraries (54 from MCI 0453, 59 from MCI 0489 and 89 from MCI 0507). Sequencing was carried out in one direction, using the T7 forward primer. The unique sequences identified in the genomes of MCI 0489, 0453 and 0507 are summarised in Tables 6.3, 6.4 and 6.5 respectively. Sequences returning a BLAST hit of less than 90% identity to sequences in the NCBI database are given in full in Appendix VI.

Pie charts presented in Figure 6.6 summarises the predicted functions of sequences returned for each of the three tester strains. Predicted bacteriophage-encoded genes were most prevalent among the sequences obtained for each strain. Of the 32, 40 and 42 sequences returned for MCI 0453, MCI 0489 and MCI 0507 respectively 18, 32 and 19 sequences returned hits related to bacteriophage or prophage genetic elements.

In addition to bacteriophage-related elements, sequences returned from the MCI 0453 subtracted library also included three sequences which are homologous to Type IV secretion or conjugation apparatus. Nine matched proteins of unknown function and two sequences were identified which returned no hits by BLAST-N or BLAST-X searching, which could represent previously undescribed genomic regions present in the genome of this isolate.

The sequences returned for MCI 0489 also include three which have similarity with proteins involved in DNA recombination, methylation and repair, one transcriptional regulator, one proposed to be involved in the ABC transport system and one matching a predicted protein of undetermined function.

Among the sequences identified among the MCI 0507 subtracted library, five sequences corresponded to miscellaneous metabolic functions. These included starvation sensing proteins including *rsp*A and *rsp*B. RspA senses homoserine lactone, which is produced during bacterial starvation and the starvation response renders the bacterium resistant to environmental stresses (Huisman and Kolter, 1994). Three were related to nucleotide binding and phosphorylation which is important in many energy-dependent cellular processes. Two sequences were identified that are likely to be related to conjugative transfer of plasmid DNA and two matched sequences of putative transcriptional regulators. Two sequences matching putative membrane proteins were identified, including one matching an autotransporter protein encoded by *E. coli* HS (a human commensal *E. coli* isolate). Sequence encoding *efa*-1 was identified. Efa-1 (EHEC factor for adherence-1) has been previously described as a factor which increases the adherence of EPEC to cultured cells and has been implicated as a factor which inhibits lymphocyte proliferation (Nicholls *et al.*, 2000;Klapproth *et al.*, 2000). It has also previously been identified as a factor encoded by German isolates of sorbitol-fermenting *E. coli* O157:H- (Janka *et al.*, 2005).

Name	Size (bp)	% ID	BLASTN Hit	Organism	Accession	Sequence begin	Sequence end	Protein ID
w1A8	288	95	hypothetical protein: (putative Fels-1 prophage DNA or RNA helicases of superfamily II)	*Shigella dysenteriae* Sd197	CP000034.1	1751820	1752108	ABB62028.1
w1A9	308	97	"gpQ"	*Yersinia* phage L-413C	AY251033.1	1013	705	AAP04438.1
w1C12	401	100	capsid protein small subunit, hypothetical protein	*E. coli* O157:H7 strain EC970520	EF174158.1	7316	7717	ABM53631.1 ABM53632.1
w1C4	575	99	bacteriophage P4 DNA primease	*E. coli* O157:H7 strain EC970520	EF174158.1	3513	2939	ABM53623.1
w1D10	497	99	site-specific DNA-methyltransferase	*S. dysenteriae* Sd197	CP000034.1	1754612	1755109	ABB62032.1
w1D11	>1200	93	1-188: putative open reading frame	Enterobacteria phage Nil2	AJ413274.1	8719	8907	CAC95099.1
		100	271-699: hypothetical protein EcolO15_21403 (X)	*E. coli* O157:H7 str.EC4501	-	1	143	ZP_02788527
		98	701-1029:hypothetical protein, hypothetical protein,adenine methylase, hypothetical protein	Stx2-converting bacteriophage 86	AB255436.1	39988	39749	BAF34069.1 BAF34070.1 BAF34071.1 BAF34072.1
w1E10	318	99	"orf31" (unknown product)	*Shigella flexneri* bacteriophage V	U82619.2	23381	23064	AAL89433.1
w1E8	491	98	Flanked by: ISSfl3 orfA and exonuclease I, 3 --> 5 specific deoxyribophosphodiesterase. Contains: IntA insertion element	*S. flexneri* 2a str. 2457T	AE014073.1	2070449	2070939	AAP17440.1 AAP17439.1 AAP16001.1
w2C8	454	99	major capsid protein	*E. coli* O157:H7 strain EC970520	EF174158.1	6298	6752	ABM53630.1
w2E9	601	79	1-272: bacteriophage lambda NinG protein (X)	*E. coli* O157:H7 str. EC508	-	333	638	ZP_02823579
			283-588:hypothetical protein EcolO_07489 (X)	*E. coli* O157:H7 str. EC508	-	59	149	ZP_02823580
w2F3	249	90	hypothetical proteins (2). Phage p_Rha.	*S. dysenteriae* Sd197	CP000034.1	1758074	1757827	ABB62034.1 ABB62035.1
w2F4	216	99	1-105: Present in EDL933					

			106-216: "gpQ"	*Yersinia* phage L-413C	AY251033.1	593	708	AAP04438.1
w2G10	688	85	1-93: Present in EDL933.					
			94-688: "hkaK" (unknown function)	Enterobacteria phage HK620	AF335538.1	5059	4527	AAK28859.1
w2H7	250	100	repressor protein CI	*S. dysenteriae* Sd197	CP000034.1	1753860	1754109	ABB62031.1
w3A11	427	95	1-201: hypothetical protein: (putative Fels-1 prophage DNA or RNA helicases of superfamily II)	*S. dysenteriae* Sd197,	CP000034.1	1751829	1751628	ABB62028.1
		72	"unnamed protein product"	*Photorhabdus luminescens* subsp. Laumondii TTO1	BX571868.1	315959	315698	CAE15290.1
w3B2	285	91[a], 98	3' flanking region (24bp deletion), hypothetical protein: phage p_Rha	*S. dysenteriae* Sd197	CP000034.1	1757511 1757592	1757568 1757831	ABB62034.1
w3B4	469	97	phage late control gene D protein, phage transcriptional activator (Ogr/Delta)	*E. coli* E24377A (ETEC)	CP000800.1	1016222	1016691	ABV17526.1 ABV16998.1
w3C4	692	92	1-236:"eae-like protein"	Enterobacteria phage phiV10 (of O157:H7)	DQ126339.2	35895	36131	AAZ95934.1
		72	245-687 putative methyltransferase	*Salmonella typhi* strain CT18	AL627268.1	259815	260181	CAD05420.1
w3C6	437	100	hypothetical protein EcolO_07509 (X)	*E. coli* O157:H7 str. EC508		16	125	ZP_02823583
w3E6	297	98	site-specific DNA-methyltransferase	*S. dysenteriae* Sd197	CP000034.1	1754357	1754616	ABB62032.1
w1B4	581	79	1-300: conserved hypothetical protein (X)	*E. coli* O157:H7 str. EC508		58	149	ZP_02823580
			448-581: Present in EDL933					
w1B11	>969	100	1-660: RecT family protein (X)	*E. coli* O157:H7 str. EC508		78	316	ZP_02825666
		82	744-895: Present in EDL933					
w1C10	500	99	putative phage DNA primase (X)	*E. coli* O157:H7 str. EC508		180	323	ZP_02823581.1
w1D6	493	99	DnaB analogue	Enterobacteria phage Nil2	AJ413274.1	5697	5204	CAC95089.1
w1F12	454	100	major capsid protein	*E. coli* O157:H7 strain EC970520	EF174158.1	6298	6752	ABM53630.1

w1G2	306	97	1-237: "gpA"	*Yersinia* phage L-413C	AY251033.1	28546	28783	AAP04475.1
			237-306: no significant hits (X)					
w1H6	568	97	flanked by: type II site-specific deoxyribonuclease and hypothetical protein, phage p_Rha	*S. dysenteriae* Sd197	CP000034.1	1757514	1756946	ABB62033.1 ABB62034.1
w1H8	>900	99	hypothetical protein, adenine methylase	Stx2-converting bacteriophage 86 DNA	AB255436.1	39111	39988	BAF34070.1 BAF34071.1
w2A4	>1000	99	predicted kinase inhibitor, predicted DNA-binding transcriptional regulator	*E. coli* W3110 DNA	AP009048.1	570305	571309	
w2B6	288	95	hypothetical protein: putative Fels-1 prophage DNA or RNA helicases of superfamily II	*S. dysenteriae* Sd197	CP000034.1	1752108	1751821	ABB62028.1
w2C11	>1000	93	1-188: antA	Enterobacteria phage Nil2	AJ413274.1	8719	8907	CAC95099.1
		99	253-685: hypothetical protein ECH7EC4501_4951 (X)	*E. coli* O157:H7 strain EC4501		1	143	ZP_02788527.1
		99	643-954: hypothetical protein, adenine methylase	Stx2-converting phage 86	AB255436.1	39988	39676	BAF34072.1 BAF34071.1
w2D3	402	100	1-306: conserved hypothetical protein (X)	*E. coli* O157:H7 strain EC508				ZP_02823583.1
		97	307-402: hypothetical proteins (2) putative phage related helicases	*S. dysenteriae* Sd197	CP000034.1	1752300	1752204	ABB62028.1 ABB62029.1
w2G2	200	98	putative baseplate assembly protein	*E. coli* APEC O1	CP000468.1	939608	939736	ABJ00305.1
w2G12	245	100	PTS-dependent dihydroxyacetone kinase operon regulatory protein	*E. coli* E24377A	CP000800.1	1341847	1342091	ABV20847.1
w3B8	455	100	major capsid protein	*E. coli* O157:H7 strain EC970520	EF174158.1	6298	6752	ABM53630.1
w3E2	409	96	1-201 hypothetical protein	*S. dysenteriae* Sd197	CP000034.1	1751829	1751628	ABB62028.1
		73	148-409: unnamed protein product (both are phage related helicases)	*P. luminescens* subsp. laumondii TTO1	BX571868.1	315959	315698	CAE15291.1

w3F2	699	99	phage DNA packaging protein NU1-like protein	*E. coli* O157:H7 strain EC970520	EF174158.1	7713	8412	ABM53633.1
w3G10	245		1-63: Present in EDL933					
		96	64-245: flanking sequence/Ogr (phage zinc binding transcriptional regulator)	*E. coli* APEC O1	CP000468.1	2259200	2259444	ABJ00319.1
w3H1	574	100	bacteriophage P4 DNA primease	*E. coli* O157:H7 strain EC970520	EF174158.1	2939	3514	ABM53623.1
w3H4	>1000	100	"w0048" (DNA breaking and rejoining enzyme)	*E. coli* O157:H- plasmid pSFO157[b]	AF401292.1	66718	67691	AAR00433.1

a: % identity is calculated disregarding the 24bp deletion

b: Also present in *E. coli* O157:H7 Sakai genome

Table 6.3: Sequences identified in the SSH library of MCI 0453: A summary of the best BLASTN hits (or BLASTX hits where the hit is followed by the letter 'X') for each sequence in the subtracted library of MCI 0453 genomic fragments. Sequences which were identified multiple times were only included as a single entry on this table. Results are further summarised in pie charts (Figure 6.6). Where the best BLAST hit has less than 90% identity with the sequence obtained these sequences are given in full in Appendix VI.

Name	Size (bp)	% ID	BLASTN Hit	Organism	Accession	Sequence begin	Sequence end	Protein ID
d1A3	574	100	bacteriophage P4 DNA primease	*E. coli* O157:H7 strain EC970520	EF174158.1	3513	2939	ABM53623.1
d1C9	300	100	bacteriophage P4 DNA primease, hypothetical protein	*E. coli* O157:H7 strain EC970520	EF174158.1	2622	2926	ABM53623.1 ABM53624.1
d1D8	399	100	putative endolysin	*E. coli* O157:H7 Sakai	BA000007.2	5051934	5051524	BAB38385.1
d1F9	121	99	hypothetical protein	*E. coli* O157:H7 strain EC970520	EF174158.1	6019	5898	ABM53628.1
d1G4	614	0	1-251: No significant hits	-	-	-	-	-
		100	260-614: hypothetical protein EcSMS35_A0078 (X)	*E. coli* SECEC SMS-3-5		39	154	YP_001739966
		99	252-614: putative protein	*E. coli* plasmid p1658/97	AF550679.1	104777	105141	AAO49623.1
d1G9	250	94	hypothetical protein, putative virion morphogenesis protein	*E. coli* O157:H7 Sakai	BA000007.2	5058623	5058841	BAB38394.1 BAB38395.1
d1H10	500	80	1-103: rep protein	*Corynebacterium renale* plasmid pCR1	X99132.2	613	716	CAA67573.2
		0	104-500: no significant hits (X)	-	-	-	-	-
d1H5	239	99	putative DNA circulation protein (phage-related)	*E. coli* O157:H7 Sakai	BA000007.2	5067720	5067971	BAB38406.1
d2B7	205	100	hypothetical protein	*E. coli* O157:H7 Sakai	BA000007.2	5075563	5075768	BAB36395.1 BAB34630.1
d2G6		100	1-320: type IV secretion system protein VirB1	*E. coli* O157:H7 str. EC4115	-	97	203	ZP_02771522
		100	315-531 hypothetical protein EschecoliO157_30031, (X)	*E. coli* O157:H7 str. EC4115	-	1	60	ZP_02771521
d2G9	600	75	Type IV conjugal transfer protein	*Aeromonas veronii*	DQ890522.1	3375	2996	ABI83640.1
d2H2	830	93	hypothetical protein, hypothetical protein (related to Gp11, bacteriophage Mu)	*E. coli* O157:H7 Sakai	BA000007.2	5048005	5048889	BAB38376.1 BAB38377.1
d3B10	882	99	1-264: present in EDL933					
			265-882 hypothetical protein	*E. coli* O157:H7 Sakai	BA000007.2	5077068	5077950	BAB38417.1

d3F10	455	100	major capsid protein	*E. coli* O157:H7 strain EC970520	EF174158.1	6298	6752	ABM53630.1
d3G3	467	99	putative transcriptional regulator, putative endolysin	*E. coli* O157:H7 Sakai	BA000007.2	5051528	5051062	BAB38384.1 BAB38385.1
d3H2	284	100	phage transposase	*E. coli* O157:H7 Sakai	BA000007.2	5043529	5043813	BAB38368.1
d3H8	281	99	putative cI repressor protein, Stx2 converting phage II	*E. coli* O157:H7 Sakai	BA000007.2	1257594	1257313	BAB34608.1
d1A1	300	100	putative DNA circulation protein	*E. coli* O157:H7 Sakai	BA000007.2	5067720	5067971	BAB38406.1
d1A2	397	99	unnamed protein product; psu gene product	Bacteriophage P4	X51522.1	10773	11170	CAA35906.1
d1B12	439	99	1-152 Present in EDL933					
			152-440 hypothetical protein	*E. coli* O157:H7 Sakai	BA000007.2	5076427	5076866	BAB38416.1
d1C3	589	99	major capsid protein	*E. coli* O157:H7 strain EC970520	EF174158.1	6298	6887	ABM53630.1
d1D1	209	71	"traH"	*E. coli* plasmid R721	AP002527.1	59100	58889	BAB12652.1
d1D2	>975	96	locus 17899…18799	*Salmonella enterica* serovar Dublin plasmid pOU1115	DQ115388.1	17899	18799	-
d1F3	>900	95	143-676: gene 23 protein	Enterobacteria phage Sf6 (*Shigella flexneri*)	AF547987.1	20706	20173	AAQ12213.1
		96	676- conserved hypothetical protein, hypothetical protein	*E. coli* HS	CP000802.1	295965	295579	ABV04665.1 ABV04666.1
d1F7	>1048	76	1-607: “locus 690...1297”	*E. coli* retronphage (phi)R73,	M64113.1	690	1297	
		77	691-940 unnamed protein product, possible helicase.	*Photorhabdus luminescens* subsp. laumondii TTO1	BX571859.1	157011	157295	CAE12448.1
d2B4	281	100	hypothetical proteins (2)	*E. coli* O157:H7 Sakai	BA000007.2	5045477	5045758	BAB38371.1 BAB38372.1
d2B6	466	99	putative transcription regulator, putative endolysin	*E. coli* O157:H7 Sakai	BA000007.2	5051528	5051062	BAB38384.1 BAB38385.1
d2C2	314	99	phage transposase	*E. coli* O157:H7 Sakai	BA000007.2	5043529	5043813	BAB38368.1

d2C7	401	100	hypothetical proteins (3)	*E. coli* O157:H7 strain EC970520	EF174158.1	2107	2508	ABM53621.1 ABM53622.1 ABM53624.1
d2G1	615	94	1-364: putative protein, putative protein	*E. coli* plasmid p1658/97	AF550679.1	105141	104777	AAO49623.1 AAO49624.1
		0	365-615: NO SIGNIFICANT HITS (X)					
d2H12	>720	0	NO SIGNIFICANT HITS (X)					
d3D1	300	98	putative protein	*E. coli* plasmid p1658/97	AF550679.1	105350	105138	AAO49623.1

Table 6.4: Sequences identified in the SSH library of MCI 0489: A summary of the best BLASTN hits (or BLASTX hits where the hit is followed by the letter 'X') for each sequence in the subtracted library of MCI 0489 genomic fragments. Sequences which were identified multiple times were only included as a single entry on this table. Results are further summarised in pie charts (Figure 6.6). Where the best BLAST hit has less than 90% identity with the sequence obtained these sequences are given in full in Appendix VI.

Name	bp	% ID	homologue	organism	Accession number	Sequence begin	Sequence end	Protein ID
sf1-9	739	98	starvation sensing protein RspB (alcohol dehydrogenase-like)	*E. coli* E24377A	CP000800.1	1778877	1779615	ABV20572.1
sf1-52	688	99	site-specific recombinase, phage integrase family	*E. coli* E24377A	CP000800.1	1001566	1000879	ABV17174.1
sf1-54	468	97	putative bacteriophage protein	*S. dysenteriae* Sd197	CP000034.1	553010	553477	ABB60777.1
sf1-72	314	100	Efa1-Lymphostatin-like protein	*E. coli* O157:H- 493/89	AJ459584.1	7357	7670	CAD30848.1
sf1-79	656	99	predicted inner membrane protein, hypothetical protein with nucleoside triphosphate hydrolase domain	*E. coli* W3110	AP009048.1	3131557	3130902	BAE77044.1 BAE77045.1
sf1-83	508	99	"w0003"	*E. coli* O157:H- pSFO157	AF401292.1	2675	2168	AAR00439.1
sfA-10	457	94	1-35:putative phage tail collar domain protein	*E. coli* E24377A	CP000800.1,	852438	852473	ABV19732.1
		80	192-256: putative phage tail domain protein			1287369	1287433	ABV21111.1
		98	257-369: (present in EDL933) putative phage tail collar domain protein					
		81	369-457: Gifsy-1 prophage proteins	*S. typhimurium* LT2	AE008818.1	6044	5948	AAL21482.1 AAL21483.1
sfA-17	223	97	Predicted phosphatase homologous to the C-terminal domain of histone macroH2A1 (X)	*Shigella dysenteriae* 1012		116	200	ZP_00921526
sfA-30	>1000	94	putative membrane protein, hypothetical protein	*E. coli* E24377A	CP000800.1	1309747	1310729	ABV19698.1 ABV18070.1
sfA-55	244	89	putative bacteriophage protein	*S. flexneri* 2a str. 301	AE005674.1	914044	913801	AAN42512.1
sfA-61	261	98	1-243: gpF1	*E. coli* APEC O1	CP000468.1	948432	948189	ABJ00313.1
sfA-65	744	99	hypothetical proteins (x3)	*E. coli* strain EC970520	EF174158.1	1367	2110	ABM53619.1 ABM53620.1 ABM53621.1
sfA-68	437	99	putative outer membrane autotransporter	*E. coli* HS	CP000802.1	377328	376892	ABV04751.1

sfA-83	284	97	antitermination protein Q	*S. flexneri* bacteriophage V	U82619.2	33167	33450	AAL89448.1
sfB-12	>1008	97	conserved hypothetical proteins (x2)	*E. coli* E24377A	CP000800.1	1306392	1307399	ABV20518.1 ABV17372.1
sfB-61	521	95	putative capsid protein of prophage	*E. coli* APEC O1	CP000468.1	1205015	1205535	ABJ00554.1
sfB-77	>930	100	w0070, w0071	*E. coli* O157:H- plasmid pSFO157	AF401292.1	89907	90836	AAR00469.1 AAR00487.1
sfB-89	290	99	w0034	*E. coli* O157:H- plasmid pSFO157	AF401292.1	47832	48121	AAR00444.1
sfB-96	520		1-470: NADH-ubiquinone oxidoreductase	*Roseobacter* sp. SK209-2-6		118	225	ZP_01756926
			481-520: present in EDL933					
sf1-85	341		1-150: dihydroxyacetone kinase, 160-320 dihydroxyacetone kinase subunit M	*E. coli* B7A,				ZP_00714922
			99-341: present in EDL933					
sfP-2	780	97	hypothetical protein, hypothetical protein	*Citrobacter koseri* ATCC BAA-895	CP000822.1	3274935	3274156	ABV14592.1 ABV14593.1
sfP-10	506	89	hypothetical protein	*C. koseri* ATCC BAA-895	CP000822.1	3279503	3278998	ABV14597.1
sf1-53	544	99	putative phage protein, predicted major capsid protein	*E. coli* UTI89	CP000243.1	930348	929805	ABE06421.1 ABE06422.1
sf1-70	798	100	1-184: present in EDL933					
		98	185-798:starvation sensing protein RspB, starvation-sensing protein RspA	*E. coli* E24377A	CP000800.1	1780225	1779612	ABV20572.1 ABV17062.1
sf1-73	714	96	putative phage protein, hypothetical protein	*E. coli* UTI89	CP000243.1	935066	934354	ABE06425.1 ABE06426.1
sfP-5	814		1-292: Present in EDL933 292-814: ORF1, ORF2, ORF3, ORF4 and partial st55	*E. coli* O157:H (non-motile) strain 493/89	AJ534392.1	3259	2743	
sf1-3	>822	99	PerC protein, hypothetical protein, hypothetical protein			5901	5083	ABM53627.1 ABM53628.1 ABM53629.1
			274-822: present in EDL933					
sf1-80	331	97	CI protein, hypothetical protein	*E. coli* O157:H7 Morioka V526 (Stx2 converting phage II DNA)	AP005154.1	43520	43190	BAC78103.1, BAC78104.1

sf1-37	415	98	putative capsid protein gp7 of prophage	*E. coli* UTI89	CP000243.1	1268235	1268649	ABE06799.1
sf1-86	412	100	orf 31: unknown product	*S. flexneri* bacteriophage V		23900	23489	AAL89433.1
sf1-21	389		1-150: hypothetical protein gp80 EcolO15_30564	*E. coli* O157:H7 str. EC4501		171	395	ZP_02790314
			150-389:present in EDL933					
sf1-29	>1400	25	30-470: hypothetical protein	*Methylobacterium nodulans*		451	640	ZP_02119661
			600-750 present in EDL933					
		86	779-810: putative protein encoded by prophage	*Shigella boydii* Sb227		51	76	YP_408524
sf1-38	295	99	1-230: putative repressor protein	*E. coli* E24377A (ETEC)	CP000800.1	833710	833480	ABV17234.1
		100	226-295 present in EDL933					
sf1-5	699	99	hypothetical protein, phage DNA packaging protein NU1-like protein.	*E. coli* O157:H7 strain EC970520	EF174158.1	7713	8412	ABM53632.1 ABM53633.1
sf1-66	380	98	e14 prophage; predicted DNA-binding transcriptional regulator	*E. coli* APEC O1	CP000468.1	2949655	2949276	ABJ02081.1
sfA-62	>1200	100	hypothetical protein EcolO15_29435 (X)	*E. coli* O157:H7 str. EC4501		1	151	ZP_02790094
sfA-77	>1200	86	65-1053: putative cytoplasmic protein (possibly zinc-chelating)	*S. typhimurium* LT2	AE008824.1	17732	16857	AAL21628.1
sfA-78	262	98	putative polysaccharide biosynthesis protein	*E. coli* HS	CP000802.1	3176740	3177002	ABV07398.1
sfB-33	283	97	antitermination protein Q	*S. flexneri* bacteriophage V	U82619.2	33167	33450	AAL89448.1
sfB-43	431	99	isocitrate dehydrogenase	*E. coli* BL21(DE3)	AB064593.1	4926	4533	BAB61874.1
sfB-56	489	99	Qin prophage; predicted protein, predicted S lysis protein	*E. coli* K12 MG1655	U00096.2	1638627	1638138	AAC74628.1 AAC74629.2
sfB-86	870	99	putative lipoprotein, conserved hypothetical protein	*E. coli* E24377A (ETEC)	CP000800.1	3466493	3465623	ABV20031.1

Table 6.5: Sequences identified in the SSH library of MCI 0507: A summary of the best BLASTN hits (or BLASTX hits where the hit is followed by the letter 'X') for each sequence in the subtracted library of MCI 0507 genomic fragments. Sequences which were identified multiple times were only included as a single entry on this table. Results are further summarised in pie charts (Figure 6.6). Where the best BLAST hit has less than 90% identity with the sequence obtained these sequences are given in full in Appendix VI.

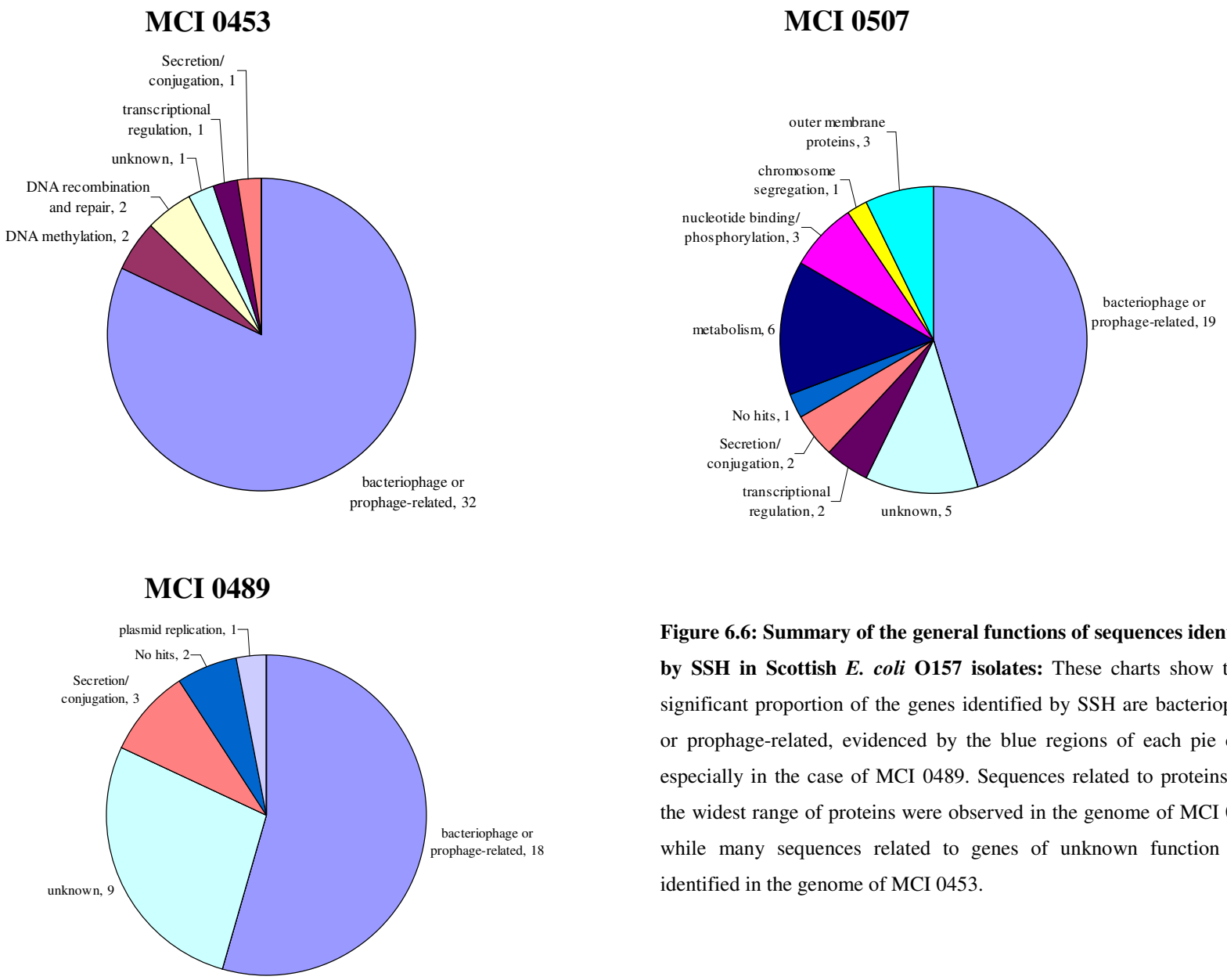


Figure 6.6: Summary of the general functions of sequences identified by SSH in Scottish *E. coli* O157 isolates: These charts show that a significant proportion of the genes identified by SSH are bacteriophage or prophage-related, evidenced by the blue regions of each pie chart, especially in the case of MCI 0489. Sequences related to proteins with the widest range of proteins were observed in the genome of MCI 0507, while many sequences related to genes of unknown function were identified in the genome of MCI 0453.

6.4 Discussion

Data presented in this chapter highlights the diversity of EHEC strains related to human infection in Scotland. Typing of two of the established EHEC virulence factor-encoding genes by PCR-RFLP shows that the *espP* gene types appear to show serotype specificity, in that *E. coli* O157 isolates almost universally encode *espP*α type O157, whereas *E. coli* O26 isolates yielded a different pattern (corresponding to *espP*α type O26). *espP* genes were not detected in the genomes of the three sorbitol-fermenting *E. coli* O157 isolates. This distribution patterns of *espP* subtypes is in agreement with that observed by Brockmeyer *et al.* (2007) except that no amplicon was generated from one *E. coli* O157 isolate (MCI 0239) and that another *E. coli* O157:H7 isolate was found to encode an *espP* allele which gave a RFLP-pattern which did not match any predicted pattern, namely, MCI 0064 (*E. coli* O157:H7 NCTC 12900). As a naturally occurring *stx*-negative isolate, MCI 0064 has been used extensively as a model organism of disease associated *E. coli* O157:H7 (Woodward *et al.*, 2003;Best *et al.*, 2005;La Ragione *et al.*, 2005;Belfiore *et al.*, 2007). As this work shows that the *espP* gene carried by this strain is atypical, it calls into question the applicability of this strain as a model for human EHEC, although this work does not address the expression or functionality of the *espP* type encoded by this isolate. Nevertheless, this result highlights the care which must be taken in selecting an isolate for use in animal studies. Any strain used as a model of human EHEC infection should be well characterised to verify that the strain is representative of the infectious agent that it is designed to model.

Genes encoding shiga-like toxin exhibit more heterogeneous carriage among Scottish EHEC isolates than that discovered for *espP* alleles. Among non-sorbitol-fermenting *E. coli* O157 isolates seven different *stx*-genotypes were identified, the most common profile obtained was one suggesting that the isolates carried *stx*2 along with *stx*2_c or *stx*2_d. In total, 18 of the human EHEC isolates were predicted to carry *stx*$2_{c/d}$-encoding genes, making this the most common allele detected. *stx*-genotypes appeared to associate with phage-types among the *E. coli* O157 isolates analysed, with the majority of phage-type 21/28 carrying *stx*2 with *stx*$2_{c/d}$ whereas all isolates of phage type 8 exhibited a pattern corresponding to *stx*1 with *stx*$2_{c/d}$.

Unfortunately, the PCR-RFLP assay could not distinguish *stx*2_c from *stx*2_d. Analysis of publicly available *stx*2_c and *stx*2_d gene sequences (accession numbers EU086525.1 and

EF584538.1) suggest that *Rsa*I or *Alu*I could be used to distinguish between these two alleles and resolve this issue. $Stx2_c$ and $Stx2_d$ differ in that $Stx2_d$ is activatable by elastase in intestinal mucous to a form which is 100-fold more verotoxic than conventional Stx2, whereas $Stx2_c$ has a similar level of toxicity as Stx2 (Melton-Celsa & O'Brien 1996, Melton-Celsa *et al* 2002). The prevalence of *E. coli* O157 isolates carrying *stx*2 and $stx2_c$ has been noted by others (Ziebell *et al.*, 2008;Leotta *et al.*, 2008;Uhlich *et al.*, 2008) and the carriage of *stx*2 and $stx2_c$ genes has been associated with increased disease severity in cases of *E. coli* O157:H7 infection (Caprioli *et al.*, 1995;Pierard *et al.*, 1998;Eklund *et al.*, 2002;Friedrich *et al.*, 2002;Persson *et al.*, 2007) whereas $Stx2_d$ is generally encoded by non-O157 STEC such as *E. coli* O91:H21 (Gobius *et al.*, 2003). Therefore, it is more likely that these strains carry the $stx2_c$ allele than the $stx2_d$ allele. The acquisition of a gene encoding $stx2_c$ in addition to *stx*2 may have implications for the pathogenicity of *E. coli* O157:H7 and may be responsible for the increased pathogenicity of phage type 21/28 *E. coli* O157 isolates, noted by Lynn *et al.* (2005). Phage type 21/28 are also prevalent among the bovine *E. coli* O157 population which could contribute to their increased association with severe cases of human EHEC infection (Halliday *et al.*, 2006). The association of phage type 8 with *stx*1/$stx2_c$ fits with two published observations. The first is that PT8 is associated with a lower relative risk of the development of HUS compared with other serotypes (Lynn *et al.*, 2005) and secondly that *stx*1 is less toxic than *stx*2 or $stx2_c$ (Melton-Celsa and O'Brien, 1998).

Genomic microarray analysis of *E. coli* O157 isolates shows the clonal nature of *E. coli* O157. However, certain regions of heterogeneity, especially among bacteriophage or prophage-related genes were found, highlighting the role of bacteriophages as mediators of genomic diversification in *E. coli* O157; however, bacteriophages form a large part of the *E. coli* O157:H7 genome and therefore are likely to feature in the diverse component regardless of their proposed role in eliciting such diversification. Twenty-four bacteriophage and prophage-related elements were identified in the genome of *E. coli* O157:H7, ranging in size from 7.5kbp to 61.6kbp and bacteriophages are proposed to constitute approximately half of the genomic content present in *E. coli* O157:H7 that is absent from the *E. coli* K12 MG1655 genome (Perna *et al.*, 2001;Hayashi *et al.*, 2001b). Genomic subtractive hybridisation allowed the detection of additional genes present in the genomes of certain *E. coli* O157 isolates related to cases of human disease. As many of these genomic regions are also bacteriophage-related this provides further evidence to support the assertion that these are regions which are

involved in genome diversification. The SSH results for MCI 0507 included genes associated with a more diverse range of biological functions than the resulting subtracted sequences from the two non-sorbitol-fermenting isolates (MCI 0489 and MCI 0453), highlighting additional differences between the genomes of sorbitol-fermenting *E. coli* O157 isolates and reference (non-sorbitol-fermenting) *E. coli* O157:H7 strains. Many of the sequences identified in the genome of MCI 0507 during this study were also identified in German isolates of sorbitol-fermenting *E. coli* O157:H- by similar methods (Janka *et al.*, 2005). These data, coupled with the conservation of *stx* types and the lack of *espP* genes, suggest that this strain of Scottish sorbitol-fermenting *E. coli* O157 are clonally related to European sorbitol-fermenting *E. coli* O157:H-.

Genes encoding type IV secretion or conjugation apparatus were identified in the genome of MCI 0453. Type IV conjugation has been shown to mediate the horizontal transfer of genomic islands between bacteria (Juhas *et al.*, 2007). Type IV secretion is also employed by organisms such as *Helicobacter pylori*, *Bordatella pertussis* and *Legionella pneumophila* for the secretion of virulence factors (Backert and Meyer, 2006). Either of these two features may aid the pathogenic function of the strain, either by facilitating protein export or increasing likelihood of horizontal gene transfer. Two sequences were also identified which relate to membrane proteins, including an autotransporter protein present in the genome of human commensal *E. coli* strain HS. The presence of this sequence in the genome of a human commensal *E. coli* isolates make the role of this protein in pathogenesis less likely; however, it has not been determined whether *E. coli* HS may cause disease opportunistically. Autotransporter proteins related to virulence have been identified in many Gram negative bacterial pathogens (reviewed in Henderson *et al.*, 2004) and it is possible that this protein may contribute to the virulence of this MCI 0453. Extensive expression and functional analysis would be required to test this possibility.

As potential virulence factors were not apparent in the results of the SSH-screen, it is unlikely that increased incidence of disease in Scotland is due to acquisition of novel virulence factor-encoding genes; however, this analysis does not rule out differences in the expression of virulence factors. Also single or multiple nucleotide polymorphisms are not identified using the methods described here, but could have significant effects on the pathogenic functions of a microorganism. Several sequences were identified in the SSH-screen which were related to

proteins of unknown function or returned no BLAST hits; hence, the possibility that these genes may have a bearing on bacterial pathogenesis may not be ruled-out.

In summary, the EHEC strains analysed in this study exhibit some diversity with respect to the carriage of virulence factor gene subtypes and gene-gains and gene-losses identified by microarray and SSH. Small-scale genome variation among *E. coli* O157 isolates was identified and that variation was largely composed of phage and prophage-related genetic elements, further supporting the role of bacteriophages in genome diversification.

Chapter 7: General Discussion

Enterohaemorrhagic *E. coli* (EHEC) represents a group of human pathogens which pose a significant threat to human health, of which *E. coli* serotype O157:H7 is the most frequently associated with human EHEC infection and also often resides in the ruminant gut. The objectives of this work were to:

1) Characterise the roles of bacterial exported molecules in the modulation of host immunity, focusing on interactions with the intestinal epithelium (Chapter 3).
2) Characterise the genotypes of *E. coli* of the bovine gastrointestinal tract and associate *E. coli* genotypes with preference for particular gastrointestinal locations (Chapter 4).
3) Evaluate the potential of *E. coli* of the bovine gastrointestinal tract for inhibition of pathogenic *E. coli* O157:H7 (Chapter 5).
4) Perform genomic characterisation of *E. coli* strains significant in human EHEC infection in Scotland (Chapter 6).

7.1 The role of EHEC-exported products in interaction with the host.

Modulation of the host immune response is a feature of many bacterial pathogens and is also a feature of bacteria which exist in the host without pathogenesis or even benefit the host organism (Lodinova-Zadnikova and Sonnenborn, 1997;Lodinova-Zadnikova *et al.*, 2003;Kamada *et al.*, 2008). Differential immune responses can restrict proliferation of a pathogenic organism within the host but may exacerbate the tissue damage caused by a pathogen; hence, understanding of the host immune responses to *E. coli* O157:H7 could lead to explanation of the differential levels of pathogenicity of the bacterium between that observed in the human compared with the asymptomatic tolerance observed in the bovine host (Karmali *et al.*, 1983b;Borczyk *et al.*, 1987;Cray, Jr. and Moon, 1995).

Data presented in Chapter 3 reaffirms the role of bacterial flagellin in the inflammatory response of cells of a human intestinal epithelial cell line (T84). Although the results of initial experiments suggested a suppressive role for substances present in bacterial supernatants, subsequent investigation revealed that this initial finding is likely to be anomalous and confirmed that flagellin is indeed the major proinflammatory ligand secreted by EHEC, but also not the only factor exported by EHEC which induces IL-8 production by the human T84 cells. This has been noted by others for a variety of enteric pathogens (Sharma *et al.*, 2005;Khan *et al.*, 2008); however, the identity of further proinflammatory ligands are yet to be determined. Furthermore, when a bovine epithelial cell line was assessed for responsiveness to bacterial products, the proinflammatory response appeared to be independent of flagellin. However, there are several key points to note in the interpretation of these data. Firstly, embryonic bovine lung (EBL) cells were chosen as a bovine epithelial cell line model. The responses of the lung epithelium compared with gastrointestinal responses are likely to differ, especially as *E. coli* is not generally tolerated in the lung (Russo and Johnson, 2000). Secondly, the assay used to determine IL-8 production is based on a weak cross-reactivity between anti-human IL-8 antibodies provided in the IL-8 DuoSet ELISA (R & D Systems) and bovine IL-8. Consequently, the observed levels of IL-8 were at the lower limit of detection using the ELISA kit, meaning that variation between replicates was often extensive. These two points highlight major difficulties faced by researchers whose goal is characterisation of cellular responses of the bovine host or bovine cell line models. At the time

of this study, no well-characterised bovine gastrointestinal epithelial cell line was available, compared with the plethora of human and mouse cell lines currently in circulation. Also, there are few commercially available antibodies for the analysis of bovine cellular responses. Given the number of publicly funded research groups dedicated to the analysis of ruminant cellular biology and immunology, it is hoped that a concerted collaborative effort will be made to make such reagents widely available.

As a protease secreted by *E. coli* O157:H7 with some well-characterised functions in the human host, preliminary analysis was conducted to attempt to further characterise the function of StcE in bacterial interaction with the epithelium and predict the function of StcE protease in the bovine host. Confirmation of the activity of recombinant StcE (kindly provided by Rodney Welch of the University of Wisconsin, Madison) against its proposed substrates (human C1-INH and salivary glycoproteins) (Lathem *et al.*, 2002, Grys *et al.*, 2005) was attempted. Although digestion of human C1-INH was observed, its previously reported effects on the salivary proteome were not detected in this study. In order to determine if StcE affects bovine C1-INH; incubation of FBS with StcE was performed followed by Western blotting the using the anti-human C1-INH antibody. Both cross specificity of a human antibody with the bovine homologue and the presence of this homologue in commercially available FBS were required to visualise the result. As bovine C1-INH was not identifiable in the resultant image this suggested either that the antibody failed to bind or that bovine C1-INH was not present; hence, it was not possible to determine whether StcE affects bovine C1-INH. Data obtained by Lathem *et al.* (2002) suggest that the presence of FBS is sufficient to allow StcE-mediated aggregation of Jurkat cells and that this aggregation was due to StcE's effect on C1-INH. Therefore, it is reasonable to postulate that StcE affects bovine C1-INH in the same way as human C1-INH. Although this analysis could not resolve the function of StcE in the bovine host, detection of anti-StcE IgA in mucosal scrapings from calves challenged with *E. coli* O157:H7 could suggest that StcE is expressed by the bacterium and recognised by the bovine immune system during colonisation. However, further investigation would be required to confirm this effect.

In summary: the roles of bacterial products in mediation of the proinflammatory response are incompletely characterised. Data shown in Chapter 3 reaffirms the role of flagellin in the human epithelial proinflammatory response but leaves the possibility of the presence of other

proinflammatory mediators in bacterial exported products. The role of the StcE protease in EHEC-interactions with the bovine host remain undetermined, although a secretory antibody response could suggest that it is expressed by the bacterium and recognised by the host immune system in colonisation of the bovine gastrointestinal tract.

7.2 *E. coli* of the bovine gastrointestinal tract

As explained previously, *E. coli* O157:H7 is regularly carried in the bovine gut. Data presented in Chapter 4 shows that many non-O157 strains which carry genes encoding EHEC-related virulence factors (especially *stx* and *espP*) are isolable from the bovine gut (Aktan *et al.*, 2004;Shaw *et al.*, 2004). No *E. coli* strains bearing an O-RFLP-pattern which matched that of *E. coli* O157 were isolated from animals used in this study. This would suggest that the prevalence of *E. coli* O157:H7 in incidence of human infection is not due to its prevalence in cattle above other serotypes which harbour EHEC-related genes (which were prevalent among the cattle analysed), suggesting that characteristics other than simply the carriage of the genes for the conventionally accepted virulence factors are involved in the increased association of *E. coli* O157:H7 with human infection. However, this study does not address the expression, secretion and regulation of these virulence factors by bovine isolates carrying these genes and in all cases the alteration of the functions of each virulence factor by single or multiple nucleotide polymorphisms are possible. Furthermore, the animals analysed here represented a small sample from a restricted geographic area and could not therefore be considered representative of the Scottish cattle population as a whole. It is also feasible that many of the isolates identified as carrying EHEC-related virulence factors may be capable of human infection, as has been noted for many non-O157 EHEC serotypes (as discussed in Section 1.3) and prevalence of non-O157:H7 serotypes could be underestimated due to isolation methods which may be biased toward the isolation and identification of *E. coli* O157:H7, such as methods relying on cefixime-tellurite resistance and non-sorbitol fermentation (March and Ratnam, 1986) or those which rely on binding of the O-antigen such as latex agglutination or immunomagnetic separation (IMS) (March and Ratnam, 1989;Bennett *et al.*, 1996).

An interesting feature of the carriage of *E. coli* O157:H7 by the bovine host is that the bacterium shows a preference for colonisation of the terminal rectum (Naylor *et al.*, 2003). In order to predict whether this feature was specific to *E. coli* O157:H7 or if other *E. coli* strains also show preference for this site, isolation and enumeration of *E. coli* from selected sites in the bovine GIT was coupled with genotyping methods in order to identify associations between genotypes and colonisation patterns. Overall analysis of total numbers of *E. coli* present at each site suggested that *E. coli* presence was not uniform at the sites analysed and that the terminal rectum and proximal colon were the most heavily colonised of the sites

analysed. When enumeration data were combined with genotypic analysis it was revealed that strains exhibiting EHEC-related genotypes were significantly associated with colonisation at the rectum in greater numbers than other sites whereas those carrying ExPEC-related genes and those in which no virulence factors were identified (suggesting similarity with laboratory *E. coli* strains) exhibited an even distribution at each of the sites analysed. Other studies have related EHEC related genotypes with colonisation at the rectum; however, others have not considered strains other than EHEC-related genotypes colonising without apparent site-preference (Naylor *et al.*, 2003;Low *et al.*, 2005;Sheng *et al.*, 2005).

A limitation of the sampling methods employed in this analysis is that *E. coli* was sampled only at a single timepoint from each animal. As a temporal pattern of *E. coli* colonisation has been suggested for *E. coli* O157:H7, with initial colonisation observed at multiple sites and the greatest persistence observed at the rectum (Grauke *et al.*, 2002;Naylor *et al.*, 2003), high levels of *E. coli* genotypes recovered from the rectum could represent organisms at later stages of colonisation; however, in this study one particular genotype (*E. coli* ONT (Or7):HNT2: *cdtB*+, *cnf*+, *fimA*+, *iutA*+, *gafD*+) was recovered at high numbers at all sites sampled in five animals. Another genotype (*E. coli* Or37:HNT1 *stx*2+, *ehx*: type 319, *fimA*+) present only at the rectum of three animals suggests that genotype may be important in the definition of the gastrointestinal localisation of *E. coli* in the bovine host.

There are a number of options for increasing the understanding of the issues addressed by this analysis. Methods for quantification of certain genotypes at certain locations could perhaps be enhanced by employing quantitative-PCR-based methodology, whereby total DNA would be obtained from a fixed region of tissue and the copies of bacterial genomes estimated by detection of certain genetic markers of representative genotypes (for example *stx*, *espP*, *ehxA* or *eae* for EHEC-related organisms or *gafD*, *cdt* or *cnf* for ExPEC-related organisms). Total numbers *E. coli*, using primer/probe sets for genes common to all *E. coli* (based on rRNA-encoding genes), could be compared against numbers of *E. coli* of certain genotypes from a single sample. Results presented in Chapter 4 highlight the genes associated with particular colonisation patterns and these genes may be employed as targets for such an assay.

To confirm that strains which were observed to be prevalent at the terminal rectum have a genuine preference for that site, conducting oral challenges of calves is an option in order to

satisfy Koch's postulates regarding the tropism of selected strains. This could be compared against a similar challenge using a strain which exhibited even colonisation at all of the sites.

In order to identify the genes involved in definition of bacterial tropism, strains with defined colonisation patterns may be subjected to mutagenesis screening and tested for altered colonisation patterns. The problem with this approach is that no robust methodology exists for the analysis of gastrointestinal colonisation patterns short of conducting animal challenges. If an *in vitro* assay could be developed for tissue tropism, perhaps employing *in vitro* organ culture (IVOC) coupled with an adherence assay, this would considerably aid such analysis. A similar method has been employed for the analysis of human tissue tropism of *E. coli* strains and may potentially be applied for analysis of *E. coli*-bovine host interactions (Chong *et al.*, 2007;Mundy *et al.*, 2007). Identification of targets by this method would rely on a defined bacterial molecule-host cell receptor affinity and accounting for temporal and physiological reasons for rectal tropism (such as aeration at the site) would require modifications to such a technique which may prove impractical to incorporate.

7.3 Probiotic potential of *E. coli*

Several studies have suggested a case for the application of probiotic strains of *E. coli* in order to reduce the prevalence of *E. coli* O157:H7 in cattle (Zhao *et al.*, 1998;Tkalcic *et al.*, 2003;Zhao *et al.*, 2003;Schamberger *et al.*, 2004). It was envisaged that strains isolated from the bovine host during this study may have applicability as probiotic organisms and that data provided in Chapter 4 would aid selection of a strain which is able to colonise effectively at the terminal rectum and may therefore compete with *E. coli* O157:H7 at its preferred site *in vivo*. To this end, bovine *E. coli* isolates were tested *in vitro* for inhibition of *E. coli* O157:H7 strains. Data presented in Chapter 5 shows that bacterial products from certain strains acted to inhibit *E. coli* O157:H7; however, of all strains which inhibited at least one strain of *E. coli* O157:H7, none were observed to inhibit all of the *E. coli* O157:H7 strains tested. This suggests that susceptibility to allelopathic molecules produced by these strains varies among *E. coli* O157:H7 subtypes. The precise identities of the allelopathic molecules produced are as yet undetermined as PCR for genes encoding microcins and colicins known to inhibit *E. coli* O157:H7 largely returned negative results. This could indicate that previously undescribed inhibitory molecules are produced by the strains identified in this study and further investigation would be required to characterise these molecules in detail.

It was envisaged that isolates which exhibited apparent rectal tropism (Chapter 4) may have applicability as they may compete with *E. coli* O157:H7 at the preferred site of colonisation in the bovine host; however, it became clear that the most promising rectal-tropic strains carried genes encoding EHEC-related virulence factors, especially Shiga-like toxin. Many were excluded from further analysis due to this fact, particularly as those encoding *stx* may pose a significant threat to human health and must be handled under higher containment levels meaning that their use as probiotic organisms would not be advisable. Although certain isolates among those not encoding *stx* were found to inhibit strains of *E. coli* O157:H7, it is not yet known whether these strains may potentially undergo lysogeny by *stx*-encoding bacteriophages if they were released into the environment. The acquisition of virulence factor-encoding genes could increase the likelihood that isolates intended to be probiotic may emerge as pathogens themselves.

In summary, data presented in Chapter 5 shows that some of the strains used in this study have inhibitory activity against *E. coli* O157:H7 although this is unlikely to lead directly to a probiotic organism for application to reduce the prevalence of *E. coli* O157:H7. Further resolution of the issues raised in this chapter could be gained firstly by identification of the allelopathic molecules produced by the inhibitory strains. This could be facilitated by fractionation of bacterial products to identify inhibitory fractions, coupled with proteomic techniques such as MALDI-TOF in order to identify the molecules responsible. The isolation of further *E. coli* strains from cattle would provide a larger panel with an increased likelihood of containing one applicable as a probiotic agent. If a strain were to be identified which fills the criteria of (i) effective colonisation of cattle (especially at the terminal rectum) (ii) absence of virulence factors, (iii) resistance to acquisition of *stx*-encoding genes by phage transduction, (iv) specificity for inhibition of *E. coli* O157/EHEC, progression to identification of *in vivo* effectiveness is an option. These criteria eliminate the vast majority of naturally-occurring *E. coli* strains meaning that the search for a probiotic *E. coli* isolate becomes more complex.

7.4 Genomic character of Scottish EHEC isolates

The results of the characterisation of *espP* genes in EHEC isolates were largely as expected. It was notable that the majority of human EHEC isolates carrying an *espP* gene carried a subtype likely to encode an active form of the EspP protease, whereas a more diverse range of alleles were identified among the bovine *E. coli* isolates (although these data are likely to be affected by the inclusion of many *E. coli* O157 and *E. coli* O26 isolates in the analysis). A large proportion of the non-O157 EHEC isolates were negative for the *espP* gene, which fits with the observations of Brockmeyer *et al.* (2007). It could then be suggested that the EspP protease is not absolutely necessary for EHEC pathogenicity; however, the possibility remains that the isolates found to be negative for this gene could encode a protease with similar function which is not detected by the PCR-assay described here.

The analysis of *stx* genes revealed that *stx*-encoding genes were almost ubiquitously carried by human EHEC isolates; however, the diversity observed among the *stx*-genotypes was extensive. The selective pressure for the emergence of variant *stx* genes may be maintained due to its antigenicity, as neutralising antibodies against Stx have been found to counteract the action of the toxin (Mukherjee *et al.*, 2002;Orth *et al.*, 2008). Many of the variant groups of Stx are antigenically non-cross-reactive, which could mean that production of a variant or multiple variants of Stx may allow evasion of host immune defences against EHEC infection.

SSH and CGH microarray data presented in Chapter 6 suggest that the genomes of Scottish isolates of *E. coli* O157:H7 do not differ greatly from reference isolates, including the genome-sequenced American and Japanese isolates (EDL933 and Sakai) in terms of the gains and losses of entire genes or large gene fragments. It is hence unlikely that Scottish EHEC have acquired significant virulence factors compared with strains from other geographical locations. This could support the notion that the increased observed prevalence of *E. coli* O157 infection in Scotland may depend on other epidemiological factors, such as the increased likelihood for contact with farm animals (Mead *et al.*, 1997;Locking *et al.*, 2001;O'Brien *et al.*, 2001;Strachan *et al.*, 2006) coupled with an increased observed prevalence of *E. coli* O157 in farm animals in Scotland (Paiba *et al.*, 2002).

It is envisaged that genotypic data presented here may be compared against clinical data from each incidence of infection in order to identify associations between genotypic traits and the severity of EHEC-induced infection. This process may be aided by extension and refinement of the genotyping techniques utilised in this study. Other studies have been successful in identifying lineages of *E. coli* O157:H7 more closely associated with human disease (Kim *et al.*, 1999;Lynn *et al.*, 2005;Zhang *et al.*, 2007), but have yet to identify the functions necessary for their varied association with human disease.

7.5 Conclusions and Future Directions

In summary: data presented in the above highlights several important features of *E. coli* as a bovine colonist and as a human pathogen. As delineated in Section 7.1 the mechanisms by which the human host recognises and responds to *E. coli* O157:H7 and the mechanisms by which *E. coli* O157:H7 acts to subvert the human host are only partially understood. The interactions between EHEC and the bovine host are even less well-characterised. Resolution of the nature of these interactions would be aided by the development and distribution of reagents for the study of bovine responses to EHEC, including immortalised cell lines and specific antibodies.

Data presented in Chapter 4 highlights the genomic diversity of resident *E. coli* of the bovine gastrointestinal tract. Some evidence was observed that there is a general preference for the terminal rectum among *E. coli* with EHEC-related genotypes; however its is yet to be establish what factors either (i) generally restrict EHEC-related *E. coli* to the bovine rectum (prevent colonisation at other sites) or (ii) augment adherence or proliferation of EHEC-related organisms at the rectum above other sites. It is possible that a combination of the two occurs and that multiple bacterial and host factors are involved.

A related objective of this research was to identify a strain likely to have applicability as a probiotic organism. Although no such strain has yet been identified the findings presented in Chapter 4 regarding the virulence genes encoded by bovine resident *E. coli*, coupled with the abilities of *E. coli* to transfer genes from one strain to another (via bacteriophages or conjugative plasmids) provide a warning against proceeding with this type of work without conducting extensive characterisation of any candidate strain for administration to cattle.

Data presented in Chapter 6 highlights the clonal nature of *E. coli* O157:H7, evidenced by the low level of detected genomic diversity. The observed diversity was generally identified in bacteriophage or prophage-related elements which reaffirms the role of these mobile elements in genome-diversification.

References

Abercrombie,J.G., Paynter,M.J.B. and Hayasaka,S.S. (2006). Ability of Colicin V to Control *Escherichia coli* O157:H7 in Ground Beef. *Journal of Food Safety.* **26:** 103-114

Abreu,M.T., Vora,P., Faure,E., Thomas,L.S., Arnold,E.T. and Arditi,M. (2001). Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *Journal of Immunology.* **167:** 1609-1616.

Acres,S.D. (1985). Enterotoxigenic *Escherichia coli* infections in newborn calves: a review. *Journal of Dairy Science*. **68:** 229-256.

Adamo,R., Sokol,S., Soong,G., Gomez,M.I. and Prince,A. (2004). *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. *American Journal of Respiratory Cellular and Molecular Biology.* **30:** 627-634.

Adler,J. and Tso,W.W. (1974). "Decision"-making in bacteria: chemotactic response of *Escherichia coli* to conflicting stimuli. *Science.* **184:** 1292-1294.

Adlerberth,I., Hanson,L.A., Svanborg,C., Svennerholm,A.M., Nordgren,S. and Wold,A.E. (1995). Adhesins of *Escherichia coli* associated with extra-intestinal pathogenicity confer binding to colonic epithelial cells. *Microbial Pathogenesis*. **18:** 373-385.

Adlerberth,I., Svanborg,C., Carlsson,B., Mellander,L., Hanson,L.A., Jalil,F., Khalil,K. and Wold,A.E. (1998). P fimbriae and other adhesins enhance intestinal persistence of *Escherichia coli* in early infancy. *Epidemiology and Infection*. **121:** 599-608.

Adu-Bobie,J., Frankel,G., Bain,C., Goncalves,A.G., Trabulsi,L.R., Douce,G., Knutton,S. and Dougan,G. (1998). Detection of intimins alpha, beta, gamma and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. *Journal of Clinical Microbiology.* **36:** 662-668.

Agin,T.S. and Wolf,M.K. (1997). Identification of a family of intimins common to *Escherichia coli* causing attaching-effacing lesions in rabbits, humans and swine. *Infection & Immunity*. **65:** 320-326.

Ahmed,A., Li,J., Shiloach,Y., Robbins,J.B. and Szu,S.C. (2006). Safety and immunogenicity of *Escherichia coli* O157 O-specific polysaccharide conjugate vaccine in 2-5-year-old children. *Journal of Infectious Diseases* **193:** 515-521.

Ahmed,R., Bopp,C., Borczyk,A. and Kasatiya,S. (1987). Phage-typing scheme for *Escherichia coli* O157:H7. *Journal of Infectious Diseases* **155:** 806-809.

Aidar-Ugrinovich,L., Blanco,J., Blanco,M., Blanco,J.E., Leomil,L., Dahbi,G., Mora,A., Onuma,D.L., Silveira,W.D. and Pestana de Castro,A.F. (2007). Serotypes, virulence genes and intimin types of Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) isolated from calves in Sao Paulo, Brazil. *International Journal of Food Microbiology* **115:** 297-306.

Akiba,M., Sameshima,T. and Nakazawa,M. (2000). Clonal turnover of enterohemorrhagic *Escherichia coli* O157:H7 in experimentally infected cattle. *FEMS Microbiology Letters* **184:** 79-83.

Aktan,I., La Ragione,R.M. and Woodward,M.J. (2007). Colonization, persistence and tissue tropism of *Escherichia coli* O26 in conventionally reared weaned lambs. *Applied and Environmental Microbiology* **73:** 691-698.

Aktan,I., Sprigings,K.A., La Ragione,R.M., Faulkner,L.M., Paiba,G.A. and Woodward,M.J. (2004). Characterisation of attaching-effacing *Escherichia coli* isolated from animals at slaughter in England and Wales. *Veterinary Microbiology* **102:** 43-53.

Alexopoulou,L., Thomas,V., Schnare,M., Lobet,Y., Anguita,J., Schoen,R.T., Medzhitov,R., Fikrig,E. and Flavell,R.A. (2002). Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice. *Nature Medicine* **8:** 878-884.

Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389-3402.

Anjum,M.F., Mafura,M., Slickers,P., Ballmer,K., Kuhnert,P., Woodward,M.J. and Ehricht,R. (2007). Pathotyping *Escherichia coli* by using miniaturized DNA microarrays. *Applied and Environmental Microbiology* **73:** 5692-5697.

Aranda,K.R., Fagundes-Neto,U. and Scaletsky,I.C. (2004). Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic *Escherichia coli* and *Shigella* spp. *Journal of Clinical Microbiology* **42:** 5849-5853.

Asensio C. and Pérez-Díaz J.C. (1976). A new family of low molecular weight antibiotics from enterobacteria. *Biochemical and Biophysical Research Communications.* **69:** 7-14.

Backert,S. and Meyer,T.F. (2006). Type IV secretion systems and their effectors in bacterial pathogenesis. *Current Opinion in Microbiology*. **9:** 207-217.

Backhed,F., Ding,H., Wang,T., Hooper,L.V., Koh,G.Y., Nagy,A., Semenkovich,C.F. and Gordon,J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Science U.S.A.* **101:** 15718-15723.

Ball,H.J., Finlay,D., Burns,L. and MacKie,D.P. (1994). Application of monoclonal antibody-based sandwich ELISAs to detect verotoxins in cattle faeces. *Research in Veterinary Science* **57:** 225-232.

Ballmer,K., Korczak,B.M., Kuhnert,P., Slickers,P., Ehricht,R. and Hachler,H. (2007). Fast DNA serotyping of *Escherichia coli* by use of an oligonucleotide microarray. *Journal of Clinical Microbiology.* **45:** 370-379.

Banatvala,N., Griffin,P. M. Greene,K. D., Barrett,T. J. Bibb,W. F. Green,J. H. and Wells,J. G. (2001). The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings. J. Infect. Dis. **183:**1063-1070

Bastian,S.N., Carle,I. and Grimont,F. (1998). Comparison of 14 PCR systems for the detection and subtyping of stx genes in Shiga-toxin-producing *Escherichia coli*. *Research in Microbiology.* **149:** 457-472.

Bauer,M.E. and Welch,R.A. (1996). Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* O157:H7. *Infection & Immunity*. **64:** 167-175.

Baumler,A.J., Tsolis,R.M. and Heffron,F. (1996). The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. *Proceedings of the National Academy of Science U.S.A.* **93:** 279-283.

Belfiore,C., Castellano,P. and Vignolo,G. (2007). Reduction of *Escherichia coli* population following treatment with bacteriocins from lactic acid bacteria and chelators. *Food Microbiology.* **24:** 223-229.

Bennett,A.R., MacPhee,S. and Betts,R.P. (1996). The isolation and detection of *Escherichia coli* O157 by use of immunomagnetic separation and immunoassay procedures. *Letters in Applied Microbiology.* **22:** 237-243.

Bentley,R. and Meganathan,R. (1983). Vitamin K biosynthesis in bacteria--precursors, intermediates, enzymes and genes. *Journal of Natural Products.* **46:** 44-59.

Berg,H.C. and Anderson,R.A. (1973). Bacteria swim by rotating their flagellar filaments. *Nature* **245:** 380-382.

Berin,M.C., Darfeuille-Michaud,A., Egan,L.J., Miyamoto,Y. and Kagnoff,M.F. (2002). Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. *Cellular Microbiology* **4:** 635-648.

Bernheiden,M., Heinrich,J.M., Minigo,G., Schutt,C., Stelter,F., Freeman,M., Golenbock,D. and Jack,R.S. (2001). LBP, CD14, TLR4 and the murine innate immune response to a peritoneal *Salmonella* infection. *Journal of Endotoxin Research* **7:** 447-450.

Bertin,Y., Boukhors,K., Pradel,N., Livrelli,V. and Martin,C. (2001). Stx2 subtyping of Shiga toxin-producing *Escherichia coli* isolated from cattle in France: detection of a new Stx2 subtype and correlation with additional virulence factors. *Journal of Clinical Microbiology* **39:** 3060-3065.

Bertin,Y., Girardeau,J.P., Darfeuille-Michaud,A. and Contrepois,M. (1996a). Characterization of 20K fimbria, a new adhesin of septicemic and diarrhea-associated *Escherichia coli* strains, that belongs to a family of adhesins with N-acetyl-D-glucosamine recognition. *Infection & Immunity* **64:** 332-342.

Bertin,Y., Martin,C., Oswald,E. and Girardeau,J.P. (1996b). Rapid and specific detection of F17-related pilin and adhesin genes in diarrheic and septicemic *Escherichia coli* strains by multiplex PCR. *Journal of Clinical Microbiology* **34:** 2921-2928.

Besser,R.E., Lett,S.M., Weber,J.T., Doyle,M.P., Barrett,T.J., Wells,J.G. and Griffin,P.M. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *JAMA*. **269:** 2217-2220.

Besser,T.E., Shaikh,N., Holt,N.J., Tarr,P.I., Konkel,M.E., Malik-Kale,P., Walsh,C.W., Whittam,T.S. and Bono,J.L. (2007). Greater diversity of Shiga toxin-encoding bacteriophage insertion sites among *Escherichia coli* O157:H7 isolates from cattle than in those from humans. *Applied and Environmental Microbiology.* **73:** 671-679.

Best,A., La Ragione,R.M., Sayers,A.R. and Woodward,M.J. (2005). Role for flagella but not intimin in the persistent infection of the gastrointestinal tissues of specific-pathogen-free chicks by shiga toxin-negative *Escherichia coli* O157:H7. *Infection & Immunity* **73:** 1836-1846.

Beutin,L., Aleksic,S., Zimmermann,S. and Gleier,K. (1994). Virulence factors and phenotypical traits of verotoxigenic strains of *Escherichia coli* isolated from human patients in Germany. *Medical Microbiology and Immunology (Berlin)* **183:** 13-21.

Beutin,L., Geier,D., Steinruck,H., Zimmermann,S. and Scheutz,F. (1993). Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *Journal of Clinical Microbiology.* **31:** 2483-2488.

Beutin,L., Montenegro,M.A., Orskov,I., Orskov,F., Prada,J., Zimmermann,S. and Stephan,R. (1989). Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. *Journal of Clinical Microbiology.* **27:** 2559-2564.

Bielaszewska,M., Dobrindt,U., Gärtner,J., Gallitz,I., Hacker,J., Karch,H., Müller,D., Schubert,S., Alexander Schmidt M., Sorsa L.J. and Zdziarski J (2007a). Aspects of genome plasticity in pathogenic *Escherichia coli*. *International Journal of Medical Microbiology* **297:** 625-639.

Bielaszewska,M., Janda,J., Bláhová,K., Minaríková,H., Jíková,E., Karmali,M.A., Laubová,J., Sikulová,J., Preston,M.A., Khakhria,R., Karch,H., Klazarová,H., Nyc,O. (1997). Human *Escherichia coli* O157:H7 infection associated with the consumption of unpasteurized goat's milk. *Epidemiology & Infection*. **119:** 299-305.

Bielaszewska,M. and Karch,H. (2005). Consequences of enterohaemorrhagic *Escherichia coli* infection for the vascular endothelium. *Thrombosis & Haemostasis.* **94:** 312-318.

Bielaszewska,M., Sinha,B., Kuczius,T. and Karch,H. (2005). Cytolethal distending toxin from Shiga toxin-producing *Escherichia coli* O157 causes irreversible G2/M arrest, inhibition of proliferation and death of human endothelial cells. *Infection & Immunity* **73:** 552-562.

Bielaszewska,M., Sonntag,A.K., Schmidt,M.A. and Karch,H. (2007b). Presence of virulence and fitness gene modules of enterohemorrhagic *Escherichia coli* in atypical enteropathogenic *Escherichia coli* O26. *Microbes & Infection* **9:** 891-897.

Bingen,E., Picard,B., Brahimi,N., Mathy,S., Desjardins,P., Elion,J. and Denamur,E. (1998). Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. *Journal of Infectious Diseases.* **177:** 642-650.

Bischoff,K.M., Shi,L. and Kennelly,P.J. (1998). The detection of enzyme activity following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Analytical Biochemistry.* **260:** 1-17.

Blanco,M., Blanco,J.E., Mora,A. and Blanco,J. (1998a). Distribution and characterization of faecal necrotoxigenic *Escherichia coli* CNF1+ and CNF2+ isolated from healthy cows and calves. *Veterinary Microbiology*. **59:** 183-192.

Blanco,M., Blanco,J.E., Blanco,J., Alonso,M.P., Balsalobre,C., Mouriño,M., Madrid,C. and Juarez,A. (1996). Polymerase chain reaction for detection of *Escherichia coli* strains producing cytotoxic necrotizing factor type 1 and 2 (CNF1 and CNF2). *Journal of Microbiological Methods.* **26:** 95-101.

Blanco,M., Blanco,J.E., Mora,A. and Blanco,J. (1998b). Prevalence and characteristics of necrotoxigenic *Escherichia coli* CNF1+ and CNF2+ in healthy cattle. *Research in Microbiology.* **149:** 47-53.

Blanco,M. Padola,N.L., Krüger,A., Sanz,M.E., Blanco,J.E., González,E.A., Dahbi,G., Mora,A., Bernárdez,M.I., Etcheverría,A.I., Arroyo,G.H., Lucchesi,P.M., Parma,A.E. and Blanco J. (2004). Virulence genes and intimin types of Shiga-toxin-producing *Escherichia coli* isolated from cattle and beef products in Argentina. *International Microbiology.* **7:** 269-276.

Blanco,M., Schumacher,S., Tasara,T., Zweifel,C., Blanco,J.E., Dahbi,G., Blanco,J. and Stephan,R. (2005). Serotypes, intimin variants and other virulence factors of eae positive *Escherichia coli* strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (eae-eta2). *BMC Microbiology*. **5:** 23.

Boerlin,P., McEwen,S.A., Boerlin-Petzold,F., Wilson,J.B., Johnson,R.P. and Gyles,C.L. (1999). Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *Journal of Clinical Microbiology* **37:** 497-503.

Bollinger,R.R., Everett,M.L., Palestrant,D., Love,S.D., Lin,S.S. and Parker,W. (2003). Human secretory immunoglobulin A may contribute to biofilm formation in the gut. *Immunology* **109:** 580-587.

Bollinger,R.R., Everett,M.L., Wahl,S.D., Lee,Y.H., Orndorff,P.E. and Parker,W. (2006). Secretory IgA and mucin-mediated biofilm formation by environmental strains of *Escherichia coli*: role of type 1 pili. *Molecular Immunology* **43:** 378-387.

Borczyk,A.A., Karmali,M.A., Lior,H. and Duncan,L.M. (1987). Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. *Lancet* **1:** 98.

Bray,J. (1945). Isolation of antigenically homogeneous strains of *Bact coli neapolitanum* from summer diarrhoea of infants. *Journal of Pathology Bacteriology* **57:** 239-247.

Brett,K.N., Hornitzky,M.A., Bettelheim,K.A., Walker,M.J. and Djordjevic,S.P. (2003). Bovine non-O157 Shiga toxin 2-containing *Escherichia coli* isolates commonly possess stx2-EDL933 and/or stx2vhb subtypes. *Journal of Clinical Microbiology.* **41:** 2716-2722.

Brockmeyer,J., Bielaszewska,M., Fruth,A., Bonn,M.L., Mellmann,A., Humpf,H.U. and Karch,H. (2007). Subtypes of the plasmid-encoded serine protease EspP in Shiga toxin-producing *Escherichia coli*: distribution, secretion and proteolytic activity. *Applied and Environmental Microbiology.* **73:** 6351-6359.

Brown,C.A., Harmon,B.G., Zhao,T. and Doyle,M.P. (1997). Experimental *Escherichia coli* O157:H7 carriage in calves. *Applied and Environmental Microbiology.* **63:** 27-32.

Brunder,W., Karch,H. and Schmidt,H. (2006). Complete sequence of the large virulence plasmid pSFO157 of the sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:H- strain 3072/96. *International Journal of Medical Microbiology.* **296:** 467-474.

Brunder,W., Khan,A.S., Hacker,J. and Karch,H. (2001). Novel type of fimbriae encoded by the large plasmid of sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:H(-). *Infection & Immunity*. **69:** 4447-4457.

Brunder,W., Schmidt,H., Frosch,M. and Karch,H. (1999). The large plasmids of Shiga-toxin-producing *Escherichia coli* (STEC) are highly variable genetic elements. *Microbiology*. **145:** 1005-1014.

Brunder,W., Schmidt,H. and Karch,H. (1997). EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Molecular Microbiology* **24:** 767-778.

Burland,V., Shao,Y., Perna,N.T., Plunkett,G., Sofia,H.J. and Blattner,F.R. (1998). The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucleic Acids Research*. **26:** 4196-4204.

Callaway,T.R., Elder,R.O., Keen,J.E. anderson,R.C. and Nisbet,D.J. (2003). Forage feeding to reduce preharvest *Escherichia coli* populations in cattle, a review. *Journal of Dairy Science.* **86:** 852-860.

Campellone,K.G., Rankin,S., Pawson,T., Kirschner,M.W., Tipper,D.J. and Leong,J.M. (2004a). Clustering of Nck by a 12-residue Tir phosphopeptide is sufficient to trigger localized actin assembly. *Journal of Cellular Biology.* **164:** 407-416.

Campellone,K.G., Robbins,D. and Leong,J.M. (2004b). EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Developmental Cell* **7:** 217-228.

Cantarelli,V.V., Takahashi,A., Akeda,Y., Nagayama,K. and Honda,T. (2000). Interaction of enteropathogenic or enterohemorrhagic *Escherichia coli* with HeLa cells results in translocation of cortactin to the bacterial adherence site. *Infection & Immunity* **68:** 382-386.

Caprioli,A., Luzzi,I., Gianviti,A., Russmann,H. and Karch,H. (1995). Pheno-genotyping of verotoxin 2 (VT2)-producing *Escherichia coli* causing haemorrhagic colitis and haemolytic uraemic syndrome by direct analysis of patients' stools. *Journal of Medical Microbiology* **43:** 348-353.

Caprioli,A., Morabito,S., Brugereb,H. and Oswald,E. (2005). Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Veterinary Research* **36:** 289-311.

Carter,B., Wu,G., Woodward,M.J. and Anjum,M.F. (2008). A process for analysis of microarray comparative genomics hybridisation studies for bacterial genomes. *BMC Genomics* **9:** 53.

Cascales,E., Buchanan,S.K., Duche,D., Kleanthous,C., Lloubes,R., Postle,K., Riley,M., Slatin,S. and Cavard,D. (2007). Colicin biology. *Microbiology & Molecular Biology Reviews.* **71:** 158-229.

Cassar C.A., Ottaway M., Paiba G.A., Futter R., Newbould S. and Woodward M.J. (2004). Absence of enteroaggregative Escherichia coli in farmed animals in Great Britain. *Veterinary Record.* **154:** 237-9

Cerqueira,A.M.F., Guth,B.E.C., Joaquim,R.M. and Andrade,J.R.C. (1999). High occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in healthy cattle in Rio de Janeiro State, Brazil. *Veterinary Microbiology* **70:** 111-121.

Chapman,P.A., Siddons,C.A., Zadik,P.M. and Jewes,L. (1991). An improved selective medium for the isolation of *Escherichia coli* O157. *Journal of Medical Microbiology.* **35:** 107-110.

Chart,H., Jenkins,C., Smith,H.R., Hedges,D. and Rowe,B. (1998). Haemolysin production by strains of Verocytotoxin-producing *Escherichia coli*. *Microbiology*. **144:** 103-107.

Chase-Topping,M.E. McKendrick,I.J., Pearce,M.C., MacDonald,P., Matthews,L., Halliday,J., Allison,L., Fenlon,D., Low,J.C., Gunn,G. and Woolhouse,M.E. (2007). Risk factors for the presence of high-level shedders of *Escherichia coli* O157 on Scottish farms. *Journal of Clinical Microbiology* **45:** 1594-1603.

China,B., Goffaux,F., Pirson,V. and Mainil,J. (1999). Comparison of eae, tir, *espA* and espB genes of bovine and human attaching and effacing *Escherichia coli* by multiplex polymerase chain reaction. *FEMS Microbiology Letters.* **178:** 177-182.

Chong,Y., Fitzhenry,R., Heuschkel,R., Torrente,F., Frankel,G. and Phillips,A.D. (2007). Human intestinal tissue tropism in *Escherichia coli* O157 : H7--initial colonization of terminal ileum and Peyer's patches and minimal colonic adhesion ex vivo. *Microbiology.* **153:** 794-802.

Clermont,O., Bonacorsi,S. and Bingen,E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied & Environmental Microbiology* **66:** 4555-4558.

Cobbold,R. and Desmarchelier,P. (2000). A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC) prevalence in three Australian diary herds. *Veterinary Microbiology.* **71:** 125-137.

Cobbold,R.N., Hancock,D.D., Rice,D.H., Berg,J., Stilborn,R., Hovde,C.J. and Besser,T.E. (2007). Rectoanal junction colonization of feedlot cattle by *Escherichia coli* O157:H7 and its association with supershedders and excretion dynamics. *Applied & Environmental Microbiology* **73:** 1563-1568.

Coimbra,R.S., Grimont,F., Lenormand,P., Burguiere,P., Beutin,L. and Grimont,P.A. (2000). Identification of *Escherichia coli* O-serogroups by restriction of the amplified O-antigen gene cluster (rfb-RFLP). *Research in Microbiology*. **151:** 639-654.

Comayras,C., Tasca,C., Peres,S.Y., Ducommun,B., Oswald,E. and De,R.J. (1997). *Escherichia coli* cytolethal distending toxin blocks the HeLa cell cycle at the G2/M transition by preventing cdc2 protein kinase dephosphorylation and activation. *Infection & Immunity* **65:** 5088-5095.

Contrepois,M., Bertin,Y., Pohl,P., Picard,B. and Girardeau,J.P. (1998). A study of relationships among F17 a producing enterotoxigenic and non-enterotoxigenic *Escherichia coli* strains isolated from diarrheic calves. *Veterinary Microbiology* **64:** 75-81.

Cookson,A.L., Bennett,J., Thomson-Carter,F. and Attwood,G.T. (2007). Molecular subtyping and genetic analysis of the enterohemolysin gene (*ehxA*) from Shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *E. coli*. *Applied & Environmental Microbiology* **73:** 6360-6369.

Coombes,B.K., Wickham,M.E., Mascarenhas,M., Gruenheid,S., Finlay,B.B. and Karmali,M.A. (2008). Molecular analysis as an aid to assess the public health risk of non-O157 Shiga toxin-producing *Escherichia coli* strains. *Applied & Environmental Microbiology* **74:** 2153-2160.

Cornick,N.A., Booher,S.L. and Moon,H.W. (2002). Intimin facilitates colonization by *Escherichia coli* O157:H7 in adult ruminants. *Infection & Immunity* **70:** 2704-2707.

Corthesy,B. (2007). Roundtrip ticket for secretory IgA: role in mucosal homeostasis? *Journal of Immunology* **178:** 27-32.

Crane,J.K., McNamara,B.P. and Donnenberg,M.S. (2001). Role of EspF in host cell death induced by enteropathogenic *Escherichia coli. Cellular Microbiology* **3:** 197-211.

Cray,W.C., Jr. and Moon,H.W. (1995). Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Applied & Environmental Microbiology* **61:** 1586-1590.

Cursino,L., Šmarda,J., Chartone-Souza,E. and Nascimento,A.M.A. (2002). Recent updated aspects of colicins of Enterobacteriaceae. *Brazilian Journal of Microbiology* **33:** 185-195.

Dahan,S., Busuttil,V., Imbert,V., Peyron,J.F., Rampal,P. and Czerucka,D. (2002). Enterohemorrhagic *Escherichia coli* infection induces interleukin-8 production via activation of mitogen-activated protein kinases and the transcription factors NF-kappaB and AP-1 in T84 cells. *Infection & Immunity* **70:** 2304-2310.

Dam,H. (1935). The antihaemorrhagic vitamin of the chick. *Biochemical Journal* **29:** 1273-1285.

Daniels,M.J., Hutchings,M.R. and Greig,A. (2003). The risk of disease transmission to livestock posed by contamination of farm stored feed by wildlife excreta. *Epidemiology & Infection* **130:** 561-568.

de Boer,E. and Heuvelink,A.E. (2000). Methods for the detection and isolation of Shiga toxin-producing *Escherichia coli*. *Symposium Series, Society for Applied Microbiology* 133S-143S.

De Rycke,J., Gonzalez,E.A., Blanco,J., Oswald,E., Blanco,M. and Boivin,R. (1990). Evidence for two types of cytotoxic necrotizing factor in human and animal clinical isolates of *Escherichia coli*. *Journal of Clinical Microbiology* **28:** 694-699.

De Rycke,J., Milon,A. and Oswald,E. (1999). Necrotoxic *Escherichia coli* (NTEC): two emerging categories of human and animal pathogens. *Veterinary Research* **30:** 221-233.

Dean-Nystrom,E.A., Bosworth,B.T., Moon,H.W. and O'Brien,A.D. (1998b). *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. *Infection & Immunity* **66:** 4560-4563.

Dean-Nystrom,E.A., Bosworth,B.T., Moon,H.W. and O'Brien,A.D. (1998a). Bovine Infection With Shiga Toxin Producing *Escherichia coli*. In: *Escherichi coli* O157:H7 and Other Shiga Toxin-Producing *E. coli* Strains, ed. J.B.Kaper and A.D.O'Brien. American Society for Microbiology, Washington, D.C., 261-267.

Dean-Nystrom,E.A., Gansheroff,L.J., Mills,M., Moon,H.W. and O'Brien,A.D. (2002). Vaccination of pregnant dams with intimin (O157) protects suckling piglets from *Escherichia coli* O157:H7 infection. *Infection & Immunity* **70:** 2414-2418.

DeGrandis,S., Law,H., Brunton,J., Gyles,C. and Lingwood,C.A. (1989). Globotetraosylceramide is recognized by the pig edema disease toxin. *Journal of Biological Chemistry*. **264:** 12520-12525.

Deibel,C., Kramer,S., Chakraborty,T. and Ebel,F. (1998). EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein. *Molecular Microbiology* **28:** 463-474.

Delbridge,L.M. and O'Riordan,M.X. (2007). Innate recognition of intracellular bacteria. *Current Opinions in Immunology* **19:** 10-16.

Delgado,M.A., Solbiati,J.O., Chiuchiolo,M.J., Farias,R.N. and Salomon,R.A. (1999). *Escherichia coli* outer membrane protein TolC is involved in production of the peptide antibiotic microcin J25. *Journal of Bacteriology* **181:** 1968-1970.

Deng,W., Puente,J.L., Gruenheid,S., Li,Y., Vallance,B.A., Vázquez,A., Barba,J., Ibarra,J.A., O'Donnell,P., Metalnikov,P., Ashman,K., Lee,S., Goode,D., Pawson,T. and Finlay,B.B. (2004). Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proceedings of the National Academy of Science U.S.A.* . **101:** 3597-3602.

Desjardins,P., Picard,B., Kaltenbock,B., Elion,J. and Denamur,E. (1995). Sex in *Escherichia coli* does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism. *Journal of Molecular Evolution.* **41:** 440-448.

DeVinney,R., Puente,J.L., Gauthier,A., Goosney,D. and Finlay,B.B. (2001). Enterohaemorrhagic and enteropathogenic *Escherichia coli* use a different Tir-based mechanism for pedestal formation. *Molecular Microbiology.* **41:** 1445-1458.

Diez-Gonzalez,F., Callaway,T.R., Kizoulis,M.G. and Russell,J.B. (1998). Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science.* **281:** 1666-1668.

Dobrindt,U. and Hacker,J. (2001). Whole genome plasticity in pathogenic bacteria. *Current Opinions in Microbiology* **4:** 550-557.

Dodd,C.C., Sanderson,M.W., Sargeant,J.M., Nagaraja,T.G., Oberst,R.D., Smith,R.A. and Griffin,D.D. (2003). Prevalence of *Escherichia coli* O157 in cattle feeds in Midwestern feedlots. *Applied & Environmental Microbiology* **69:** 5243-5247.

Dodson,K.W., Jacob-Dubuisson,F., Striker,R.T. and Hultgren,S.J. (1993). Outer-membrane PapC molecular usher discriminately recognizes periplasmic chaperone-pilus subunit complexes. *Proceedings of the National Academy of Science U.S.A.* **90:** 3670-3674.

Donnelly,M.A. and Steiner,T.S. (2002). Two nonadjacent regions in enteroaggregative *Escherichia coli* flagellin are required for activation of toll-like receptor 5. *Journal of Biological Chemistry*. **277:** 40456-40461.

Donnenberg,M.S., Giron,J.A., Nataro,J.P. and Kaper,J.B. (1992). A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. *Molecular Microbiology* **6:** 3427-3437.

Donohue-Rolfe,A., Keusch,G.T., Edson,C., Thorley-Lawson,D. and Jacewicz,M. (1984). Pathogenesis of Shigella diarrhea. IX. Simplified high yield purification of Shigella toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. *Journal of Experimental Medicine* **160:** 1767-1781.

Doyle,M.P. and Schoeni,J.L. (1987). Isolation of *Escherichia coli* O157:H7 from retail fresh meats and poultry. *Applied & Environmental Microbiology*. **53:** 2394-2396.

Duquesne,S., Petit,V., Peduzzi,J. and Rebuffat,S. (2007). Structural and functional diversity of microcins, gene-encoded antibacterial peptides from enterobacteria. *Journal of Molecular Microbiology & Biotechnology*. **13:** 200-209.

Dyce,K.M., Sack,W.O. and Wensing,C.J.G. (1987). Textbook of Veterinary Anatomy, W.B. Saunders Company, Philadelphia, Pennyslvania. p650.

Dytoc,M., Soni,R., Cockerill,F., III, De Azavedo,J., Louie,M., Brunton,J. and Sherman,P. (1993). Multiple determinants of verotoxin-producing *Escherichia coli* O157**:**H7 attachment-effacement. *Infection & Immunity*. **61:** 3382-3391.

Dziva,F., Mahajan,A., Cameron,P., Currie,C., McKendrick,I.J., Wallis,T.S., Smith,D.G. and Stevens,M.P. (2007). EspP, a Type V-secreted serine protease of enterohaemorrhagic *Escherichia coli* O157:H7, influences intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. *FEMS Microbiology Letters*. **271:** 258-264.

Dziva,F., van Diemen,P.M., Stevens,M.P., Smith,A.J. and Wallis,T.S. (2004). Identification of *Escherichia coli* O157:H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis. *Microbiology.* **150:** 3631-3645.

Eaves-Pyles,T.D., Wong,H.R., Odoms,K. and Pyles,R.B. (2001). *Salmonella* flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. *Journal of Immunology*. **167:** 7009-7016.

Eckburg,P.B., Bik,E.M., Bernstein,C.N., Purdom,E., Dethlefsen,L., Sargent,M., Gill,S.R., Nelson,K.E. and Relman,D.A. (2005). Diversity of the human intestinal microbial flora. *Science.* **308:** 1635-1638.

Eklund,M., Leino,K. and Siitonen,A. (2002). Clinical *Escherichia coli* strains carrying stx genes: stx variants and stx-positive virulence profiles. *Journal of Clinical Microbiology*. **40:** 4585-4593.

Elam,N.A., Gleghorn,J.F., Rivera,J.D., Galyean,M.L., Defoor,P.J., Brashears,M.M. and Younts-Dahl,S.M. (2003). Effects of live cultures of *Lactobacillus acidophilus* (strains NP45 and NP51) and *Propionibacterium freudenreichii* on performance, carcass and intestinal characteristics and *Escherichia coli* strain O157 shedding of finishing beef steers. *Journal of Animal Science.* **81:** 2686-2698.

Elder,R.O., Keen,J.E., Wittum,T.E., Callaway,T.R., Edrington,T.S. anderson,R.C. and Nisbet,D.J (2008). Intervention to reduce fecal shedding of enterohemorrhagic *Escherichia coli* O157:H7 in naturally infected cattle using neomycin sulfate. *Journal of Animal Science.* **80:** 15

Elliott,S.J., Yu,J. and Kaper,J.B. (1999). The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* O157:H7 is unable to confer the attaching and effacing phenotype upon *E. coli* K-12. *Infection & Immunity* **67:** 4260-4263.

Endo,Y., Tsurugi,K., Yutsudo,T., Takeda,Y., Ogasawara,T. and Igarashi,K. (1988). Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *European Journal of Biochemistry.* **171:** 45-50.

Erdem,A.L., Avelino,F., Xicohtencatl-Cortes,J. and Giron,J.A. (2007). Host protein binding and adhesive properties of H6 and H7 flagella of attaching and effacing *Escherichia coli*. *Journal of Bacteriology*. **189:** 7426-7435.

Erickson,A.K., Baker,D.R., Bosworth,B.T., Casey,T.A., Benfield,D.A. and Francis,D.H. (1994). Characterization of porcine intestinal receptors for the K88ac fimbrial adhesin of *Escherichia coli* as mucin-type sialoglycoproteins. *Infection & Immunity*. **62:** 5404-5410.

Escobar-Paramo,P., Clermont,O., Blanc-Potard,A.B., Bui,H., Le Bouguénec,C. and Denamur,E. (2004). A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. *Molecular Biology and Evolution.* **21:** 1085-1094.

Evaldson,G., Heimdahl,A., Kager,L. and Nord,C.E. (1982). The normal human anaerobic microflora. Scand. *Journal of Infectious Diseases Suppl.* **35:** 9-15.

Faith,N.G., Shere,J.A., Brosch,R., Arnold,K.W., Ansay,S.E., Lee,M.S., Luchansky,J.B. and Kaspar,C.W. (1996). Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. *Applied and Environmental Microbiology* **62:** 1519-1525.

Falguieres,T., Mallard,F., Baron,C., Hanau,D., Lingwood,C., Goud,B., Salamero,J. and Johannes,L. (2001). Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. *Molecular Biology of the Cell.* **12:** 2453-2468.

Farrell,R.J. and LaMont,J.T. (2002). Microbial factors in inflammatory bowel disease. *Gastroenterology Clinics of North America* **31:** 41-62.

Fenselau,C. and Demirev,P.A. (2001). Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrometry Reviews.* **20:** 157-171.

Fields,P.I., Blom,K., Hughes,H.J., Helsel,L.O., Feng,P. and Swaminathan,B. (1997). Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-restriction fragment length polymorphism test for identification of *E. coli* O157:H7 and O157:NM. *Journal of Clinical Microbiology*. **35:** 1066-1070.

Fitzhenry,R., Dahan,S., Torres,A.G., Chong,Y., Heuschkel,R., Murch,S.H., Thomson,M., Kaper,J.B., Frankel,G. and Phillips,A.D. (2006). Long polar fimbriae and tissue tropism in *Escherichia coli* O157:H7. *Microbes and Infection* **8:** 1741-1749.

Fitzhenry,R.J., Pickard,D.J., Hartland,E.L., Reece,S., Dougan,G., Phillips,A.D. and Frankel,G. (2002). Intimin type influences the site of human intestinal mucosal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7. *Gut* **50:** 180-185.

Fogh,J., Wright,W.C. and Loveless,J.D. (1977). Absence of HeLa cell contamination in 169 cell lines derived from human tumors. *Journal of National Cancer Institute.* **58:** 209-214.

Franchi,L, Amer,A., Body-Malapel,M., Kanneganti,TD., Ozören,N., Jagirdar,R., Inohara,N., Vandenabeele,P., Bertin,J., Coyle,A., Grant,EP. and Núñez,G. (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in *Salmonella*-infected macrophages. *Nature Immunology* **7:** 576-582.

Franck,S.M., Bosworth,B.T. and Moon,H.W. (1998). Multiplex PCR for enterotoxigenic, attaching and effacing and Shiga toxin-producing *Escherichia coli* strains from calves. *Journal of Clinical Microbiology* **36:** 1795-1797.

Fraser,M.E., Fujinaga,M., Cherney,M.M., Melton-Celsa,A.R., Twiddy,E.M., O'Brien,A.D. and James,M.N. (2004). Structure of shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *Journal of Biological Chemistry* **279:** 27511-27517.

Frey,J. and Kuhnert,P. (2002). RTX toxins in Pasteurellaceae. International *Journal of Medical Microbiology* **292:** 149-158.

Friedberg,D., Umanski,T., Fang,Y. and Rosenshine,I. (1999). Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli*. *Molecular Microbiology.* **34:** 941-952.

Friedrich,A.W., Bielaszewska,M., Zhang,W.L., Pulz,M., Kuczius,T., Ammon,A. and Karch,H. (2002). *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *Journal of Infectious Diseases.* **185:** 74-84.

Fukushima,H. and Seki,R. (2004). High numbers of Shiga toxin-producing *Escherichia coli* found in bovine faeces collected at slaughter in Japan. *FEMS Microbiology Letters.* **238:** 189-197.

Galan,J.E., Ginocchio,C. and Costeas,P. (1992). Molecular and functional characterization of the *Salmonella* invasion gene invA: homology of InvA to members of a new protein family. *Journal of Bacteriology*. **174:** 4338-4349.

Gansheroff,L.J. and O'Brien,A.D. (2000). *Escherichia coli* O157:H7 in beef cattle presented for slaughter in the U.S.: higher prevalence rates than previously estimated. *Proceedings of the National Academy of Science U.S.A.* **97:** 2959-2961.

Garber,L., Wells,S., Schroeder-Tucker,L. and Ferris,K. (1999). Factors associated with fecal shedding of verotoxin-producing *Escherichia coli* O157 on dairy farms. *Journal of Food Protection*. **62:** 307-312.

Garmendia,J.Phillips,A.D., Carlier,M.F., Chong,Y., Schüller,S., Marches,O., Dahan,S., Oswald,E., Shaw,R.K., Knutton,S. and Frankel,G. (2004). TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cellular Microbiology* **6:** 1167-1183.

Garmendia,J., Frankel,G. and Crepin,V.F. (2005) Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: translocation, translocation, translocation. *Infection & Immunity.* **73:** 2573-2585

Gerner-Smidt,P., Hise,K., Kincaid,J., Hunter,S., Rolando,S., Hyytia-Trees,E., Ribot,E.M. and Swaminathan,B. (2006). PulseNet USA: a five-year update. *Foodborne Pathogens and Disease.* **3:** 9-19.

Gewirtz,A.T., Navas,T.A., Lyons,S., Godowski,P.J. and Madara,J.L. (2001a). Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *Journal of Immunology*. **167:** 1882-1885.

Gewirtz,A.T., Simon,P.O., Jr., Schmitt,C.K., Taylor,L.J., Hagedorn,C.H., O'Brien,A.D., Neish,A.S. and Madara,J.L. (2001b). *Salmonella* typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *Journal of Clinical Investigation.* **107:** 99-109.

Gill,D.M. and Richardson,S.H. (1980). Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of *Escherichia coli*: comparison with cholera toxin. *Journal of Infectious Diseases.* **141:** 64-70.

Girardeau,J.P., Dalmasso,A., Bertin,Y., Ducrot,C., Bord,S., Livrelli,V., Vernozy-Rozand,C. and Martin,C. (2005). Association of virulence genotype with phylogenetic background in comparison to different

seropathotypes of Shiga toxin-producing *Escherichia coli* isolates. *Journal of Clinical Microbiology*. **43:** 6098-6107.

Girardin,S.E., Boneca,I.G., Carneiro,L.A., Antignac,A., Jéhanno,M., Viala,J., Tedin,K., Taha,MK., Labigne,A., Zähringer,U., Coyle,A.J., DiStefano,P.S., Bertin,J., Sansonetti,P.J. and Philpott,D.J. (2003a). Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science.* **300:** 1584-1587.

Girardin,S.E., Boneca,I.G., Viala,J., Chamaillard,M., Labigne,A., Thomas,G., Philpott,D.J. and Sansonetti,P.J. (2003b). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *Journal of Biological Chemistry.* **278:** 8869-8872.

Girardin,S.E., Travassos,L.H., Herve,M., Blanot,D., Boneca,I.G., Philpott,D.J., Sansonetti,P.J. and Mengin-Lecreulx,D. (2003c). Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *Journal of Biological Chemistry* **278:** 41702-41708.

Giron,J.A., Torres,A.G., Freer,E. and Kaper,J.B. (2002). The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Molecular Microbiology.*. **44:** 361-379.

Gobert,A.P., Coste,A., Guzman,C.A., Vareille,M., Hindre,T., de Sablet.T., Girardeau,J.P. and Martin,C. (2008). Modulation of chemokine gene expression by Shiga-toxin producing *Escherichia coli* belonging to various origins and serotypes. *Microbes & Infection.* **10:** 159-165.

Gobius,K.S., Higgs,G.M. and Desmarchelier,P.M. (2003). Presence of activatable Shiga toxin genotype (stx(2d)) in Shiga toxigenic *Escherichia coli* from livestock sources. *Journal of Clinical Microbiology*. **41:** 3777-3783.

Goldwater,P.N. and Bettelheim,K.A. (1998). New perspectives on the role of *Escherichia coli* O157:H7 and other enterohaemorrhagic *E. coli* serotypes in human disease. *Journal of Medical Microbiology*. **47:** 1039-1045.

Goldwater,P.N. and Bettelheim,K.A. (2002). Role of Non-O157:H7 *Escherichia coli* in hemolytic uremic syndrome. *Clinical Infectious Diseases.* **35:** 346-347.

Goosney,D.L., DeVinney,R. and Finlay,B.B. (2001). Recruitment of cytoskeletal and signaling proteins to enteropathogenic and enterohemorrhagic *Escherichia coli* pedestals. *Infection & Immunity*. **69:** 3315-3322.

Gordon,D.M. and O'Brien,C.L. (2006). Bacteriocin diversity and the frequency of multiple bacteriocin production in *Escherichia coli*. *Microbiology.* **152:** 3239-3244.

Gordon,D.M., Riley,M.A. and Pinou,T. (1998). Temporal changes in the frequency of colicinogeny in *Escherichia coli* from house mice. *Microbiology*. **144:** 2233-2240.

Grauke,L.J., Kudva,I.T., Yoon,J.W., Hunt,C.W., Williams,C.J. and Hovde,C.J. (2002). Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. *Applied & Environmental Microbiology* **68:** 2269-2277.

Greenquist,M.A., Drouillard,J.S., Sargeant,J.M., Depenbusch,B.E., Shi,X., Lechtenberg,K.F. and Nagaraja,T.G. (2005). Comparison of rectoanal mucosal swab cultures and fecal cultures for determining prevalence of *Escherichia coli* O157:H7 in feedlot cattle. *Applied & Environmental Microbiology* **71:** 6431-6433.

Griffin,P.M. and Tauxe,R.V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli* and the associated hemolytic uremic syndrome. *Epidemiology Reviews.* **13:** 60-98.

Gruenheid,S. Sekirov,I., Thomas,N.A., Deng,W., O'Donnell,P., Goode,D., Li,Y., Frey,E.A., Brown,N.F., Metalnikov,P., Pawson,T., Ashman,K. and Finlay,B.B. (2004). Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic *Escherichia coli* O157:H7. *Molecular Microbiology.* **51:** 1233-1249.

Grutzkau,A., Hanski,C., Hahn,H. and Riecken,E.O. (1990). Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by Yersinia enterocolitica and other enteroinvasive bacteria. *Gut.* **31:** 1011-1015.

Grys,T.E., Siegel,M.B., Lathem,W.W. and Welch,R.A. (2005). The StcE protease contributes to intimate adherence of enterohemorrhagic *Escherichia coli* O157:H7 to host cells. *Infection & Immunity* **73:** 1295-1303.

Grys,T.E., Walters,L.L. and Welch,R.A. (2006). Characterization of the StcE protease activity of *Escherichia coli* O157:H7. *Journal of Bacteriology*. **188:** 4646-4653.

Güler,L., Gunduz,K. and Ok,U. (2008). Virulence Factors and Antimicrobial Susceptibility of *Escherichia coli* Isolated from Calves in Turkey. *Zoonoses & Public Health.* **55:** 249-257.

Guo,H., Yi,W., Song,J.K. and Wang,P.G. (2008). Current understanding on biosynthesis of microbial polysaccharides. *Current Topics in Medical Chemistry.* **8:** 141-151.

Gyles,C.L. (2007). Shiga toxin-producing *Escherichia coli:* an overview. *Journal of Animal Science* **85:** E45-E62.

Halliday,J.E., Chase-Topping,M.E., Pearce,M.C., McKendrick,I.J., Allison,L., Fenlon,D., Low,C., Mellor,D.J., Gunn,G.J. and Woolhouse,M.E. (2006). Herd-level risk factors associated with the presence of Phage type 21/28 *E. coli* O157 on Scottish cattle farms. *BMC Microbiology* **6:** 99.

Han,M.J. and Lee,S.Y. (2006). The *Escherichia coli* proteome: past, present and future prospects. *Microbiology & Molecular Biology Reviews* **70:** 362-439.

Hardy,K.G., Meynell,G.G., Dowman,J.E. and Spratt,B.G. (1973). Two major groups of colicin factors: their evolutionary significance. *Molecular & General Genetics.* **125:** 217-230.

Hartland,E.L., Huter,V., Higgins,L.M., Goncalves,N.S., Dougan,G., Phillips,A.D., MacDonald,T.T. and Frankel,G. (2000). Expression of intimin gamma from enterohemorrhagic *Escherichia coli* in Citrobacter rodentium. *Infection & Immunity* **68:** 4637-4646.

Hauf,N. and Chakraborty,T. (2003). Suppression of NF-kappa B activation and proinflammatory cytokine expression by Shiga toxin-producing *Escherichia coli*. *Journal of Immunology* **170:** 2074-2082.

Hayashi,F., Smith,K.D., Ozinsky,A., Hawn,T.R., Yi,E.C., Goodlett,D.R., Eng,J.K., Akira,S., Underhill,D.M. and Aderem,A. (2001a). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature.* **410:** 1099-1103.

Hayashi,H., Sakamoto,M. and Benno,Y. (2002). Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. *Microbiology & Immunology.* **46:** 535-548.

Hayashi,T., Makino,K., Ohnishi,M., Kurokawa,K., Ishii,K., Yokoyama,K., Han,C.G., Ohtsubo,E., Nakayama,K., Murata,T., Tanaka,M., Tobe,T., Iida,T., Takami,H., Honda,T., Sasakawa,C., Ogasawara,N., Yasunaga,T., Kuhara,S., Shiba,T., Hattori,M. and Shinagawa,H. (2001b). Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Research* **8:** 11-22.

He,X.S., Rivkina,M., Stocker,B.A. and Robinson,W.S. (1994). Hypervariable region IV of *Salmonella* gene *fliC*d encodes a dominant surface epitope and a stabilizing factor for functional flagella. *Journal of Bacteriology*. **176:** 2406-2414.

Health Protection Scotland (2007). *HPS Weekly Report* **41:** 204-205.

Henderson,I.R., Navarro-Garcia,F., Desvaux,M., Fernandez,R.C. and Ala'Aldeen,D. (2004). Type V protein secretion pathway: the autotransporter story. *Microbiology & Molecular Biology Reviews.* **68:** 692-744.

Herschman,H.R. and Helsinki,D.R. (1967). Comparative study of the events of colicin induction. *Journal of Bacteriology* **94:** 691-699.

Herzer,P.J., Inouye,S., Inouye,M. and Whittam,T.S. (1990). Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. *Journal of Bacteriology*. **172:** 6175-6181.

Heuvelink,A.E., van Heerwaarden,C., Zwartkruis-Nahuis,J.T., van Oosterom,R., Edink,K., van Duynhoven,Y.T. and de Boer, E. (2002). *Escherichia coli* O157 infection associated with a petting zoo. *Epidemiology & Infection* **129:** 295-302.

Higgs,H.N. and Pollard,T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annual Reviews in Biochemistry.* **70:** 649-676.

Hoey,D.E., Sharp,L., Currie,C., Lingwood,C.A., Gally,D.L. and Smith,D.G. (2003). Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. *Cellular Microbiology* **5:** 85-97.

Hogan,J. and Smith,K.L. (2003). Coliform mastitis. *Veterinary Research.* **34:** 507-519.

Holland,R.D., Wilkes,J.G., Rafii,F., Sutherland,J.B., Persons,C.C., Voorhees,K.J. and Lay,J.O., Jr. (1996). Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **10:** 1227-1232.

Hovde,C.J., Austin,P.R., Cloud,K.A., Williams,C.J. and Hunt,C.W. (1999). Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. *Applied & Environmental Microbiology.* **65:** 3233-3235.

Hudault,S., Guignot,J. and Servin,A.L. (2001). *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. *Gut* **49:** 47-55.

Huisman,G.W. and Kolter,R. (1994). Sensing starvation: a homoserine lactone--dependent signaling pathway in *Escherichia coli*. *Science* **265:** 537-539.

Hull,R.A., Gill,R.E., Hsu,P., Minshew,B.H. and Falkow,S. (1981). Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. *Infection & Immunity* **33:** 933-938.

Hurley,B.P., Thorpe,C.M. and Acheson,D.W. (2001). Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. *Infection & Immunity* **69:** 6148-6155.

Hwang,J., Zhong,X. and Tai,P.C. (1997). Interactions of dedicated export membrane proteins of the colicin V secretion system: CvaA, a member of the membrane fusion protein family, interacts with CvaB and TolC. *Journal of Bacteriology*. **179:** 6264-6270.

Hyatt,D.R., Galland,J.C. and Gillespie,J.R. (2001). Usefulness of a commercially available enzyme immunoassay for Shiga-like toxins I and II as a presumptive test for the detection of *Escherichia coli* O157:H7 in cattle feces. *Journal of Veterinary Diagnostic Investigation.* **13:** 71-73.

Iino,T., Komeda,Y., Kutsukake,K., Macnab,R.M., Matsumura,P., Parkinson,J.S., Simon,M.I. and Yamaguchi,S. (1988). New unified nomenclature for the flagellar genes of *Escherichia coli* and *Salmonella* typhimurium. *Microbiology Reviews* **52:** 533-535.

Inohara,N., Ogura,Y., Fontalba,A., Gutierrez,O., Pons,F., Crespo,J., Fukase,K., Inamura,S., Kusumoto,S., Hashimoto,M., Foster,S.J., Moran,A.P., Fernandez-Luna,J.L. and Nuñez,G (2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *Journal of Biological Chemistry* **278:** 5509-5512.

Ishii,S., Meyer,K.P. and Sadowsky,M.J. (2007). Relationship between phylogenetic groups, genotypic clusters and virulence gene profiles of *Escherichia coli* strains from diverse human and animal sources. *Applied & Environmental Microbiology* **73:** 5703-5710.

Ismaili,A., Philpott,D.J., Dytoc,M.T. and Sherman,P.M. (1995). Signal transduction responses following adhesion of verocytotoxin-producing *Escherichia coli*. *Infection & Immunity* **63:** 3316-3326.

Izumiya,H., Masuda,T., Ahmed,R., Khakhria,R., Wada,A., Terajima,J., Itoh,K., Johnson,W.M., Konuma,H., Shinagawa,K., Tamura,K. and Watanabe,H. (1998). Combined use of bacteriophage typing and pulsed-field gel

electrophoresis in the epidemiological analysis of Japanese isolates of enterohemorrhagic *Escherichia coli* O157:H7. *Microbiology & Immunology* **42:** 515-519.

James,B.W. and Keevil,C.W. (1999). Influence of oxygen availability on physiology, verocytotoxin expression and adherence of *Escherichia coli* O157. *Journal of Applied Microbiology.* **86:** 117-124.

Janeway,C.A., Travers,P., Walport,M. and Shlomchik,M. (2001). Immunobiology, Garland Science.

Janka,A., Becker,G., Sonntag,A.K., Bielaszewska,M., Dobrindt,U. and Karch,H. (2005). Presence and characterization of a mosaic genomic island which distinguishes sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:H- from *E. coli* O157:H7. *Applied & Environmental Microbiology* **71:** 4875-4878.

Janka,A., Bielaszewska,M., Dobrindt,U., Greune,L., Schmidt,M.A. and Karch,H. (2003). Cytolethal distending toxin gene cluster in enterohemorrhagic *Escherichia coli* O157:H- and O157:H7: characterization and evolutionary considerations. *Infection & Immunity* **71:** 3634-3638.

Janka,A., Bielaszewska,M., Dobrindt,U. and Karch,H. (2002). Identification and distribution of the enterohemorrhagic *Escherichia coli* factor for adherence (efa1) gene in sorbitol-fermenting *Escherichia coli* O157: H-. *International Journal of Medical Microbiology* **292:** 207-214.

Jarvis,K.G., Giron,J.A., Jerse,A.E., McDaniel,T.K., Donnenberg,M.S. and Kaper,J.B. (1995). Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proceedings of the National Academy of Science U.S.A.* **92:** 7996-8000.

Jenkins,C., Pearce,M.C., Chart,H., Cheasty,T., Willshaw,G.A., Gunn,G.J., Dougan,G., Smith,H.R., Synge,B.A. and Frankel,G. (2002). An eight-month study of a population of verocytotoxigenic *Escherichia coli* (VTEC) in a Scottish cattle herd. *Journal of Applied Microbiology.* **93:** 944-953.

Jensen,V.B., Harty,J.T. and Jones,B.D. (1998). Interactions of the invasive pathogens *Salmonella typhimurium, Listeria monocytogenes* and *Shigella flexneri* with M cells and murine Peyer's patches. *Infection & Immunity* **66:** 3758-3766.

Jerse,A.E., Yu,J., Tall,B.D. and Kaper,J.B. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Science U.S.A.* **87:** 7839-7843.

Johansen,F.E., Pekna,M., Norderhaug,I.N., Haneberg,B., Hietala,M.A., Krajci,P., Betsholtz,C. and Brandtzaeg,P. (1999). Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. *Journal of Experimental Medicine* **190:** 915-922.

John,M., Kudva,I.T., Griffin,R.W., Dodson,A.W., McManus,B., Krastins,B., Sarracino,D., Progulske-Fox,A., Hillman,J.D., Handfield,M., Tarr,P.I. and Calderwood,S.B. (2005). Use of in vivo-induced antigen technology for identification of *Escherichia coli* O157:H7 proteins expressed during human infection. *Infection & Immunity* **73:** 2665-2679.

Johnson,J.R., Oswald,E., O'Bryan,T.T., Kuskowski,M.A. and Spanjaard,L. (2002). Phylogenetic distribution of virulence-associated genes among *Escherichia coli* isolates associated with neonatal bacterial meningitis in the Netherlands. *Journal of Infectious Disease* **185:** 774-784.

Johnson,J.R. and Stell,A.L. (2000). Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *Journal of Infectious Disease* **181:** 261-272.

Johnson,R.P., Cray,W.C., Jr. and Johnson,S.T. (1996). Serum antibody responses of cattle following experimental infection with *Escherichia coli* O157:H7. *Infection & Immunity* **64:** 1879-1883.

Johnson,T.J., Kariyawasam,S., Wannemuehler,Y., Mangiamele,P., Johnson,S.J., Doetkott,C., Skyberg,J.A., Lynne,A.M., Johnson,J.R. and Nolan,L.K. (2007). The genome sequence of avian pathogenic *Escherichia coli* strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. *Journal of Bacteriology.* **189:** 3228-3236.

Jones,B.D., Ghori,N. and Falkow,S. (1994). *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *Journal of Experimental Medicine* **180:** 15-23.

Jordan,D.M., Cornick,N., Torres,A.G., Dean-Nystrom,E.A., Kaper,J.B. and Moon,H.W. (2004). Long polar fimbriae contribute to colonization by *Escherichia coli* O157:H7 *in vivo*. *Infection & Immunity* **72:** 6168-6171.

Jores,J., Zehmke,K., Eichberg,J., Rumer,L. and Wieler,L.H. (2003). Description of a novel intimin variant (type zeta) in the bovine O84:NM verotoxin-producing *Escherichia coli* strain 537/89 and the diagnostic value of intimin typing. *Experimental Biological Medicine.* **228:** 370-376.

Juhas,M., Crook,D.W., Dimopoulou,I.D., Lunter,G., Harding,R.M., Ferguson,D.J. and Hood,D.W. (2007). Novel type IV secretion system involved in propagation of genomic islands. *Journal of Bacteriology*. **189:** 761-771.

Kaipainen,T., Pohjanvirta,T., Shpigel,N.Y., Shwimmer,A., Pyorala,S. and Pelkonen,S. (2002). Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. *Veterinary Microbiology.* **85:** 37-46.

Kalman,D., Weiner,O.D., Goosney,D.L., Sedat,J.W., Finlay,B.B., Abo,A. and Bishop,J.M. (1999). Enteropathogenic *E. coli* acts through WASP and Arp2/3 complex to form actin pedestals. *Nature Cell Biology.* **1:** 389-391.

Kamada,N., Maeda,K., Inoue,N., Hisamatsu,T., Okamoto,S., Hong,KS., Yamada,T., Watanabe,N., Tsuchimoto,K., Ogata,H., Hibi,T. (2008). Nonpathogenic *Escherichia coli* strain Nissle 1917 inhibits signal transduction in intestinal epithelial cells. *Infection & Immunity* **76:** 214-220.

Kanack,K.J., Crawford,J.A., Tatsuno,I., Karmali,M.A. and Kaper,J.B. (2005). SepZ/EspZ is secreted and translocated into HeLa cells by the enteropathogenic *Escherichia coli* type III secretion system. *Infection & Immunity* **73:** 4327-4337.

Kang,S.J., Ryu,S.J., Chae,J.S., Eo,S.K., Woo,G.J. and Lee,J.H. (2004). Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhoea. *Veterinary Microbiology.* **98:** 323-328.

Karch,H. and Bielaszewska,M. (2001). Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H(-) strains: epidemiology, phenotypic and molecular characteristics and microbiological diagnosis. *Journal of Clinical Microbiology*. **39:** 2043-2049.

Karch,H., Wiss,R., Gloning,H., Emmrich,P., Aleksic,S. and Bockemuhl,J. (1990). [Hemolytic-uremic syndrome in infants due to verotoxin-producing *Escherichia coli*]. *Dtsch. Med. Wochenschr.* **115:** 489-495.

Karmali,M.A., Mascarenhas,M., Shen,S., Ziebell,K., Johnson,S., Reid-Smith,R., Isaac-Renton,J., Clark,C., Rahn,K. and Kaper,J.B. (2003). Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *Journal of Clinical Microbiology* **41:** 4930-4940.

Karmali,M.A., Petric,M., Lim,C., Fleming,P.C. and Steele,B.T. (1983a). *Escherichia coli* cytotoxin, haemolytic-uraemic syndrome and haemorrhagic colitis. *Lancet* **2:** 1299-1300.

Karmali,M.A., Steele,B.T., Petric,M. and Lim,C. (1983b). Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* **1:** 619-620.

Kassenborg,H.D., Hedberg,C.W., Hoekstra,M., Evans,M.C., Chin,A.E., Marcus,R., Vugia,D.J., Smith,K., Ahuja,S.D., Slutsker,L. and Griffin,P.M. (2004). Farm visits and undercooked hamburgers as major risk factors for sporadic *Escherichia coli* O157:H7 infection: data from a case-control study in 5 FoodNet sites. *Clinical Infectious Disease*. **38:** S271-S278.

Kauffmann,F. (1947). The Serology of the Coli Group. *Journal of Immunology* **57:** 71-100.

Kaye,S.A., Louise,C.B., Boyd,B., Lingwood,C.A. and Obrig,T.G. (1993). Shiga toxin-associated hemolytic uremic syndrome: interleukin-1 beta enhancement of Shiga toxin cytotoxicity toward human vascular endothelial cells in vitro. *Infection & Immunity* **61:** 3886-3891.

Kenny,B., DeVinney,R., Stein,M., Reinscheid,D.J., Frey,E.A. and Finlay,B.B. (1997). Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell.* **91:** 511-520.

Kenny,B., Ellis,S., Leard,A.D., Warawa,J., Mellor,H. and Jepson,M.A. (2002). Co-ordinate regulation of distinct host cell signalling pathways by multifunctional enteropathogenic *Escherichia coli* effector molecules. *Molecular Microbiology.* **44:** 1095-1107.

Khakhria,R., Duck,D. and Lior,H. (1990). Extended phage-typing scheme for *Escherichia coli* O157:H7. *Epidemiology & Infection* **105:** 511-520.

Khan,M.A., Bouzari,S., Ma,C., Rosenberger,C.M., Bergstrom,K.S., Gibson,D.L., Steiner,T.S. and Vallance,B.A. (2008). Flagellin-dependent and -independent inflammatory responses following infection by enteropathogenic *Escherichia coli* and *Citrobacter rodentium*. *Infection & Immunity* **76:** 1410-1422.

Khan,M.A., Kang,J. and Steiner,T.S. (2004). Enteroaggregative *Escherichia coli* flagellin-induced interleukin-8 secretion requires Toll-like receptor 5-dependent p38 MAP kinase activation. *Immunology.* **112:** 651-660.

Kilian, M., and P. Bulow. 1979. Rapid identification of Enterobacteriaceae.II. Use of β-glucuronidase detecting agar medium (PGUA agar) for the identification of *E. coli* in primary cultures of urine samples. Acta pathologica, microbiologica, et immunologica Scandinavica Section B **87:** 271-276.

Kim,J., Nietfeldt,J. and Benson,A.K. (1999). Octamer-based genome scanning distinguishes a unique subpopulation of *Escherichia coli* O157:H7 strains in cattle. *Proceedings of the National Academy of Science U.S.A.* **96:** 13288-13293.

Kim J., Thanabalasuriar A., Chaworth-Musters T., Fromme J.C., Frey E.A., Lario P.I., Metalnikov P., Rizg K., Thomas N.A., Lee S.F., Hartland E.L., Hardwidge P.R., Pawson T., Strynadka N.C., Finlay B.B., Schekman R. and Gruenheid S. (2007) The bacterial virulence factor NleA inhibits cellular protein secretion by disrupting mammalian COPII function. *Cell Host & Microbe.* **13:** 160-71.

Klapproth,J.M., Scaletsky,I.C., McNamara,B.P., Lai,L.C., Malstrom,C., James,S.P. and Donnenberg,M.S. (2000). A large toxin from pathogenic *Escherichia coli* strains that inhibits lymphocyte activation. *Infection & Immunity* **68:** 2148-2155.

Konadu,E., Donohue-Rolfe,A., Calderwood,S.B., Pozsgay,V., Shiloach,J., Robbins,J.B. and Szu,S.C. (1999). Syntheses and immunologic properties of *Escherichia coli* O157 O-specific polysaccharide and Shiga toxin 1 B subunit conjugates in mice. *Infection & Immunity* **67:** 6191-6193.

Konowalchuk,J., Speirs,J.I. and Stavric,S. (1977). Vero response to a cytotoxin of *Escherichia coli*. *Infection & Immunity* **18:** 775-779.

Korhonen,T.K., Vaisanen-Rhen,V., Rhen,M., Pere,A., Parkkinen,J. and Finne,J. (1984). *Escherichia coli* fimbriae recognizing sialyl galactosides. *Journal of Bacteriology*. **159:** 762-766.

Krishnamurthy,T. and Ross,P.L. (1996). Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Communications in Mass Spectrometry.* **10:** 1992-1996.

Krishnan,C., Fitzgerald,V.A., Dakin,S.J. and Behme,R.J. (1987). Laboratory investigation of outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7. *Journal of Clinical Microbiology* **25:** 1043-1047.

Krogfelt,K.A., Bergmans,H. and Klemm,P. (1990). Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. *Infection & Immunity* **58:** 1995-1998.

Kudva,I.T., Krastins,B., Sheng,H., Griffin,R.W., Sarracino,D.A., Tarr,P.I., Hovde,C.J., Calderwood,S.B. and John,M. (2006). Proteomics-based expression library screening (PE**LS):** a novel method for rapidly defining microbial immunoproteomes. *Molecular & Cellular Proteomics* **5:** 1514-1519.

Kuwajima,G. (1988). Flagellin domain that affects H antigenicity of *Escherichia coli* K-12. *Journal of Bacteriology*. **170:** 485-488.

Kuwajima,G., Asaka,J., Fujiwara,T., Fujiwara,T., Node,K. and Kondo,E. (1986). Nucleotide sequence of the hag gene encoding flagellin of *Escherichia coli*. *Journal of Bacteriology*. **168:** 1479-1483.

Kyaw,C.M., De Araujo,C.R., Lima,M.R., Gondim,E.G., Brigido,M.M. and Giugliano,L.G. (2003). Evidence for the presence of a type III secretion system in diffusely adhering *Escherichia coli* (DAEC). Infect. Genet. Evol**. 3:** 111-117.

La Ragione,R.M., Ahmed,N.M., Best,A., Clifford,D., Weyer,U., Cooley,W.A., Johnson,L., Pearson,G.R. and Woodward,M.J. (2005). Colonization of 8-week-old conventionally reared goats by *Escherichia coli* O157:H7 after oral inoculation. *Journal of Medical Microbiology* **54:** 485-492.

Lan,R. and Reeves,P.R. (2002). *Escherichia coli* in disgu**ise:** molecular origins of *Shigella*. *Microbes & Infection* **4:** 1125-1132.

Lanzavecchia,A. and Sallusto,F. (2007). Toll-like receptors and innate immunity in B-cell activation and antibody responses. *Current Opinions in Immunology* **19:** 268-274.

Larkin,M.A., Blackshields,G., Brown,N.P., Chenna,R., McGettigan,P.A., McWilliam,H., Valentin,F., Wallace,I.M., Wilm,A., Lopez,R., Thompson,J.D., Gibson,T.J., Higgins,D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23:** 2947-2948.

Lathem,W.W., Bergsbaken,T. and Welch,R.A. (2004). Potentiation of C1 esterase inhibitor by StcE, a metalloprotease secreted by *Escherichia coli* O157:H7. *Journal of Experimental Medicine* **199:** 1077-1087.

Lathem,W.W., Bergsbaken,T., Witowski,S.E., Perna,N.T. and Welch,R.A. (2003). Acquisition of StcE, a C1 esterase inhibitor-specific metalloprotease, during the evolution of *Escherichia coli* O157:H7. *Journal of Infectious Diseases.* **187:** 1907-1914.

Lathem,W.W., Grys,T.E., Witowski,S.E., Torres,A.G., Kaper,J.B., Tarr,P.I. and Welch,R.A. (2002). StcE, a metalloprotease secreted by *Escherichia coli* O157:H7, specifically cleaves C1 esterase inhibitor. *Molecular Microbiology.* **45:** 277-288.

Laven,R.A., Ashmore,A. and Stewart,C.S. (2003). *Escherichia coli* in the rumen and colon of slaughter cattle, with particular reference to *E. coli* O157. *The Veterinary Journal* **165:** 78-83.

Lea,N., Lord,J.M. and Roberts,L.M. (1999). Proteolytic cleavage of the A subunit is essential for maximal cytotoxicity of *Escherichia coli* O157:H7 Shiga-like toxin-1. *Microbiology.* **145:** 999-1004.

Lejeune,J.T., Besser,T.E., Merrill,N.L., Rice,D.H. and Hancock,D.D. (2001). Livestock drinking water microbiology and the factors influencing the quality of drinking water offered to cattle. Journal of Dairy Science **84:** 1856-1862.

Lejeune,J.T., Besser,T.E., Rice,D.H., Berg,J.L., Stilborn,R.P. and Hancock,D.D. (2004). Longitudinal study of fecal shedding of *Escherichia coli* O157:H7 in feedlot cattle: predominance and persistence of specific clonal types despite massive cattle population turnover. *Applied & Environmental Microbiology* **70:** 377-384.

Leotta,G.A., Miliwebsky,E.S., Chinen,I., Espinosa,E.M., Azzopardi,K., Tennant,S.M., Robins-Browne,R.M. and Rivas,M. (2008). Characterisation of Shiga toxin-producing *Escherichia coli* O157 strains isolated from humans in Argentina, Australia and New Zealand. *BMC Microbiology***. 8:** 46.

Leveille,S., Caza,M., Johnson,J.R., Clabots,C., Sabri,M. and Dozois,C.M. (2006). Iha from an *Escherichia coli* urinary tract infection outbreak clonal group A strain is expressed in vivo in the mouse urinary tract and functions as a catecholate siderophore receptor. *Infection & Immunity* **74:** 3427-3436.

Levine,M.M., Xu,J.G., Kaper,J.B., Lior,H., Prado,V., Tall,B., Nataro,J., Karch,H. and Wachsmuth,K. (1987). A DNA probe to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. *Journal of Infectious Diseases.* **156:** 175-182.

Licence,K., Oates,K.R., Synge,B.A. and Reid,T.M. (2001). An outbreak of *E. coli* O157 infection with evidence of spread from animals to man through contamination of a private water supply. *Epidemiology & Infection* **126:** 135-138.

Liebler-Tenorio,E.M. and Pabst,R. (2006). MALT structure and function in farm animals. *Veterinary Research* **37:** 257-280.

Lim,J.Y., Li,J., Sheng,H., Besser,T.E., Potter,K. and Hovde,C.J. (2007). *Escherichia coli* O157:H7 colonization at the rectoanal junction of long-duration culture-positive cattle. *Applied & Environmental Microbiology* **73:** 1380-1382.

Lin,Z., Kurazono,H., Yamasaki,S. and Takeda,Y. (1993). Detection of various variant verotoxin genes in *Escherichia coli* by polymerase chain reaction. *Microbiology & Immunology.* **37:** 543-548.

Lindberg,A.A., Brown,J.E., Stromberg,N., Westling-Ryd,M., Schultz,J.E. and Karlsson,K.A. (1987). Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. *Journal of Biological Chemistry* **262:** 1779-1785.

Lindgren,S.W., Samuel,J.E., Schmitt,C.K. and O'Brien,A.D. (1994). The specific activities of Shiga-like toxin type II (SLT-II) and SLT-II-related toxins of enterohemorrhagic *Escherichia coli* differ when measured by Vero cell cytotoxicity but not by mouse lethality. *Infection & Immunity* **62:** 623-631.

Lingwood,C.A. (1996). Role of verotoxin receptors in pathogenesis. *Trends in Microbiolog***y 4:** 147-153.

Lintermans,PF., Pohl,P., Bertels,A., Charlier,G., Vandekerckhove,J., Van Damme,J., Schoup,J., Schlicker,C. Korhonen,T., De Greve,H., *et al.* (1988). Characterization and purification of the F17 adhesin on the surface of bovine enteropathogenic and septicemic *Escherichia coli*. *American Journal of Veterinary Research* **49:** 1794-1799.

Liu,D. and Reeves,P.R. (1994). *Escherichia coli* K12 regains its O-antigen. *Microbiology.* **140:** 49-57.

Locking,M., Browning,L., Smith-Palmer,A. and Brownlie,S. (2006). HPS Weekly Report **40:** 1-4.

Locking,M.E., O'Brien,S.J., Reilly,W.J., Wright,E.M., Campbell,D.M., Coia,J.E., Browning,L.M. and Ramsay,C.N. (2001). Risk factors for sporadic cases of *Escherichia coli* O157 infection: the importance of contact with animal excreta. *Epidemiology & Infection* **127:** 215-220.

Lodinova-Zadnikova,R., Cukrowska,B. and Tlaskalova-Hogenova,H. (2003). Oral administration of probiotic *Escherichia coli* after birth reduces frequency of allergies and repeated infections later in life (after 10 and 20 years). *International Archives of Allergy Immunology* **131:** 209-211.

Lodinova-Zadnikova,R. and Sonnenborn,U. (1997). Effect of preventive administration of a nonpathogenic *Escherichia coli* strain on the colonization of the intestine with microbial pathogens in newborn infants. *Biology of the Neonate.* **71:** 224-232.

Lopez,E.L., Diaz,M., Grinstein,S., Devoto,S., Mendilaharzu,F., Murray,B.E., Ashkenazi,S., Rubeglio,E., Woloj,M., Vasquez,M., *et al.* (1989). Hemolytic uremic syndrome and diarrhea in Argentine children: the role of Shiga-like toxins. *Journal of Infectious Diseases.* **160:** 469-475.

Louise,C.B., Kaye,S.A., Boyd,B., Lingwood,C.A. and Obrig,T.G. (1995). Shiga toxin-associated hemolytic uremic syndrome: effect of sodium butyrate on sensitivity of human umbilical vein endothelial cells to Shiga toxin. *Infection & Immunity* **63:** 2766-2769.

Louise,C.B. and Obrig,T.G. (1991). Shiga toxin-associated hemolytic-uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1 beta and tumor necrosis factor alpha on human vascular endothelial cells in vitro. *Infection & Immunity* **59:** 4173-4179.

Low,J.C., McKendrick,I.J., McKechnie,C., Fenlon,D., Naylor,S.W., Currie,C., Smith,D.G., Allison,L. and Gally,D.L. (2005). Rectal carriage of enterohemorrhagic *Escherichia coli* O157 in slaughtered cattle. *Applied & Environmental Microbiology* **71:** 93-97.

Lund,B., Marklund,B.I., Stromberg,N., Lindberg,F., Karlsson,K.A. and Normark,S. (1988). Uropathogenic *Escherichia coli* can express serologically identical pili of different receptor binding specificities. *Molecular Microbiology***. 2:** 255-263.

Lynn,R.M., O'Brien,S.J., Taylor,C.M., Adak,GK., Chart,H., Cheasty,T., Coia,J.E., Gillespie,I.A., Locking,M.E., Reilly,W.J., Smith,H.R., Waters,A. and Willshaw,G.A. (2005). Childhood hemolytic uremic syndrome, United Kingdom and Ireland. *Emerging Infectious Diseases* **11:** 590-596.

Macnab,R.M. (2003). How bacteria assemble flagella. *Annual Reviews in Microbiology.* **57:** 77-100.

Macpherson,A.J. and Uhr,T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* **303:** 1662-1665.

Mann,K.G. (1999). Biochemistry and physiology of blood coagulation. *Thrombosis & Haemostasis* **82:** 165-174.

March,S.B. and Ratnam,S. (1986). Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *Journal of Clinical Microbiology*. **23:** 869-872.

March,S.B. and Ratnam,S. (1989). Latex agglutination test for detection of *Escherichia coli* serotype O157. *Journal of Clinical Microbiology*. **27:** 1675-1677.

Marchès,O., Ledger,TN., Boury,M., Ohara,M., Tu,X., Goffaux,F., Mainil,J., Rosenshine,I., Sugai,M., De Rycke,J. and Oswald,E. (2003). Enteropathogenic and enterohaemorrhagic *Escherichia coli* deliver a novel effector called Cif, which blocks cell cycle G2/M transition. *Molecular Microbiology.* **50:** 1553-1567.

Matise,I., Cornick,N.A., Booher,S.L., Samuel,J.E., Bosworth,B.T. and Moon,H.W. (2001). Intervention with Shiga toxin (Stx) antibody after infection by Stx-producing *Escherichia coli*. *Journal of Infectious Diseases.* **183:** 347-350.

Matsuzawa,T., Kuwae,A., Yoshida,S., Sasakawa,C. and Abe,A. (2004). Enteropathogenic *Escherichia coli* activates the RhoA signaling pathway via the stimulation of GEF-H1. *EMBO Journal***. 23:** 3570-3582.

Matthews,L., Low,J.C., Gally,D.L., Pearce,M.C., Mellor,D.J., Heesterbeek,J.A., Chase-Topping,M., Naylor,S.W., Shaw,D.J., Reid,S.W., Gunn,G.J. and Woolhouse,M.E. (2006a). Heterogeneous shedding of *Escherichia coli* O157 in cattle and its implications for control. *Proceedings of the National Academy of Science U.S.A.* . **103:** 547-552.

Matthews,L., McKendrick,I.J., Ternent,H., Gunn,G.J., Synge,B. and Woolhouse,M.E. (2006b). Super-shedding cattle and the transmission dynamics of *Escherichia coli* O157. *Epidemiology & Infection* **134:** 131-142.

McDaniel,T.K., Jarvis,K.G., Donnenberg,M.S. and Kaper,J.B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of the National Academy of Science U.S.A.* **92:** 1664-1668.

McDaniel,T.K. and Kaper,J.B. (1997). A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Molecular Microbiology.* **23:** 399-407.

McNally,A., Roe,A.J., Simpson,S., Thomson-Carter,F.M., Hoey,D.E., Currie,C., Chakraborty,T., Smith,D.G. and Gally,D.L. (2001). Differences in levels of secreted locus of enterocyte effacement proteins between human disease-associated and bovine *Escherichia coli* O157. *Infection & Immunity* **69:** 5107-5114.

McNamara,B.P. and Donnenberg,M.S. (1998). A novel proline-rich protein, EspF, is secreted from enteropathogenic *Escherichia coli* via the type III export pathway. *FEMS Microbiology Letters* **166:** 71-78.

McNamara,B.P., Koutsouris,A., O'Connell,C.B., Nougayrede,J.P., Donnenberg,M.S. and Hecht,G. (2001a). Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *Journal of Clinical Investigation* **107:** 621-629.

McNamara,N., Khong,A., McKemy,D., Caterina,M., Boyer,J., Julius,D. and Basbaum,C. (2001b). ATP transduces signals from ASGM1, a glycolipid that functions as a bacterial receptor. *Proceedings of the National Academy of Science U.S.A.* **98:** 9086-9091.

McNeilly,T.N., Naylor,S.W., Mahajan,A., Mitchell,M.C., McAteer,S., Deane,D., Smith,D.G., Low,J.C., Gally,D.L. and Huntley,J.F. (2008). *Escherichia coli* O157:H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin. *Infection & Immunity* **76:** 2594-2602

Mead,P.S., Finelli,L., Lambert-Fair,M.A., Champ,D., Townes,J., Hutwagner,L., Barrett,T., Spitalny,K. and Mintz,E. (1997). Risk factors for sporadic infection with *Escherichia coli* O157:H7. Archives of Internal Medicine **157:** 204-208.

Medzhitov,R., Preston-Hurlburt,P. and Janeway,C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388:** 394-397.

Mellies,J.L., Elliott,S.J., Sperandio,V., Donnenberg,M.S. and Kaper,J.B. (1999). The Per regulon of enteropathogenic *Escherichia coli* : identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Molecular Microbiology.* **33:** 296-306.

Mellmann,A., Cloud,J., Maier,T., Keckevoet,U., Ramminger,I., Iwen,P., Dunn,J., Hall,G., Wilson,D., LaSala, P., Kostrzewa, M. and Harmsen D. (2008). Evaluation of Matrix-Assisted laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in Comparison to 16S rRNA Gene Sequencing for Species Identification of Nonfermenting Bacteria. *Journal of Clinical Microbiology*. epub ahead of print: doi:10.1128/JCM.00157-08

Melmed,G., Thomas,L.S., Lee,N., Tesfay,S.Y., Lukasek,K., Michelsen,K.S., Zhou,Y., Hu,B., Arditi,M. and Abreu,M.T. (2003). Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *Journal of Immunology* **170:** 1406-1415.

Melton-Celsa,A.R., Darnell,S.C. and O'Brien,A.D. (1996). Activation of Shiga-like toxins by mouse and human intestinal mucus correlates with virulence of enterohemorrhagic *Escherichia coli* O91**:**H21 isolates in orally infected, streptomycin-treated mice. *Infection & Immunity* **64:** 1569-1576.

Melton-Celsa,A.R. and O'Brien,A.D. (1998). Structure, Biology and Relative Toxicity of Shiga Toxin Family Members for Cells and Animals. In: *Escherichia coli* O157:H7 and Other Shiga Toxin-Producing *E. coli* Strains, ed. J.B.Kaper and A.D.O'Brien ASM Press, Washington D.C..

Menge,C., Blessenohl,M., Eisenberg,T., Stamm,I. and Baljer,G. (2004). Bovine ileal intraepithelial lymphocytes represent target cells for Shiga toxin 1 from *Escherichia coli*. *Infection & Immunity* **72:** 1896-1905.

Menge,C., Wieler,L.H., Schlapp,T. and Baljer,G. (1999). Shiga toxin 1 from *Escherichia coli* blocks activation and proliferation of bovine lymphocyte subpopulations in vitro. *Infection & Immunity* **67:** 2209-2217.

Milford,D.V., Taylor,C.M., Guttridge,B., Hall,S.M., Rowe,B. and Kleanthous,H. (1990). Haemolytic uraemic syndromes in the British Isles 1985-8: association with verocytotoxin producing *Escherichia coli*. Part 1: Clinical and epidemiological aspects. *Archives of Disease in Childhood* **65:** 716-721.

Miyamoto,Y., Iimura,M., Kaper,J.B., Torres,A.G. and Kagnoff,M.F. (2006). Role of Shiga toxin versus H7 flagellin in enterohaemorrhagic *Escherichia coli* signalling of human colon epithelium *in vivo*. *Cellular Microbiology* **8:** 869-79

Miyao,Y., Kataoka,T., Nomoto,T., Kai,A., Itoh,T. and Itoh,K. (1998). Prevalence of verotoxin-producing *Escherichia coli* harbored in the intestine of cattle in Japan. *Veterinary Microbiology.* **61:** 137-143.

Molofsky,A.B., Byrne,B.G., Whitfield,N.N., Madigan,C.A., Fuse,E.T., Tateda,K. and Swanson,M.S. (2006). Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *Journal of Experimental Medicine* **203:** 1093-1104.

Morgan,G.M., Newman,C., Palmer,S.R., Allen,J.B., Shepherd,W., Rampling,A.M., Warren,R.E., Gross,R.J., Scotland,S.M. and Smith,H.R. (1988). First recognized community outbreak of haemorrhagic colitis due to verotoxin-producing *Escherichia coli* O157.H7 in the UK. *Epidemiology & Infection* **101:** 83-91.

Mouricout,M., Petit,J.M., Carias,J.R. and Julien,R. (1990). Glycoprotein glycans that inhibit adhesion of *Escherichia coli* mediated by K99 fimbriae: treatment of experimental colibacillosis. *Infection & Immunity* **58:** 98-106.

Mukherjee,J., Chios,K., Fishwild,D., Hudson,D., O'Donnell,S., Rich,S.M., Donohue-Rolfe,A. and Tzipori,S. (2002). Human Stx2-specific monoclonal antibodies prevent systemic complications of *Escherichia coli* O157:H7 infection. *Infection & Immunity* **70:** 612-619.

Mundy,R., MacDonald,T.T., Dougan,G., Frankel,G. and Wiles,S. (2005). *Citrobacter rodentium* of mice and man. *Cellular Microbiology***. 7:** 1697-1706.

Mundy,R., Schuller,S., Girard,F., Fairbrother,J.M., Phillips,A.D. and Frankel,G. (2007). Functional studies of intimin *in vivo* and *ex vivo*: implications for host specificity and tissue tropism. *Microbiology.* **153:** 959-967.

Muniesa,M., Blanco,J.E., De,S.M., Serra-Moreno,R., Blanch,A.R. and Jofre,J. (2004). Diversity of stx2 converting bacteriophages induced from Shiga-toxin-producing *Escherichia coli* strains isolated from cattle. *Microbiology.* **150:** 2959-2971.

Murakami,H. and Masui,H. (1980). Hormonal control of human colon carcinoma cell growth in serum-free medium. *Proceedings of the National Academy of Science U.S.A.* **77:** 3464-3468.

Murthy,K.G., Deb,A., Goonesekera,S., Szabo,C. and Salzman,A.L. (2004). Identification of conserved domains in *Salmonella muenchen* flagellin that are essential for its ability to activate TLR5 and to induce an inflammatory response in vitro. *Journal of Biological Chemistry* **279:** 5667-5675.

Musken,A., Bielaszewska,M., Greune,L., Schweppe,C.H., Muthing,J., Schmidt,H., Schmidt,M.A., Karch,H. and Zhang,W. (2008). Anaerobic conditions promote expression of Sfp fimbriae and adherence of sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:NM to human intestinal epithelial cells. *Applied & Environmental Microbiology* **74:** 1087-1093.

Naik,S., Kelly,E.J., Meijer,L., Pettersson,S. and Sanderson,I.R. (2001). Absence of Toll-like receptor 4 explains endotoxin hyporesponsiveness in human intestinal epithelium. *Journal of Pediatriatric Gastroenterology and Nutrition*. **32:** 449-453.

Naito,Y. and Yoshikawa,T. (2002). Molecular and cellular mechanisms involved in Helicobacter pylori-induced inflammation and oxidative stress. *Free Radical Biology & Medicine* **33:** 323-336.

Nakazawa,M. and Akiba,M. (1999). Swine as a potential reservoir of shiga toxin-producing *Escherichia coli* O157:H7 in Japan. *Emerging Infectious Diseases.* **5:** 833-834.

Nandiwada,L.S., Schamberger,G.P., Schafer,H.W. and ez-Gonzalez,F. (2004). Characterization of an E2-type colicin and its application to treat alfalfa seeds to reduce *Escherichia coli* O157:H7. *International Journal of Food Microbiology.* **93:** 267-279.

Nataro,J.P. and Kaper,J.B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews.* **11:** 142-201.

Naylor,S.W., Low,J.C., Besser,T.E., Mahajan,A., Gunn,G.J., Pearce,M.C., McKendrick,I.J., Smith,D.G. and Gally,D.L. (2003). Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infection & Immunity* **71:** 1505-1512.

Naylor,S.W., Nart,P., Sales,J., Flockhart,A., Gally,D.L. and Low,J.C. (2007). Impact of the direct application of therapeutic agents to the terminal recta of experimentally colonized calves on *Escherichia coli* O157:H7 shedding. *Applied and Environmental Microbiology* **73:** 1493-1500.

Naylor,S.W., Roe,A.J., Nart,P., Spears,K., Smith,D.G., Low,J.C. and Gally,D.L. (2005). *Escherichia coli* O157: H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. *Microbiology.* **151:** 2773-2781.

Newton,S.M., Wasley,R.D., Wilson,A., Rosenberg,L.T., Miller,J.F. and Stocker,B.A. (1991). Segment IV of a *Salmonella* flagellin gene specifies flagellar antigen epitopes. *Molecular Microbiology***. 5:** 419-425.

Nicholls,L., Grant,T.H. and Robins-Browne,R.M. (2000). Identification of a novel genetic locus that is required for in vitro adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. *Molecular Microbiology.* **35:** 275-288.

Nikaido,H. (1996). Multidrug efflux pumps of gram-negative bacteria. *Journal of Bacteriology*. **178:** 5853-5859.

Nowrouzian,F., Adlerberth,I. and Wold,A.E. (2001). P fimbriae, capsule and aerobactin characterize colonic resident *Escherichia coli*. *Epidemiology & Infection* **126:** 11-18.

Nowrouzian,F., Hesselmar,B., Saalman,R., Strannegard,I.L., Aberg,N., Wold,A.E. and Adlerberth,I. (2003). *Escherichia coli* in infants' intestinal microflora: colonization rate, strain turnover and virulence gene carriage. *Pediatric Research* **54:** 8-14.

O'Brien,A.D. and Holmes,R.K. (1987). Shiga and Shiga-like toxins. *Microbiology Reviews.* **51:** 206-220.

O'Brien,A.D. and LaVeck,G.D. (1983). Purification and characterization of a Shigella dysenteriae 1-like toxin produced by *Escherichia coli*. *Infection & Immunity* **40:** 675-683.

O'Brien,A.D., Newland,J.W., Miller,S.F., Holmes,R.K., Smith,H.W. and Formal,S.B. (1984). Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. *Science* **226:** 694-696.

O'Brien,S.J., Adak,G.K. and Gilham,C. (2001). Contact with farming environment as a major risk factor for Shiga toxin (Vero cytotoxin)-producing *Escherichia coli* O157 infection in humans. *Emerging Infectious Diseases.* **7:** 1049-1051.

Ogden,I.D., Hepburn,N.F., MacRae,M., Strachan,N.J., Fenlon,D.R., Rusbridge,S.M. and Pennington,T.H. (2002). Long-term survival of *Escherichia coli* O157 on pasture following an outbreak associated with sheep at a scout camp. *Letters in Applied Microbiology* **34:** 100-104.

Ogino,T., Ohno,R., Sekiya,K., Kuwae,A., Matsuzawa,T., Nonaka,T., Fukuda,H., Imajoh-Ohmi,S. and Abe,A. (2006). Assembly of the type III secretion apparatus of enteropathogenic *Escherichia coli*. *Journal of Bacteriology.* **188:** 2801-2811.

Ogura,Y. Kurokawa,K., Ooka,T., Tashiro,K., Tobe,T., Ohnishi,M., Nakayama,K., Morimoto,T., Terajima,J., Watanabe,H., Kuhara,S., Hayashi,T. (2006). Complexity of the genomic diversity in enterohemorrhagic *Escherichia coli* O157 revealed by the combinational use of the O157 Sakai OligoDNA microarray and the Whole Genome PCR scanning. *DNA Research* **13:** 3-14.

Ogushi,K., Wada,A., Niidome,T., Okuda,T., Llanes,R., Nakayama,M., Nishi,Y., Kurazono,H., Smith,K.D., Aderem,A., Moss,J., Hirayama,T. (2004). Gangliosides act as co-receptors for *Salmonella* enteritidis FliC and

promote FliC induction of human beta-defensin-2 expression in Caco-2 cells. *Journal of Biological Chemistry* **279:** 12213-12219.

Ojeda,A., Prado,V., Martinez,J., Arellano,C., Borczyk,A., Johnson,W., Lior,H. and Levine,M.M. (1995). Sorbitol-negative phenotype among enterohemorrhagic *Escherichia coli* strains of different serotypes and from different sources. *Journal of Clinical Microbiology*. **33:** 2199-2201.

Orden,J.A., Ruiz-Santa-Quiteria,J.A., Cid,D., Garcia,S. and de la Fuente,R. (1999). Prevalence and characteristics of necrotoxigenic *Escherichia coli* (NTEC) strains isolated from diarrhoeic dairy calves. *Veterinary Microbiology.* **66:** 265-273.

Orth,D., Grif,K., Zimmerhackl,L.B. and Wurzner,R. (2008). Prevention and treatment of enterohemorrhagic *Escherichia coli* infections in humans. *Expert Reviews of Anti-infection Therapy.* **6:** 101-108.

Oswald,E., Schmidt,H., Morabito,S., Karch,H., Marches,O. and Caprioli,A. (2000). Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. *Infection & Immunity* **68:** 64-71.

Oswald,E., Sugai,M., Labigne,A., Wu,H.C., Fiorentini,C., Boquet,P. and O'Brien,A.D. (1994). Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proceedings of the National Academy of Science U.S.A.* **91:** 3814-3818.

Paiba,G.A., Gibbens,J.C., Pascoe,S.J., Wilesmith,J.W., Kidd,S.A., Byrne,C., Ryan,J.B., Smith,R.P., McLaren,M., Futter,R.J., Kay,A.C., Jones,Y.E., Chappell,S.A., Willshaw,G.A. and Cheasty,T. (2002). Faecal carriage of verocytotoxin-producing *Escherichia coli* O157 in cattle and sheep at slaughter in Great Britain. *Veterinary Record.*. **150:** 593-598.

Pallen,M.J. and Wren,B.W. (2007). Bacterial pathogenomics. *Nature.* **449:** 835-842.

Paros,M., Tarr,P.I., Kim,H., Besser,T.E. and Hancock,D.D. (1993). A comparison of human and bovine *Escherichia coli* O157:H7 isolates by toxin genotype, plasmid profile and bacteriophage lambda-restriction fragment length polymorphism profile. *Journal of Infectious Diseases.* **168:** 1300-1303.

Paton,A.W., Morona,R. and Paton,J.C. (2001a). Neutralization of Shiga toxins Stx1, Stx2c and Stx2e by recombinant bacteria expressing mimics of globotriose and globotetraose. *Infection & Immunity* **69:** 1967-1970.

Paton,A.W. and Paton,J.C. (2002). Reactivity of convalescent-phase hemolytic-uremic syndrome patient sera with the megaplasmid-encoded TagA protein of Shiga toxigenic *Escherichia coli* O157. *Journal of Clinical Microbiology*. **40:** 1395-1399.

Paton,A.W., Srimanote,P., Talbot,U.M., Wang,H. and Paton,J.C. (2004). A new family of potent AB(5) cytotoxins produced by Shiga toxigenic *Escherichia coli*. *Journal of Experimental Medicine* **200:** 35-46.

Paton,A.W., Srimanote,P., Woodrow,M.C. and Paton,J.C. (2001b). Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infection & Immunity* **69:** 6999-7009.

Pavlova,O.A. and Severinov,K.V. (2006). [Posttranslationally modified microcins]. *Genetika* **42:** 1636-1646.

Pearson,G.R., Bazeley,K.J., Jones,J.R., Gunning,R.F., Green,M.J., Cookson,A. and Woodward,M.J. (1999). Attaching and effacing lesions in the large intestine of an eight-month-old heifer associated with *Escherichia coli* O26 infection in a group of animals with dysentery. *Veterinary Record.*. **145:** 370-373.

Perdomo,O.J., Cavaillon,J.M., Huerre,M., Ohayon,H., Gounon,P. and Sansonetti,P.J. (1994). Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. *Journal of Experimental Medicine* **180:** 1307-1319.

Perelle,S., Dilasser,F., Grout,J. and Fach,P. (2003). Development of a 5'-nuclease PCR assay for detecting Shiga toxin-producing *Escherichia coli* O145 based on the identification of an 'O-island 29' homologue. *Journal of Applied Microbiology.* **94:** 587-594.

Perera,L.P., Marques,L.R. and O'Brien,A.D. (1988). Isolation and characterization of monoclonal antibodies to Shiga-like toxin II of enterohemorrhagic *Escherichia coli* and use of the monoclonal antibodies in a colony enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology*. **26:** 2127-2131.

Peres,S.Y., Marches,O., Daigle,F., Nougayrede,J.P., Herault,F., Tasca,C., De Rycke,J. and Oswald,E. (1997). A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. *Molecular Microbiology.* **24:** 1095-1107.

Perna,N.T., Plunkett,G. 3rd., Burland,V., Mau,B., Glasner,J.D., Rose,D.J., Mayhew,G.F., Evans,P.S., Gregor,J., Kirkpatrick,H.A., Pósfai,G., Hackett,J., Klink,S., Boutin,A., Shao,Y., Miller,L., Grotbeck,E.J., Davis,N.W., Lim,A., Dimalanta,E.T., Potamousis,K.D., Apodaca,J., Anantharaman,T.S., Lin,J., Yen,G., Schwartz,D.C., Welch,R.A. and Blattner,F.R. (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature.* **409:** 529-533.

Persson,S., Olsen,K.E., Ethelberg,S. and Scheutz,F. (2007). Subtyping method for *Escherichia coli* shiga toxin (verocytotoxin) 2 variants and correlations to clinical manifestations. *Journal of Clinical Microbiology* **45:** 2020-2024.

Phillips,A.D. and Frankel,G. (2000). Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with human intestinal organ cultures. *Journal of Infectious Diseases.* **181:** 1496-1500.

Phillips,A.D., Navabpour,S., Hicks,S., Dougan,G., Wallis,T. and Frankel,G. (2000). Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut*. **47:** 377-381.

Philpott,D.J., Belaid,D., Troubadour,P., Thiberge,J.M., Tankovic,J., Labigne,A. and Ferrero,R.L. (2002). Reduced activation of inflammatory responses in host cells by mouse-adapted *Helicobacter pylori* isolates. *Cellular Microbiology*. **4:** 285-296.

Picard,B., Garcia,J.S., Gouriou,S., Duriez,P., Brahimi,N., Bingen,E., Elion,J. and Denamur,E. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infection & Immunity* **67:** 546-553.

Pickering,L.K., Obrig,T.G. and Stapleton,F.B. (1994). Hemolytic-uremic syndrome and enterohemorrhagic *Escherichia coli*. *Pediatric Infectious Diseases Journal.* **13:** 459-475.

Pickett,C.L., Cottle,D.L., Pesci,E.C. and Bikah,G. (1994). Cloning, sequencing and expression of the *Escherichia coli* cytolethal distending toxin genes. *Infection & Immunity* **62:** 1046-1051.

Pierard,D., Muyldermans,G., Moriau,L., Stevens,D. and Lauwers,S. (1998). Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. *Journal of Clinical Microbiology*. **36:** 3317-3322.

Pillai,L., Sha,J., Erova,T.E., Fadl,A.A., Khajanchi,B.K. and Chopra,A.K. (2006). Molecular and functional characterization of a ToxR-regulated lipoprotein from a clinical isolate of Aeromonas hydrophila. *Infection & Immunity* **74:** 3742-3755.

Pirro,F., Wieler,L.H., Failing,K., Bauerfeind,R. and Baljer,G. (1995). Neutralizing antibodies against Shiga-like toxins from *Escherichia coli* in colostra and sera of cattle. *Veterinary Microbiology.* **43:** 131-141.

Poltorak,A., He,X., Smirnova,I., Liu,MY., Van Huffel,C., Du,X., Birdwell,D., Alejos,E., Silva,M., Galanos,C., Freudenberg,M., Ricciardi-Castagnoli,P., Layton,B. and Beutler B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282:** 2085-2088.

Potter,A.A., Klashinsky,S., Li,Y., Frey,E., Townsend,H., Rogan,D., Erickson,G., Hinkley,S., Klopfenstein,T., Moxley,R.A., Smith,D.R. and Finlay,B.B. (2004). Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins. *Vaccine* **22:** 362-369.

Pouttu,R., Puustinen,T., Kukkonen,M., Virkola,R., Laurila,M., Hacker,J., Klemm,P. and Korhonen,T.K. (2000). Functional variability of type 1 fimbriae of *Escherichia coli*. *Advances in Experimental Medical Biology.* **485:** 63-68.

Pouttu,R., Puustinen,T., Virkola,R., Hacker,J., Klemm,P. and Korhonen,T.K. (1999). Amino acid residue Ala-62 in the FimH fimbrial adhesin is critical for the adhesiveness of meningitis-associated *Escherichia coli* to collagens. *Molecular Microbiology.* **31:** 1747-1757.

Pradel,N., Livrelli,V., De,C.C., Palcoux,J.B., Reynaud,A., Scheutz,F., Sirot,J., Joly,B. and Forestier,C. (2000). Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food and children during a one-year prospective study in France. *Journal of Clinical Microbiology*. **38:** 1023-1031.

Price,P.A. (1988). Role of vitamin-K-dependent proteins in bone metabolism. *Annual Reviews in Nutrition***. 8:** 565-583.

Pritchard,G.C., Williamson,S., Carson,T., Bailey,J.R., Warner,L., Willshaw,G. and Cheasty,T. (2001). Wild rabbits--a novel vector for verocytotoxigenic *Escherichia coli* O157. *Veterinary Record.*. **149:** 567.

Pruimboom-Brees,I.M., Morgan,T.W., Ackermann,M.R., Dean-Nystrom,E., Samuel,J.E., Cornick,N.A. and Moon,H.W. (2000). Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. *Proceedings of the National Academy of Science U.S.A.* **97:** 10325-10329.

Rakoff-Nahoum,S., Paglino,J., Eslami-Varzaneh,F., Edberg,S. and Medzhitov,R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* **118:** 229-241.

Ramachandran,V., Brett,K., Hornitzky,M.A., Dowton,M., Bettelheim,K.A., Walker,M.J. and Djordjevic,S.P. (2003). Distribution of intimin subtypes among *Escherichia coli* isolates from ruminant and human sources. *Journal of Clinical Microbiology*. **41:** 5022-5032.

Ramos Moreno,A.C., Cabilio Guth,B.E. and Baquerizo,M.M. (2006). Can the *fliC* PCR-restriction fragment length polymorphism technique replace classic serotyping methods for characterizing the H antigen of enterotoxigenic *Escherichia coli* strains? *Journal of Clinical Microbiology*. **44:** 1453-1458.

Rappaport,F. and Henig,E. (1952). Media for the isolation and differentiation of pathogenic Esch. coli (serotypes O 111 and O 55). *Journal of Clinical Pathology* **5:** 361-362.

Ratnam,S., March,S.B., Ahmed,R., Bezanson,G.S. and Kasatiya,S. (1988). Characterization of *Escherichia coli* serotype O157:H7. *Journal of Clinical Microbiology*. **26:** 2006-2012.

Reeves,P.R., Hobbs,M., Valvano,M.A., Skurnik,M., Whitfield,C., Coplin,D., Kido,N., Klena,J., Maskell,D., Raetz,C.R. and Rick,P.D. (1996). Bacterial polysaccharide synthesis and gene nomenclature. *Trends in Microbiology***. 4:** 495-503.

Rendon,M.A., Saldana,Z., Erdem,A.L., Monteiro-Neto,V., Vazquez,A., Kaper,J.B., Puente,J.L. and Giron,J.A. (2007). Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. *Proceedings of the National Academy of Science U.S.A.* **104:** 10637-10642.

Renter,D.G., Sargeant,J.M., Hygnstorm,S.E., Hoffman,J.D. and Gillespie,J.R. (2001). *Escherichia coli* O157:H7 in free-ranging deer in Nebraska. *Journal of Wildlife Diseases.* **37:** 755-760.

Rescigno,M. and Borrow,P. (2001). The host-pathogen interaction: new themes from dendritic cell biology. *Cell.* **106:** 267-270.

Riley,L.W., Remis,R.S., Helgerson,S.D., McGee,H.B., Wells,J.G., Davis,B.R., Hebert,R.J., Olcott,E.S., Johnson,L.M., Hargrett,N.T., Blake,P.A. and Cohen,M.L. (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine.* **308:** 681-685.

Rios,M., Prado,V., Trucksis,M., Arellano,C., Borie,C., Alexandre,M., Fica,A. and Levine,M.M. (1999). Clonal diversity of Chilean isolates of enterohemorrhagic *Escherichia coli* from patients with hemolytic-uremic syndrome, asymptomatic subjects, animal reservoirs and food products. *Journal of Clinical Microbiology*. **37:** 778-781.

Roach,J.C., Glusman,G., Rowen,L., Kaur,A., Purcell,M.K., Smith,K.D., Hood,L.E. and Aderem,A. (2005). The evolution of vertebrate Toll-like receptors. *Proceedings of the National Academy of Science U.S.A.* **102:** 9577-9582.

Robinson,C.M., Sinclair,J.F., Smith,M.J. and O'Brien,A.D. (2006). Shiga toxin of enterohemorrhagic *Escherichia coli* type O157:H7 promotes intestinal colonization. *Proceedings of the National Academy of Science U.S.A.* **103:** 9667-9672.

Rogers,T.J., Paton,A.W., McColl,S.R. and Paton,J.C. (2003). Enhanced CXC chemokine responses of human colonic epithelial cells to locus of enterocyte effacement-negative shiga-toxigenic *Escherichia coli*. *Infection & Immunity*. **71:** 5623-5632.

Rohatgi,R., Nollau,P., Ho,H.Y., Kirschner,M.W. and Mayer,B.J. (2001). Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *Journal of Biological Chemistry* **276:** 26448-26452.

Russo,T.A. and Johnson,J.R. (2000). Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. *Journal of Infectious Diseases.* **181:** 1753-1754.

Rutter,J.M. and Luther,P.D. (1984). Cell culture assay for toxigenic *Pasteurella multocida* from atrophic rhinitis of pigs. *Veterinary Record.*. **114:** 393-396.

Saarela,S., Westerlund-Wikstrom,B., Rhen,M. and Korhonen,T.K. (1996). The GafD protein of the G (F17) fimbrial complex confers adhesiveness of *Escherichia coli* to laminin. *Infection & Immunity* **64:** 2857-2860.

Sable,S., Pons,A.M., Gendron-Gaillard,S. and Cottenceau,G. (2000). Antibacterial activity evaluation of microcin J25 against diarrheagenic *Escherichia coli*. *Applied & Environmental Microbiology* **66:** 4595-4597.

Samatey,F.A., Imada,K., Nagashima,S., Vonderviszt,F., Kumasaka,T., Yamamoto,M. and Namba,K. (2001). Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. *Nature* **410:** 331-337.

Samuel,J.E., Perera,L.P., Ward,S., O'Brien,A.D., Ginsburg,V. and Krivan,H.C. (1990). Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. *Infection & Immunity* **58:** 611-618.

Sanchez-SanMartin,C., Bustamante,V.H., Calva,E. and Puente,J.L. (2001). Transcriptional regulation of the orf19 gene and the tir-cesT-eae operon of enteropathogenic *Escherichia coli*. *Journal of Bacteriology*. **183:** 2823-2833.

Sandhu,K.S. and Gyles,C.L. (2002). Pathogenic Shiga toxin-producing *Escherichia coli* in the intestine of calves. *Canadian Journal of Veterinary Research* **66:** 65-72.

Sandvig,K., Grimmer,S., Lauvrak,S.U., Torgersen,M.L., Skretting,G., van Deurs,B. and Iversen,T.G. (2002). Pathways followed by ricin and Shiga toxin into cells. *Histochemistry and Cell Biology*. **117:** 131-141.

Savkovic,S.D., Koutsouris,A. and Hecht,G. (1996). Attachment of a noninvasive enteric pathogen, enteropathogenic *Escherichia coli*, to cultured human intestinal epithelial monolayers induces transmigration of neutrophils. *Infection & Immunity* **64:** 4480-4487.

Schamberger,G.P. and Diez-Gonzalez,F. (2004). Characterization of colicinogenic *Escherichia coli* strains inhibitory to enterohemorrhagic *Escherichia coli*. *Journal of Food Protection*. **67:** 486-492.

Schamberger,G.P., Phillips,R.L., Jacobs,J.L. and Diez-Gonzalez,F. (2004). Reduction of *Escherichia coli* O157:H7 populations in cattle by addition of colicin E7-producing *E. coli* to feed. *Applied & Environmental Microbiology* **70:** 6053-6060.

Scheutz,F. and Strockbine,N.A. (2001). Genus I: Escherichia. In: Bergey's Manual of Systematic Bacteriology, Volume 2: Part B: The Gammaproteobacteria, ed. G.Garrity, D.J.Brenner, N.R.Krieg and J.R.Staley, Baltimore: Williams & Wilkins, 607-624.

Schmidt,H., Beutin,L. and Karch,H. (1995a). Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infection & Immunity* **63:** 1055-1061.

Schmidt,H., Henkel,B. and Karch,H. (1997). A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohemorrhagic *Escherichia coli* O157 strains. *FEMS Microbiology Letters.* **148:** 265-272.

Schmidt,H., Knop,C., Franke,S., Aleksic,S., Heesemann,J. and Karch,H. (1995b). Development of PCR for screening of enteroaggregative *Escherichia coli*. *Journal of Clinical Microbiology*. **33:** 701-705.

Schmidt,H., Maier,E., Karch,H. and Benz,R. (1996). Pore-forming properties of the plasmid-encoded hemolysin of enterohemorrhagic *Escherichia coli* O157:H7. *European Journal of Biochemistry*. **241:** 594-601.

Schmidt,H., Plaschke,B., Franke,S., Russmann,H., Schwarzkopf,A., Heesemann,J. and Karch,H. (1994). Differentiation in virulence patterns of *Escherichia coli* possessing eae genes. *Medical Microbiology & Immunology (Berlin).* **183:** 23-31.

Schmidt,H., Scheef,J., Huppertz,H.I., Frosch,M. and Karch,H. (1999). *Escherichia coli* O157:H7 and O157:H(-) strains that do not produce Shiga toxin: phenotypic and genetic characterization of isolates associated with diarrhea and hemolytic-uremic syndrome. *Journal of Clinical Microbiology*. **37:** 3491-3496.

Schmidt,H., Scheef,J., Morabito,S., Caprioli,A., Wieler,L.H. and Karch,H. (2000). A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Applied and Environmental Microbiology.* **66:** 1205-1208.

Schmitt,C.K., McKee,M.L. and O'Brien,A.D. (1991). Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H- strain E32511. *Infection & Immunity* **59:** 1065-1073.

Scotland,S.M., Smith,H.R., Willshaw,G.A. and Rowe,B. (1983). Vero cytotoxin production in strain of *Escherichia coli* is determined by genes carried on bacteriophage. *Lancet* **2:** 216.

Scott,D.A. and Kaper,J.B. (1994). Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxin. *Infection & Immunity* **62:** 244-251.

Sekiya,K., Ohishi,M., Ogino,T., Tamano,K., Sasakawa,C. and Abe,A. (2001). Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. *Proceedings of the National Academy of Science U.S.A.* **98:** 11638-11643.

Selander,R.K., Caugant,D.A., Ochman,H., Musser,J.M., Gilmour,M.N. and Whittam,T.S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Applied & Environmental Microbiology* **51:** 873-884.

Serra-Moreno,R., Jofre,J. and Muniesa,M. (2007). Insertion site occupancy by stx2 bacteriophages depends on the locus availability of the host strain chromosome. *Journal of Bacteriology*. **189:** 6645-6654.

Seshadri,R., Joseph,S.W., Chopra,A.K., Sha,J., Shaw,J., Graf,J., Haft,D., Wu,M., Ren,Q., Rosovitz,MJ., Madupu,R., Tallon,L., Kim,M., Jin,S., Vuong,H., Stine,O.C., Ali,A., Horneman,A.J. and Heidelberg,J.F. (2006).

Genome sequence of Aeromonas hydrophila ATCC 7966T: jack of all trades. *Journal of Bacteriology*. **188:** 8272-8282.

Sethabutr,O., Venkatesan,M., Murphy,G.S., Eampokalap,B., Hoge,C.W. and Echeverria,P. (1993). Detection of Shigellae and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. *Journal of Infectious Diseases.* **167:** 458-461.

Shaikh,N. and Tarr,P.I. (2003). *Escherichia coli* O157:H7 Shiga toxin-encoding bacteriophages: integrations, excisions, truncations and evolutionary implications. *Journal of Bacteriology*. **185:** 3596-3605.

Sharma,R., Tesfay,S., Tomson,F.L., Kanteti,R.P., Viswanathan,V.K. and Hecht,G. (2006). Balance of Bacterial Pro- and Anti-inflammatory Mediators Dictates Net Effect of Enteropathogenic *Escherichia coli* (EPEC) on Intestinal Epithelial Cells. *American Journal of Physiology: Gastrointestinal & Liver Physiology*. **290:** G685-94

Shaw,D.J., Jenkins,C., Pearce,M.C., Cheasty,T., Gunn,G.J., Dougan,G., Smith,H.R., Woolhouse,M.E. and Frankel,G. (2004). Shedding patterns of verocytotoxin-producing *Escherichia coli* strains in a cohort of calves and their dams on a Scottish beef farm. *Applied & Environmental Microbiology* **70:** 7456-7465.

Shen,S., Mascarenhas,M., Rahn,K., Kaper,J.B. and Karmali,M.A. (2004). Evidence for a hybrid genomic island in verocytotoxin-producing *Escherichia coli* CL3 (serotype O113:H21) containing segments of EDL933 (serotype O157:H7) O islands 122 and 48. *Infection & Immunity* **72:** 1496-1503.

Sheng,H., Davis,M.A., Knecht,H.J., Hancock,D.D., Van Donkersgoed,J. and Hovde,C.J. (2005). Characterization of a shiga toxin-, intimin- and enterotoxin hemolysin-producing *Escherichia coli* ONT:H25 strain commonly isolated from healthy cattle. *Journal of Clinical Microbiology*. **43:** 3213-3220.

Sheng,H., Knecht,H.J., Kudva,I.T. and Hovde,C.J. (2006a). Application of bacteriophages to control intestinal *Escherichia coli* O157:H7 levels in ruminants. *Applied & Environmental Microbiology* **72:** 5359-5366.

Sheng,H., Lim,J.Y., Knecht,H.J., Li,J. and Hovde,C.J. (2006b). Role of *Escherichia coli* O157:H7 virulence factors in colonization at the bovine terminal rectal mucosa. *Infection & Immunity* **74:** 4685-4693.

Sheoran,A.S., Chapman,S., Singh,P., Donohue-Rolfe,A. and Tzipori,S. (2003). Stx2-specific human monoclonal antibodies protect mice against lethal infection with *Escherichia coli* expressing Stx2 variants. *Infection & Immunity* **71:** 3125-3130.

Shimazu,R., Akashi,S., Ogata,H., Nagai,Y., Fukudome,K., Miyake,K. and Kimoto,M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *Journal of Experimental Medicine* **189:** 1777-1782.

Shimizu,M., Sakano,T., Yamamoto,J. and Kitajima,K. (1987). Incidence and some characteristics of fimbriae FY and 31A of *Escherichia coli* isolates from calves with diarrhea in Japan. *Microbiology & Immunology* **31:** 417-426.

Silvestro,L., Caputo,M., Blancato,S., Decastelli,L., Fioravanti,A., Tozzoli,R., Morabito,S. and Caprioli,A. (2004). Asymptomatic carriage of verocytotoxin-producing *Escherichia coli* O157 in farm workers in Northern Italy. *Epidemiology & Infection* **132:** 915-919.

Sinclair,J.F. and O'Brien,A.D. (2002). Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157:H7. *Journal of Biological Chemistry* **277:** 2876-2885.

Smit,H., Gaastra,W., Kamerling,J.P., Vliegenthart,J.F. and de Graaf,F.K. (1984). Isolation and structural characterization of the equine erythrocyte receptor for enterotoxigenic *Escherichia coli* K99 fimbrial adhesin. *Infection & Immunity* **46:** 578-584.

Smith,D., Blackford,M., Younts,S., Moxley,R., Gray,J., Hungerford,L., Milton,T. and Klopfenstein,T. (2001). Ecological relationships between the prevalence of cattle shedding *Escherichia coli* O157:H7 and characteristics of the cattle or conditions of the feedlot pen. *Journal of Food Protection* **64:** 1899-1903.

Smith,D.L., James,C.E., Sergeant,M.J., Yaxian,Y., Saunders,J.R., McCarthy,A.J. and Allison,H.E. (2007). Short-tailed stx phages exploit the conserved YaeT protein to disseminate Shiga toxin genes among enterobacteria. *Journal of Bacteriology*. **189:** 7223-7233.

Smith,H.W., Green,P. and Parsell,Z. (1983). Vero cell toxins in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chickens and pigs. *Journal of General Microbiology*. **129:** 3121-3137.

Smith,K.D., Andersen-Nissen,E., Hayashi,F., Strobe,K., Bergman,M.A., Barrett,S.L., Cookson,B.T. and Aderem,A. (2003). Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nature Immunology*. **4:** 1247-1253.

Sokurenko,E.V., Chesnokova,V., Dykhuizen,D.E., Ofek,I., Wu,X.R., Krogfelt,K.A., Struve,C., Schembri,M.A. and Hasty,D.L. (1998). Pathogenic adaptation of *Escherichia coli* by natural variation of the FimH adhesin. *Proceedings of the National Academy of Science U.S.A.* **95:** 8922-8926.

Sperandio,V., Mellies,J.L., Delahay,R.M., Frankel,G., Crawford,J.A., Nguyen,W. and Kaper,J.B. (2000). Activation of enteropathogenic *Escherichia coli* (EPEC) LEE2 and LEE3 operons by Ler. *Molecular Microbiology.* **38:** 781-793.

Sperandio,V., Mellies,J.L., Nguyen,W., Shin,S. and Kaper,J.B. (1999). Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proceedings of the National Academy of Science U.S.A.* **96:** 15196-15201.

Sperandio,V., Torres,A.G., Jarvis,B., Nataro,J.P. and Kaper,J.B. (2003). Bacteria-host communication: the language of hormones. *Proceedings of the National Academy of Science U.S.A.* **100:** 8951-8956.

Staley,J.T. (2006). The bacterial species dilemma and the genomic-phylogenetic species concept. *Philosophical Transactions of the Royal Society of London. B: Biological Sciences.* **361:** 1899-1909.

Stappenbeck,T.S., Hooper,L.V. and Gordon,J.I. (2002). Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proceedings of the National Academy of Science U.S.A. **99:** 15451-15455.

Stein,P.E., Boodhoo,A., Tyrrell,G.J., Brunton,J.L. and Read,R.J. (1992). Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. *Nature*. **355:** 748-750.

Stevens,M.P., Roe,A.J., Vlisidou,I., van Diemen,P.M., La Ragione,R.M., Best,A., Woodward,M.J., Gally,D.L. and Wallis,T.S. (2004). Mutation of toxB and a truncated version of the efa-1 gene in *Escherichia coli* O157:H7 influences the expression and secretion of locus of enterocyte effacement-encoded proteins but not intestinal colonization in calves or sheep. *Infection & Immunity* **72:** 5402-5411.

Stevens,M.P., van Diemen,P.M., Frankel,G., Phillips,A.D. and Wallis,T.S. (2002). Efa1 influences colonization of the bovine intestine by shiga toxin-producing *Escherichia coli* serotypes O5 and O111. *Infection & Immunity* **70:** 5158-5166.

Strachan,N.J., Dunn,G.M., Locking,M.E., Reid,T.M. and Ogden,I.D. (2006). *Escherichia coli* O157: burger bug or environmental pathogen? *International Journal of Food Microbiology* **112:** 129-137.

Strockbine,N.A., Marques,L.R., Newland,J.W., Smith,H.W., Holmes,R.K. and O'Brien,A.D. (1986). Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. *Infection & Immunity.* **53:** 135-140.

Suau,A., Bonnet,R., Sutren,M., Godon,J.J., Gibson,G.R., Collins,M.D. and Dore,J. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied & Environmental Microbiology.* **65:** 4799-4807.

Swinbanks,D. (1996). Japan shuns radishes after 'possible link' to *E. coli*. *Nature* **382:** 567.

Synge,B. and Paiba,G. (2000). Verocytotoxin-producing *E coli* O157. *Veterinary Record.* **147:** 27.

Synge,B.A., Chase-Topping,M.E., Hopkins,G.F., McKendrick,I.J., Thomson-Carter,F., Gray,D., Rusbridge,S.M., Munro,F.I., Foster,G. and Gunn,G.J. (2003). Factors influencing the shedding of verocytotoxin-producing *Escherichia coli* O157 by beef suckler cows. *Epidemiology & Infection* **130:** 301-312.

Takeuchi,O., Kawai,T., Muhlradt,P.F., Morr,M., Radolf,J.D., Zychlinsky,A., Takeda,K. and Akira,S. (2001). Discrimination of bacterial lipoproteins by Toll-like receptor 6. *International Immunology.* **13:** 933-940.

Takeuchi,O., Sato,S., Horiuchi,T., Hoshino,K., Takeda,K., Dong,Z., Modlin,R.L. and Akira,S. (2002). Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *Journal of Immunology* **169:** 10-14.

Tang,P., Foubister,V., Pucciarelli,M. and Finlay,B. (1993). Methods to study bacterial invasion. *Journal of Microbiological Methods.* **18:** 227-240.

Tarr,C.L. and Whittam,T.S. (2002). Molecular evolution of the intimin gene in O111 clones of pathogenic *Escherichia coli*. *Journal of Bacteriology*. **184:** 479-487.

Tarr,P.I., Bilge,S.S., Vary,J.C., Jr., Jelacic,S., Habeeb,R.L., Ward,T.R., Baylor,M.R. and Besser,T.E. (2000). Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infection & Immunity* **68:** 1400-1407.

Taylor,P., Allison,L., Willshaw,G.A., Cheasty,T. and Hanson,M. Sorbitol-fermenting *Escherichia coli* O157 in Scotland. 5th International Symposium on Shiga Toxin (Verotoxin)-producing *E.coli* Infections.Edinburgh, Scotland , P280. 2003.

te Loo,D.M., Heuvelink,A.E., de Boer,E., Nauta,J., van der Walle,J., Schroder,C., van Hinsbergh, V, Chart,H., van de Kar,N.C. and van den Heuvel,L.P. (2001a). Vero cytotoxin binding to polymorphonuclear leukocytes among households with children with hemolytic uremic syndrome. *Journal of Infectious Diseases* **184:** 446-450.

te Loo,D.M., van Hinsbergh,V, van den Heuvel,L.P. and Monnens,L.A. (2001b). Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. *Journal of the American Society of Nephrology.* **12:** 800-806.

The Pennington Group (1998). Report on the circumstances leading to the 1996 outbreak of infection with *E.coli* O157 in Central Scotland, the implications for food safety and the lessons to be learned. . The Scottish Office.

Thompson,J.S., Hodge,D.S. and Borczyk,A.A. (1990). Rapid biochemical test to identify verocytotoxin-positive strains of *Escherichia coli* serotype O157. *Journal of Clinical Microbiology.* **28:** 2165-2168.

Thran,B.H., Hussein,H.S., Hall,M.R. and Khaiboullina,S.F. (2001a). Occurrence of verotoxin-producing *Escherichia coli* in dairy heifers grazing an irrigated pasture. *Toxicology* **159:** 159-169.

Thran,B.H., Hussein,H.S., Hall,M.R. and Khaiboullina,S.F. (2001b). Shiga toxin-producing *Escherichia coli* in beef heifers grazing an irrigated pasture. *Journal of Food Protection* **64:** 1613-1616.

Tkalcic,S., Zhao,T., Harmon,B.G., Doyle,M.P., Brown,C.A. and Zhao,P. (2003). Fecal shedding of enterohemorrhagic *Escherichia coli* in weaned calves following treatment with probiotic *Escherichia coli*. *Journal of Food Protection* **66:** 1184-1189.

Tlaskalova-Hogenova,H. Stepánková,R., Hudcovic,T., Tucková,L., Cukrowska,B., Lodinová-Zádníková,R., Kozáková,H., Rossmann,P., Bártová,J., Sokol,D., Funda,D.P., Borovská,D., Reháková,Z., Sinkora,J., Hofman,J., Drastich,P. and Kokesová,A. (2004). Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunology Letters* **93:** 97-108.

Tobe,T., Beatson,S.A., Taniguchi,H., Abe,H., Bailey,C.M., Fivian,A., Younis,R., Matthews,S., Marches,O., Frankel,G., Hayashi,T. and Pallen,M.J. (2006). An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proceedings of the National Academy of Science U.S.A.* **103:** 14941-14946.

Torres,A.G., Giron,J.A., Perna,N.T., Burland,V., Blattner,F.R., Avelino-Flores,F. and Kaper,J.B. (2002). Identification and characterization of lpfABCC'DE, a fimbrial operon of enterohemorrhagic *Escherichia coli* O157:H7. *Infection & Immunity* **70:** 5416-5427.

Torres,A.G., Kanack,K.J., Tutt,C.B., Popov,V. and Kaper,J.B. (2004). Characterization of the second long polar (LP) fimbriae of *Escherichia coli* O157:H7 and distribution of LP fimbriae in other pathogenic *E. coli* strains. *FEMS Microbiology Letters.* **238:** 333-344.

Torres,A.G. and Kaper,J.B. (2003). Multiple elements controlling adherence of enterohemorrhagic *Escherichia coli* O157:H7 to HeLa cells. *Infection & Immunity* **71:** 4985-4995.

Toth,I., Cohen,M.L., Rumschlag,H.S., Riley,L.W., White,E.H., Carr,J.H., Bond,W.W. and Wachsmuth,I.K. (1990). Influence of the 60-megadalton plasmid on adherence of *Escherichia coli* O157:H7 and genetic derivatives. *Infection & Immunity* **58:** 1223-1231.

Toth,I., Herault,F., Beutin,L. and Oswald,E. (2003). Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new cdt variant (Type IV). *Journal of Clinical Microbiology.* **41:** 4285-4291.

Tozzi,A.E., Caprioli,A., Minelli,F., Gianviti,A., De Petris,L., Edefonti,A., Montini,G., Ferretti,A., De Palo,T., Gaido,M. and Rizzoni,G. (2003). Shiga toxin-producing *Escherichia coli* infections associated with hemolytic uremic syndrome, Italy, 1988-2000. *Emerging Infectious Diseases.***. 9:** 106-108.

Trabulsi,L.R., Keller,R. and Tardelli Gomes,T.A. (2002). Typical and atypical enteropathogenic *Escherichia coli*. *Emerging Infectious Diseases.* **8:** 508-513.

Tu,X., Nisan,I., Yona,C., Hanski,E. and Rosenshine,I. (2003). EspH, a new cytoskeleton-modulating effector of enterohaemorrhagic and enteropathogenic *Escherichia coli*. *Molecular Microbiology.* **47:** 595-606.

Tullus,K., Kuhn,I., Orskov,I., Orskov,F. and Mollby,R. (1992). The importance of P and type 1 fimbriae for the persistence of *Escherichia coli* in the human gut. *Epidemiology & Infection*. **108:** 415-421.

Tzipori,S., Gunzer,F., Donnenberg,M.S., de,M.L., Kaper,J.B. and Donohue-Rolfe,A. (1995). The role of the eaeA gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. *Infection & Immunity*. **63:** 3621-3627.

Uhlich,G.A., Sinclair,J.R., Warren,N.G., Chmielecki,W.A. and Fratamico,P. (2008). Characterization of Shiga toxin-producing *Escherichia coli* isolates associated with two multistate food-borne outbreaks that occurred in 2006. *Applied & Environmental Microbiology.* **74:** 1268-1272.

Uren,T.K., Wijburg,O.L., Simmons,C., Johansen,F.E., Brandtzaeg,P. and Strugnell,R.A. (2005). Vaccine-induced protection against gastrointestinal bacterial infections in the absence of secretory antibodies. *European Journal of Immunology.* **35:** 180-188.

Usein,C.R., Damian,M., Tatu-Chitoiu,D., Capusa,C., Fagaras,R. and Mircescu,G. (2003). Comparison of genomic profiles of *Escherichia coli* isolates from urinary tract infections. *Roumanian Archives of Microbiology & Immunology.* **62:** 137-154.

van de Kar,N.C., Monnens,L.A., Karmali,M.A. and van Hinsbergh,V. (1992). Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial ce**lls:** implications for the pathogenesis of the hemolytic uremic syndrome. *Blood.* **80:** 2755-2764.

van Diemen,P.M., Dziva,F., Stevens,M.P. and Wallis,T.S. (2005). Identification of enterohemorrhagic *Escherichia coli* O26:H- genes required for intestinal colonization in calves. *Infection & Immunity* **73:** 1735-1743.

Varki,A. (1996). "Unusual" modifications and variations of vertebrate oligosaccharides: are we missing the flowers for the trees? *Glycobiology* **6:** 707-710.

Vazquez-Torres,A. and Fang,F.C. (2000). Cellular routes of invasion by enteropathogens. *Current Opinions in Microbiology*. **3:** 54-59.

Vezzi,A., Campanaro,S., D'Angelo,M., Simonato,F., Vitulo,N., Lauro,F.M., Cestaro,A., Malacrida,G., Simionati,B., Cannata,N., Romualdi,C., Bartlett,D.H. and Valle,G. (2005). Life at depth: *Photobacterium profundum* genome sequence and expression analysis. *Science.* **307:** 1459-1461.

Vicente-Suarez,I., Takahashi,Y., Cheng,F., Horna,P., Wang,H.W., Wang,H.G. and Sotomayor,E.M. (2007). Identification of a novel negative role of flagellin in regulating IL-10 production. European *Journal of Immunology* **37:** 3164-3175.

Viswanathan,V.K., Koutsouris,A., Lukic,S., Pilkinton,M., Simonovic,I., Simonovic,M. and Hecht,G. (2004). Comparative analysis of EspF from enteropathogenic and enterohemorrhagic *Escherichia coli* in alteration of epithelial barrier function. *Infection & Immunity* **72:** 3218-3227.

Vlisidou,I., Dziva,F., La Ragione,R.M., Best,A., Garmendia,J., Hawes,P., Monaghan,P., Cawthraw,S.A., Frankel,G., Woodward,M.J. and Stevens,M.P. (2006). Role of intimin-tir interactions and the tir-cytoskeleton coupling protein in the colonization of calves and lambs by *Escherichia coli* O157:H7. *Infection & Immunity* **74:** 758-764.

Wang,L. and Reeves,P.R. (1998). Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infection & Immunity* **66:** 3545-3551.

Wang,X., Heazlewood,S.P., Krause,D.O. and Florin,T.H. (2003). Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. *Journal of Applied Microbiology.* **95:** 508-520.

Watanabe,H., Wada,A., Inagaki,Y., Itoh,K. and Tamura,K. (1996). Outbreaks of enterohaemorrhagic *Escherichia coli* O157:H7 infection by two different genotype strains in Japan, 1996. *Lancet* **348:** 831-832.

Welsh,J. and McClelland,M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research.* **18:** 7213-7218.

Werts,C., Tapping,R.I., Mathison,J.C., Chuang,T.H., Kravchenko,V., Saint Girons,I., Haake,D.A., Godowski,P.J., Hayashi,F., Ozinsky,A., Underhill,D.M., Kirschning,C.J., Wagner,H., Aderem,A., Tobias,P.S. and Ulevitch,R.J. (2001). Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nature Immunology*. **2:** 346-352.

Westerholt,S., Hartung,T., Tollens,M., Gustrau,A., Oberhoffer,M., Karch,H., Klare,B., Pfeffer,K., Emmrich,P. and Oberhoffer,R. (2000). Inflammatory and immunological parameters in children with haemolytic uremic syndrome (HUS) and gastroenteritis-pathophysiological and diagnostic clues. *Cytokine.* **12:** 822-827.

Westerholt,S., Pieper,A.K., Griebel,M., Volk,H.D., Hartung,T. and Oberhoffer,R. (2003). Characterization of the cytokine immune response in children who have experienced an episode of typical hemolytic-uremic syndrome. *Clinical Diagnostic Laboratory Immunology* **10:** 1090-1095.

Whitfield,C. and Roberts,I.S. (1999). Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Molecular Microbiology.* **31:** 1307-1319.

Whittam,T.S., Ochman,H. and Selander,R.K. (1983). Multilocus genetic structure in natural populations of *Escherichia coli*. *Proceedings of the National Academy of Science U.S.A.* **80:** 1751-1755.

Wieler,L.H., Tigges,M., Ebel,F., Schaferkordt,S., Djafari,S., Schlapp,T., Baljer,G. and Chakraborty,T. (1996). The enterohemolysin phenotype of bovine Shiga-like toxin-producing *Escherichia coli* (SLTEC) is encoded by the EHEC-hemolysin gene. *Veterinary Microbiology.* **52:** 153-164.

Wijburg,O.L., Uren,T.K., Simpfendorfer,K., Johansen,F.E., Brandtzaeg,P. and Strugnell,R.A. (2006). Innate secretory antibodies protect against natural *Salmonella typhimurium* infection. *Journal of Experimental Medicine* **203:** 21-26.

Wilson,J., Spika,J., Clarke,R., McEwen,S., Johnson,R., Rahn,K., Renwick,S., Karmali,M., Lior,H., Alves,D., Gyles,C. and Sandhu,K. (1998). Verocytotoxigenic *Escherichia coli* infection in dairy farm families. *Canadian Communicable Disease Reports*. **24:** 17-20.

Wilson,J.B., Clarke,R.C., Renwick,S.A., Rahn,K., Johnson,R.P., Karmali,M.A., Lior,H., Alves,D., Gyles,C.L., Sandhu,K.S., McEwen,S.A. and Spika,J.S. (1996). Vero cytotoxigenic *Escherichia coli* infection in dairy farm families. *Journal of Infectious Diseases.* **174:** 1021-1027.

Wilson,J.B., McEwen,S.A., Clarke,R.C., Leslie,K.E., Wilson,R.A., Waltner-Toews,D. and Gyles,C.L. (1992). Distribution and characteristics of verocytotoxigenic *Escherichia coli* isolated from Ontario dairy cattle. *Epidemiology & Infection* **108:** 423-439.

Wirth,T., Falush,D., Lan,R., Colles,F., Mensa,P., Wieler,L.H., Karch,H., Reeves,P.R., Maiden,M.C., Ochman,H., Achtman,M. (2006). Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Molecular Microbiology.* **60:** 1136-1151.

Wold,A.E., Caugant,D.A., Lidin-Janson,G., de Man,M.P. and Svanborg,C. (1992). Resident colonic *Escherichia coli* strains frequently display uropathogenic characteristics. *Journal of Infectious Diseases. 165*, 46-52.

Woodward,M.J., Best,A., Sprigings,K.A., Pearson,G.R., Skuse,A.M., Wales,A., Hayes,C.M., Roe,J.M., Low,J.C. and La Ragione,R.M. (2003). Non-toxigenic *Escherichia coli* O157:H7 strain NCTC12900 causes attaching-effacing lesions and eae-dependent persistence in weaned sheep. International *Journal of Medical Microbiology* **293:** 299-308.

Wray,C., Piercy,D.W., Carroll,P.J. and Cooley,W.A. (1993). Experimental infection of neonatal pigs with CNF toxin-producing strains of *Escherichia coli*. *Research in Veterinary Science* **54:** 290-298.

Wright,D.J., Chapman,P.A. and Siddons,C.A. (1994). Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food samples. *Epidemiology & Infection* **113:** 31-39.

Wu,G., Carter,B., Mafura,M., Liebana,E., Woodward,M.J. and Anjum,M.F. (2008). Genetic diversity among *Escherichia coli* O157:H7 isolates and identification of genes linked to human infections. *Infection & Immunity* **76:** 845-856.

Yarze,J.C. and Chase,M.P. (2000). *E. coli* O157:H7--another waterborne outbreak! *American Journal of Gastroenterology* **95:** 1096.

Yona-Nadler,C., Umanski,T., Aizawa,S., Friedberg,D. and Rosenshine,I. (2003). Integration host factor (IHF) mediates repression of flagella in enteropathogenic and enterohaemorrhagic *Escherichia coli*. *Microbiology.*. **149:** 877-884.

Yonekura,K., Maki-Yonekura,S. and Namba,K. (2003). Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. *Nature*. **424:** 643-650.

Yoshioka,K., Aizawa,S. and Yamaguchi,S. (1995). Flagellar filament structure and cell motility of *Salmonella* typhimurium mutants lacking part of the outer domain of flagellin. *Journal of Bacteriology*. **177:** 1090-1093.

Zadik,P.M., Chapman,P.A. and Siddons,C.A. (1993). Use of tellurite for the selection of verocytotoxigenic *Escherichia coli* O157. *Journal of Medical Microbiology* **39:** 155-158.

Zhang,W.L., Kohler,B., Oswald,E., Beutin,L., Karch,H., Morabito,S., Caprioli,A., Suerbaum,S. and Schmidt,H. (2002). Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *Journal of Clinical Microbiology* **40:** 4486-4492.

Zhang,Y., Laing,C., Steele,M., Ziebell,K., Johnson,R., Benson,A.K., Taboada,E. and Gannon,V.P. (2007). Genome evolution in major *Escherichia coli* O157:H7 lineages. *BMC Genomics*. **8:** 121.

Zhang,Z., Schwartz,S., Wagner,L. and Miller,W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7:** 203-214.

Zhao,T., Doyle,M.P., Harmon,B.G., Brown,C.A., Mueller,P.O. and Parks,A.H. (1998). Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. *Journal of Clinical Microbiology.* **36:** 641-647.

Zhao,T., Tkalcic,S., Doyle,M.P., Harmon,B.G., Brown,C.A. and Zhao,P. (2003). Pathogenicity of enterohemorrhagic *Escherichia coli* in neonatal calves and evaluation of fecal shedding by treatment with probiotic *Escherichia coli*. *Journal of Food Protection.* **66:** 924-930.

Zhou,X., Giron,J.A., Torres,A.G., Crawford,J.A., Negrete,E., Vogel,S.N. and Kaper,J.B. (2003). Flagellin of enteropathogenic *Escherichia coli* stimulates interleukin-8 production in T84 cells. *Infection & Immunity* **71:** 2120-2129.

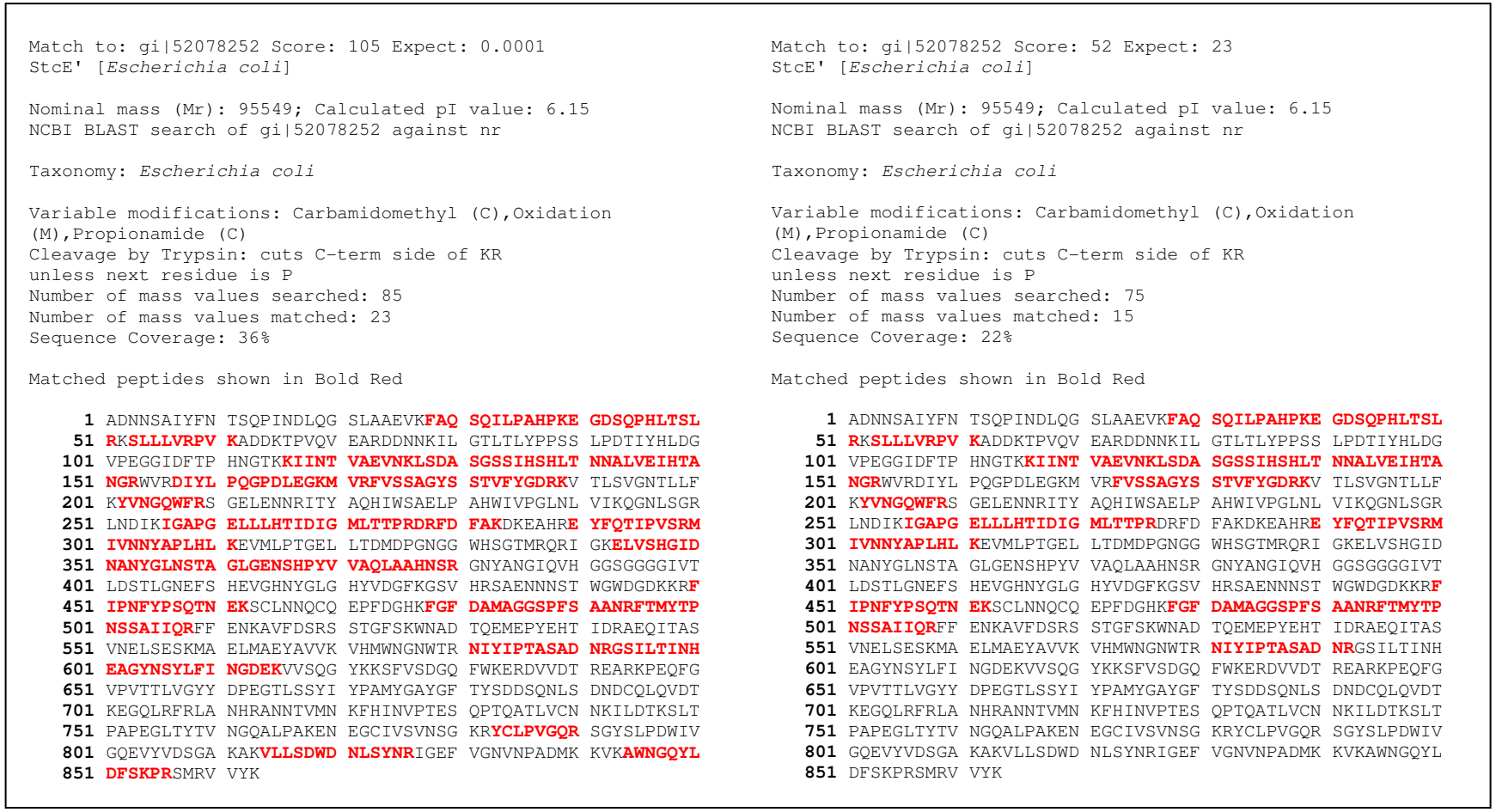
Ziebell,K., Steele,M., Zhang,Y., Benson,A., Taboada,E.N., Laing,C., McEwen,S., Ciebin,B., Johnson,R. and Gannon,V. (2008). Genotypic characterization and prevalence of virulence factors among Canadian *Escherichia coli* O157:H7 strains. *Applied & Environmental Microbiology* **74:** 4314-23

Zwiener,R.J., Belknap,W.M. and Quan,R. (1989). Severe pseudomembranous enterocolitis in a child: case report and literature review. *Pediatric Infectious Diseases Journal* **8:** 876-882.

Appendices

Appendix I Analysis of rStcE by MALDI-TOF.

Results of MALDI-TOF analysis of bands resulting from SDS-PAGE of rStcE (Figure 3.9) are presented in Figure A1.



Match to: gi|52078252 Score: 105 Expect: 0.0001
StcE' [*Escherichia coli*]

Nominal mass (Mr): 95549; Calculated pI value: 6.15
NCBI BLAST search of gi|52078252 against nr

Taxonomy: *Escherichia coli*

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 85
Number of mass values matched: 23
Sequence Coverage: 36%

Matched peptides shown in Bold Red

```
   1 ADNNSAIYFN TSQPINDLQG SLAAEVKFAQ SQILPAHPKE GDSQPHLTSL
  51 RKSLLLVRPV KADDKTPVQV EARDDNNKIL GTLTLYPPSS LPDTIYHLDG
 101 VPEGGIDFTP HNGTKKIINT VAEVNKLSDA SGSSIHSHLT NNALVEIHTA
 151 NGRWVRDIYL PQGPDLEGKM VRFVSSAGYS STVFYGDRKV TLSVGNTLLF
 201 KYVNGQWFRS GELENNRITY AQHIWSAELP AHWIVPGLNL VIKQGNLSGR
 251 LNDIKIGAPG ELLLHTIDIG MLTTPRDRFD FAKDKEAHRE YFQTIPVSRM
 301 IVNNYAPLHL KEVMLPTGEL LTDMDPGNGG WHSGTMRQRI GKELVSHGID
 351 NANYGLNSTA GLGENSHPYV VAQLAAHNSR GNYANGIQVH GGSGGGGIVT
 401 LDSTLGNEFS HEVGHNYGLG HYVDGFKGSV HRSAENNNST WGWDGDKKRF
 451 IPNFYPSQTN EKSCLNNQCQ EPFDGHKFGF DAMAGGSPFS AANRFTMYTP
 501 NSSAIIQRFF ENKAVFDSRS STGFSKWNAD TQEMEPYEHT IDRAEQITAS
 551 VNELSESKMA ELMAEYAVVK VHMWNGNWTR NIYIPTASAD NRGSILTINH
 601 EAGYNSYLFI NGDEKVVSQG YKKSFVSDGQ FWKERDVVDT REARKPEQFG
 651 VPVTTLVGYY DPEGTLSSYI YPAMYGAYGF TYSDDSQNLS DNDCQLQVDT
 701 KEGQLRFRLA NHRANNTVMN KFHINVPTES QPTQATLVCN NKILDTKSLT
 751 PAPEGLTYTV NGQALPAKEN EGCIVSVNSG KRYCLPVGQR SGYSLPDWIV
 801 GQEVYVDSGA KAKVLLSDWD NLSYNRIGEF VGNVNPADMK KVKAWNGQYL
 851 DFSKPRSMRV VYK
```

Match to: gi|52078252 Score: 52 Expect: 23
StcE' [*Escherichia coli*]

Nominal mass (Mr): 95549; Calculated pI value: 6.15
NCBI BLAST search of gi|52078252 against nr

Taxonomy: *Escherichia coli*

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 75
Number of mass values matched: 15
Sequence Coverage: 22%

Matched peptides shown in Bold Red

```
   1 ADNNSAIYFN TSQPINDLQG SLAAEVKFAQ SQILPAHPKE GDSQPHLTSL
  51 RKSLLLVRPV KADDKTPVQV EARDDNNKIL GTLTLYPPSS LPDTIYHLDG
 101 VPEGGIDFTP HNGTKKIINT VAEVNKLSDA SGSSIHSHLT NNALVEIHTA
 151 NGRWVRDIYL PQGPDLEGKM VRFVSSAGYS STVFYGDRKV TLSVGNTLLF
 201 KYVNGQWFRS GELENNRITY AQHIWSAELP AHWIVPGLNL VIKQGNLSGR
 251 LNDIKIGAPG ELLLHTIDIG MLTTPRDRFD FAKDKEAHRE YFQTIPVSRM
 301 IVNNYAPLHL KEVMLPTGEL LTDMDPGNGG WHSGTMRQRI GKELVSHGID
 351 NANYGLNSTA GLGENSHPYV VAQLAAHNSR GNYANGIQVH GGSGGGGIVT
 401 LDSTLGNEFS HEVGHNYGLG HYVDGFKGSV HRSAENNNST WGWDGDKKRF
 451 IPNFYPSQTN EKSCLNNQCQ EPFDGHKFGF DAMAGGSPFS AANRFTMYTP
 501 NSSAIIQRFF ENKAVFDSRS STGFSKWNAD TQEMEPYEHT IDRAEQITAS
 551 VNELSESKMA ELMAEYAVVK VHMWNGNWTR NIYIPTASAD NRGSILTINH
 601 EAGYNSYLFI NGDEKVVSQG YKKSFVSDGQ FWKERDVVDT REARKPEQFG
 651 VPVTTLVGYY DPEGTLSSYI YPAMYGAYGF TYSDDSQNLS DNDCQLQVDT
 701 KEGQLRFRLA NHRANNTVMN KFHINVPTES QPTQATLVCN NKILDTKSLT
 751 PAPEGLTYTV NGQALPAKEN EGCIVSVNSG KRYCLPVGQR SGYSLPDWIV
 801 GQEVYVDSGA KAKVLLSDWD NLSYNRIGEF VGNVNPADMK KVKAWNGQYL
 851 DFSKPRSMRV VYK
```

Figure A1: Results of MALDI-TOF analysis of StcE bands separated by SDS-PAGE: The frame to the left shows the MASCOT result for the larger of the two bands analysed. Peptides were detected covering the protein intermittently from 25 residues from the N-terminus N-terminus to 7 residues from the C-terminus. The frame to the right shows the results from the lower molecular weight band. No peptides were detected covering the C-terminal 271 residues.

Appendix II Bovine isolates employed during this study

Table A1 shows the full list of bovine isolates provided by Neil Paton (all isolated by rectal swab or faecal free catch) and those pro or Stuart Naylor (all isolated from tissue sections). Table A2 lists the strains isolated during this study from tissue sections. Table A3 lists the strains isolated by rectal swabbing of animals three weeks prior to euthanasia. Isolation method/Site: FFC: faecal free-catch, RS: recto-anal mucosal swab TR: terminal rectum, PR: proximal rectum, DC: distal colon, PC: proximal colon, IL: ileum (refer to Figure 4.12 and Section 2.3.2 for a full description of sites and isolation methods).

Animal	Isolation/ Site	Colony	GLUC	SOR-F	H-pattern	O-pattern	Ref. No.
7	FFC	3	ND	+	H35	Or1	MCI 0104
7	FFC	2	ND	+	H25	O116	MCI 0122
8	FFC	1	ND	+	H4	Or4	MCI 0108
8	FFC	2	ND	+	H14	Or11	MCI 0123
8	FFC	3	ND	+	H14	Or11	MCI 0124
9	FFC	2	ND	+	H34	Or2	MCI 0105
9	FFC	1	ND	+	H+(NT)	Or2	MCI 0115
9	FFC	3	ND	+	H+(NT)	Or7	MCI 0121
10	FFC	1	ND	+	H-/NT	Or3	MCI 0106
10	FFC	2	ND	+	H-/NT	Or3	MCI 0111
10	FFC	3	ND	+	H4	Or58	MCI 0112
11	FFC	3	ND	+	H4	Or4	MCI 0109
11	FFC	1	ND	+	H34	Or11	MCI 0113
11	FFC	2	ND	+	H4	Or5	MCI 0114
12	FFC	1	ND	+	H-/NT	Or3	MCI 0116
12	FFC	2	ND	+	H-/NT	Or3	MCI 0117
12	FFC	3	ND	+	H-/NT	Or3	MCI 0118
13	FFC	1	ND	+	H14	Or11	MCI 0119
13	FFC	3	ND	+	H14	Or11	MCI 0107
13	FFC	2	ND	+	H35	Or6	MCI 0120
14	FFC	1	ND	+	H11	O26	MCI 0125
14	FFC	2	ND	+	H11	O26	MCI 0126
14	FFC	3	ND	+	H11	O26	MCI 0110
100742	RS	2	ND	+	H31	Or9	MCI 0160
300395	RS	2	ND	+	H35	Or11	MCI 0166
300395	RS	1	ND	+	H35	Or14	MCI 0176
300395	RS	3	ND	+	H21	Or18	MCI 0180
300741	RS	3	ND	+	H21	Or11	MCI 0170
300741	RS	1	ND	+	H35	Or4	MCI 0171
300744	RS	2	ND	+	H21	Or16	MCI 0182
400211	RS	1	ND	+	H21	ND	MCI 0161

400211	RS	3	ND	+	H35	Or10	MCI 0162
400211	RS	2	ND	+	H35	Or10	MCI 0168
400376	RS	2	ND	+	HNT	Or11	MCI 0172
400385	RS	1	ND	+	H35	Or12	MCI 0167
400396	RS	1	ND	+	HNT	Or11	MCI 0165
500340	RS	3	ND	+	H35	Or13	MCI 0169
500340	RS	1	ND	+	H35	Or14	MCI 0173
500348	RS	2	ND	+	H14	Or11	MCI 0164
600341	RS	3	ND	+	H21	Or7	MCI 0174
600341	RS	2	ND	+	H21	Or7	MCI 0177
600747	RS	2	ND	+	H19NM	Or15	MCI 0179
600747	RS	1	ND	+	H30	Or14	MCI 0181
824	TR	1	+	ND	H8	Or17	MCI 0308
824	TR	2	+	ND	H8	Or17	MCI 0309
824	TR	3	+	ND	H8	Or17	MCI 0310
824	TR	4	+	ND	H8	Or22	MCI 0373
824	TR	5	+	ND	H8	ND	MCI 0374
824	TR	6	+	ND	H8	ND	MCI 0375
824	TR	7	+	ND	H8	ND	MCI 0382
824	TR	8	+	ND	H8	ND	MCI 0383
824	TR	9	+	ND	H8	ND	MCI 0384
540	TR	1	-	ND	H-/NT	Or18	MCI 0311
540	TR	2	-	ND	H-/NT	Or18	MCI 0312
540	TR	3	-	ND	H-/NT	Or18	MCI 0313
540	TR	1	+	ND	H21	Or8	MCI 0319
540	TR	2	+	ND	H21	Or8	MCI 0320
540	TR	3	+	ND	H21	Or8	MCI 0321
593	TR	1	+	ND	H21	Or8	MCI 0327
593	TR	2	+	ND	H21	Or8	MCI 0328
593	TR	3	+	ND	H21	Or8	MCI 0329
541	TR	1	+	ND	H8	Or17	MCI 0333
541	TR	2	+	ND	H8	Or17	MCI 0334
541	TR	3	+	ND	H8	Or17	MCI 0335
541	PC	1	+	ND	H32	Or19	MCI 0344
541	PC	2	+	ND	H35	Or18	MCI 0345
541	PC	3	+	ND	H32	Or19	MCI 0346
493	TR	1	+	ND	H29	ND	MCI 0354
710	PR	1	+	ND	H21	Or11	MCI 0356
710	PR	2	+	ND	H21	Or11	MCI 0357
710	PR	3	+	ND	H21	Or11	MCI 0358
739	TR	1	+	ND	H21	Or20	MCI 0362
739	TR	2	+	ND	H21	Or20	MCI 0363
739	TR	3	+	ND	H21	Or20	MCI 0364
740	TR	1	+	ND	H48	Or21	MCI 0368
740	TR	2	+	ND	H31	ND	MCI 0369
740	TR	3	+	ND	H48	Or21	MCI 0370
583	TR	1	+	ND	H-/NT	Or17	MCI 0377
583	TR	2	+	ND	H21	Or23	MCI 0378
583	TR	3	+	ND	H8	ND	MCI 0379
587	TR	1	+	ND	H21	Or8	MCI 0386

587	TR	2	+	ND	H21	Or8	MCI 0387
587	TR	3	+	ND	H21	Or8	MCI 0388
586	TR	1	+	ND	H19NM	Or25	MCI 0398
586	TR	2	+	ND	H19NM	Or25	MCI 0399
586	TR	3	+	ND	H19NM	Or25	MCI 0400
566	TR	1	+	ND	H21	Or24	MCI 0392
566	TR	2	+	ND	H21	Or24	MCI 0393
566	TR	3	+	ND	H21	Or24	MCI 0394
825	TR	1	+	ND	H19NM	Or26	MCI 0404
825	TR	2	+	ND	H19NM	Or26	MCI 0405
825	TR	3	+	ND	H19NM	Or26	MCI 0406
826	TR	1	+	ND	H19NM	Or27	MCI 0410
826	TR	2	+	ND	H19NM	Or27	MCI 0411
826	TR	3	+	ND	H19NM	Or27	MCI 0412

Table A1: Summary of bovine isolates provided by Neil Paton and Stuart Naylor: (GLUC: glucuronidase positive (+)/negative (-), SOR-F: sorbitol-fermenting (+)/non-fermenting (-), O-pattern: O-RFLP pattern, H-pattern: H-RFLP-pattern.
Isolation/Site: FFC: faecal free-catch, RS: recto-anal mucosal swab TR: terminal rectum, PR: proximal rectum, DC: distal colon, PC: proximal colon, IL: ileum.

Animal	Site	GLUC	SOR-F	H-pattern	O-pattern	Ref. No.	#same O/H
02757	TR	+	+	H19NM	Or29	MCI0466	4/21
02757	TR	+	+	H36	O103	MCI0465	10/21
02757	TR	+	+	H8	Or30	MCI0467	2/21
02757	TR	-	-	NA	NA	NA	5/21
02757	PR	+	+	H19NM	Or29	MCI0470	5/21
02757	PR	+	+	H36	O103	MCI0469	11/21
02757	PR	+	+	H8	Or30	MCI0471	1/21
02757	PR	+	+	H29	Or59	NA	1/21
02757	PR	-	-	NA	NA	NA	4/21
02757	PC	+	+	H36	O103	MCI0473	13/21
02757	PC	+	+	H19NM	Or29	MCI0474	7/21
02757	PC	-	-	NA	NA	NA	1/21
02757	IL	+	+	H36	O103	MCI0476	5/21
02757	IL	+	+	H19NM	Or29	MCI0477	9/21
02757	IL	-	-	NA	NA	NA	7/21
04097	TR	+	+	H19NM	Or29	MCI0454	12/21
04097	TR	+	+	H8	Or30	MCI0455	4/21
04097	TR	+	+	H10	Or31	NA	1/21
04097	TR	-	-	NA	NA	NA	4/21
04097	PR	+	+	H19NM	Or29	MCI0458	20/21
04097	PR	+	+	H29	Or32	MCI0459	1/21
04097	PC	+	+	H19NM	Or29	MCI0460	14/21
04097	PC	+	+	H8	Or30	MCI0462	1/21
04097	PC	+	+	H7	Or33	MCI0461	1/21
04097	PC	-	-	NA	NA	NA	5/21
04097	IL	+	+	H19NM	Or29	MCI0463	5/21
04097	IL	+	+	H25	Or34	MCI0464	1/21
04097	IL	-	-	NA	NA	NA	14/2
600096	TR	+	-	H+(NT)1	O26	MCI0483	14/18
600096	TR	+	+	H20	Or35	MCI0487	4/18
600096	PR	+	-	H+(NT)1	O26	MCI0484	18/18

600096	PC	+	-	H+(NT)1	O26	MCI0485	12/18
600096	PC	-	-	NA	NA	NA	6/18
600096	IL	+	-	H+(NT)1	O26	MCI0486	5/11
600096	IL	-	-	NA	NA	NA	6/11
200927	TR	+	+	H+(NT)2	Or7	MCI0490	18/18
200927	PR	+	+	H+(NT)2	Or7	MCI0491	18/18
200927	DC	+	+	H+(NT)2	Or7	MCI0493	9/18
200927	DC	+	+	H47	Or36	MCI0495	1/18
200927	DC	-	-	NA	NA	NA	8/18
200927	PC	+	+	H+(NT)2	Or7	MCI0492	15/18
200927	PC	+	+	H47	Or36	MCI0494	1/18
200927	IL	-	-	NA	NA	NA	18/18
601223	TR	+	+	H+(NT)2	Or7	MCI0496	6/14
601223	TR	-	-	NA	NA	NA	8/14
601223	PR	+	+	H+(NT)2	Or7	MCI0497	16/18
601223	PR	-	-	NA	NA	NA	2/18
601223	DC	+	+	H+(NT)2	Or7	MCI0500	9/18
601223	DC	-	-	NA	NA	NA	9/18
601223	PC	+	+	H+(NT)2	Or7	MCI0498	9/18
601223	PC	-	-	NA	NA	NA	9/18
601223	IL	+	+	H+(NT)2	Or7	MCI0499	9/18
601223	IL	-	-	NA	NA	NA	9/18
300710	TR	+	+	H+(NT)2	Or7	MCI0501	3/18
300710	TR	-	-	NA	NA	NA	15/18
300710	PR	+	+	H+(NT)2	Or7	MCI0502	2/18
300710	PR	-	-	NA	NA	NA	15/18
300710	DC	+	+	H+(NT)2	Or7	MCI0505	3/10
300710	DC	-	-	NA	NA	NA	7/10
300710	PC	+	+	H+(NT)2	Or7	MCI0503	3/18
300710	PC	-	-	NA	NA	NA	15/18
300710	IL	+	+	H+(NT)2	Or7	MCI0504	3/18
300710	IL	-	-	NA	NA	NA	15/18
300129	TR	+	+	H49	Or35	MCI0512	6/18
300129	TR	+	+	H+(NT)1	Or37	MCI0517	9/18
300129	TR	+	+	-	Or57	MCI0653	1/18
300129	PR	+	+	H49	Or35	MCI0513	12/18
300129	PR	÷	+	-	Or14	MCI0518	4/18
300129	PR	+	+	H5	Or7	MCI0521	1/18
300129	DC	+	+	H49	Or35	MCI0514	13/18
300129	DC	+	+	-	Or14	MCI0519	1/18
300129	DC	+	+	-	Or39	MCI0654	1/18
300129	DC	-	-	NA	NA	NA	2/18
300129	PC	+	+	H49	Or35	MCI0515	16/18
300129	PC	+	+	H+(NT)2	Or39	MCI0522	2/18
300129	IL	+	+	H49	Or35	MCI0516	16/18
300129	IL	+	+	-	Or14	MCI0520	1/18
300129	IL	-	-	NA	NA	NA	1/18
300136	TR	+	+	-	Or14	MCI0523	7/18
300136	TR	+	+	H+(NT)l	Or37	MCI0528	9/18
300136	TR	+	+	H+(NT)8	Or38	MCI0529	1/18
300136	TR	-	-	NA	NA	NA	1/18
300136	PR	+	+	-	Or14	MCI0524	16/18
300136	PR	+	+	H+(NT)8	Or38	MCI0530	1/18

300136	PR	-	-	NA	NA	NA	1/18
300136	DC	+	+	H+(NT)8	Or38	MCI0531	11/18
300136	DC	+	+	-	Or14	MCI0525	7/18
300136	DC	-	-	NA	NA	NA	2/18
300136	PC	+	+	-	Or14	MCI0526	14/18
300136	PC	+	+	H+(NT)8	Or38	MCI0532	3/18
300136	PC	-	-	NA	NA	NA	1/18
300136	IL	+	+	-	Or14	MCI0527	15/18
300136	IL	+	+	H+(NT)8	Or38	MCI0533	3/18
700140	TR	+	÷	H+(NT)1	Or37	MCI0534	4/18
700140	TR	+	+	H+(NT)2	Or39	MCI0535	14/18
700140	PR	+	+	H+(NT)2	Or39	MCI0536	18/18
700140	DC	+	+	H+(NT)2	Or39	MCI0537	16/18
700140	DC	+	+	H+(NT)9	Or26	MCI0540	1/18
700140	DC	-	-	NA	NA	NA	1/18
700140	PC	+	+	H+(NT)2	Or39	MCI0538	13/18
700140	PC	+	+	H47	Or36	MCI0541	5/18
700140	IL	+	+	H+(NT)2	Or39	MCI0539	16/18
700140	IL	+	+	H+(NT)3	Or40	MCI0543	1/18
700140	IL	+	+	-	Or36	NA	1/18
700217	TR	+	+	H25	Or41	MCI0544	8/18
700217	TR	+	+	H8	Or17	MCI0546	4/18
700217	TR	+	+	H2	O103	MCI0549	6/18
700217	TR	+	+	H8	Or17	MCI0550	1/18
700217	PR	+	+	H+(NT)2	Or7	MCI0551	2/18
700217	PR	+	+	H2	O103	MCI0555	3/18
700217	PR	+	+	-	~O147	MCI0657	1/18
700217	PR	+	+	H+(NT)2	Or7	MCI0556	2/18
700217	PR	+	+	H25	Or41	MCI0545	3/18
700217	PR	+	+	H2	Or10	MCI0557	2/18
700217	PR	-	-	NA	NA	NA	6/18
700217	DC	+	+	H+(NT)2	Or7	MCI0552	4/14
700217	DC	+	+	H16NM	Or42	MCI0561	2/14
700217	DC	+	+	H2	Or10	MCI0558	3/14
700217	DC	+	+	H+(NT)7	Or43	MCI0564	2/14
700217	DC	-	-	NA	NA	NA	3/14
700217	PC	+	+	H+(NT)2	Or7	MCI0553	4/18
700217	PC	+	+	H2	Or10	MCI0559	7/18
700217	PC	+	+	H16NM	Or42	MCI0562	6/18
700217	PC	+	+	H8	Or44	MCI0566	1/18
700217	IL	+	+	H2	Or10	MCI0560	6/18
700217	IL	+	+	H+(NT)2	Or7	MCI0554	2/18
700217	IL	+	+	H16NM	Or42	MCI0563	8/18
700217	IL	+	+	H+(NT)7	Or43	MCI0565	1/18
700217	IL	-	-	NA	NA	NA	1/18
400354	TR	+	+	H-	Or45	MCI0567	1/18
400354	TR	+	+	H+(NT)2	Or7	MCI0568	8/18
400354	TR	+	+	H2	Or46	MCI0573	3/18
400354	TR	+	+	H2	Or60	not assigned[a]	1/18
400354	TR	-	-	NA	NA	NA	5/18
400354	PR	+	+	H+(NT)2	Or7	MCI0569	11/18
400354	PR	+	+	H12	~O149	MCI0574	1/18
400354	PR	-	-	NA	NA	NA	6/18

400354	DC	+	+	H40	Or47	MCI0575	5/18
400354	DC	+	+	H+(NT)2	Or7	MCI0570	1/18
400354	DC	+	+	H+(NT)4	Or48	MCI0578	3/18
400354	DC	-	-	NA	NA	NA	9/18
400354	PC	+	+	H+(NT)2	Or7	MCI0571	11/18
400354	PC	+	+	H40	Or47	MCI0576	1/18
400354	PC	-	-	NA	NA	NA	6/18
400354	IL	+	+	H40	Or47	MCI0577	6/18
400354	IL	+	+	H+(NT)2	Or7	MCI0572	3/18
400354	IL	+	+	NA	NA	NA	1/18
400354	IL	+	+	H5	-	MCI0579	1/18
400354	IL	+/-	+/-	H+(NT)2	Or7	MCI0580	1/18
400354	IL	-	-	NA	NA	NA	6/18
301437	TR	+	+	H40	Or47	MCI0581	16/18
301437	TR	+	+	H12	~O149	MCI0586	1/18
301437	TR	-	-	NA	NA	NA	1/18
301437	PR	+	+	H40	Or47	MCI0582	15/18
301437	PR	+	+	H5	Or49	MCI0589	1/18
301437	PR	+/-	+	H16NM	Or50	MCI0590	1/18
301437	PR	-	-	NA	NA	NA	1/18
301437	DC	+	+	H40	Or47	MCI0583	15/18
301437	DC	+	+	H12	~O149	MCI0587	1/18
301437	DC	+	+	-	Or14	MCI0591	1/18
301437	DC	+	+	-	Or51	MCI0593	1/18
301437	PC	+	+	-	Or14	MCI0592	1/18
301437	PC	+	+	H40	Or47	MCI0584	17/18
301437	IL	+	+	H40	Or47	MCI0585	11/18
301437	IL	+	+	H12	~O149	MCI0588	1/18
301437	IL	-	-	NA	NA	NA	6/18
401473	TR	+	+	H25	Or41	MCI0594	11/18
401473	TR	+	+	H2	Or10	MCI0595	2/18
401473	TR	+	+	H2	Or46	MCI0597	1/18
401473	TR	-	-	NA	NA	NA	4/18
401473	PR	+	+	H2	Or46	MCI0598	6/18
401473	PR	+	+	H+(NT)2	Or52	MCI0602	8/18
401473	PR	+	-	H16NM	Or42	MCI0606	1/18
401473	PR	+	+	-	Or46	MCI0608	1/18
401473	PR	+	+	H9	Or53	MCI0611	1/18
401473	PR	-	-	NA	NA	NA	1/18
401473	DC	+	+	H+(NT)2	Or52	MCI0603	13/18
401473	DC	+	+	H2	Or46	MCI0599	3/18
401473	DC	+	+	H16NM	Or42	MCI0607	1/18
401473	DC	+	+	-	Or46	MCI0609	1/18
401473	PC	+	+	H+(NT)2	Or52	MCI0604	10/18
401473	PC	+	+	H2	Or46	MCI0600	3/18
401473	PC	+	+	H9	Or53	MCI0612	2/18
401473	PC	+	+	H2	Or10	MCI0596	2/18
401473	PC	+	+	-	Or46	MCI0610	1/18
401473	IL	+	+	-	Or52	MCI0613	2/18
401473	IL	+	+	H+(NT)2	Or52	MCI0605	11/18
401473	IL	+	+	H2	Or46	MCI0601	3/18
401473	IL	+	+	H8	Or17	MCI0659	1/18
401473	IL	+	+	-	O5/O113	MCI0614	1/18

200522	TR	+	+	H+(NT)2	Or7	MCI0615	11/18
200522	TR	+	+	-	Or52	MCI0619	1/18
200522	TR	+	+	H+(NT)2	Or52	MCI0660	4/18
200522	PR	+	+	H+(NT)2	Or52	MCI0621	12/18
200522	PR	+	+	H+(NT)2	Or7	MCI0661	5/18
200522	PR	-	-	NA	NA	NA	1/18
200522	DC	+	+	H+(NT)2	Or52	MCI0622	8/18
200522	DC	+	+	H25	Or54	MCI0625	1/18
200522	DC	+	+	H+(NT)2	Or7	MCI0616	7/18
200522	DC	+	+	H5	Or48	MCI0626	1/18
200522	DC	+	+	H2	Or46	MCI0627	1/18
200522	PC	+	+	H+(NT)2	Or7	MCI0617	9/18
200522	PC	+	+	H+(NT)2	Or52	MCI0623	8/18
200522	PC	-	-	NA	NA	NA	1/18
200522	IL	+	+	H+(NT)2	Or7	MCI0618	5/18
200522	IL	+	+	H+(NT)2	Or52	MCI0624	4/18
200522	IL	-	-	NA	NA	NA	9/18

Table A2: Bovine *E. coli* strains isolated by necropsy sampling during this study: Numbers of each O/H pattern combination are given as a fraction of the total number of *E. coli* colonies picked from each site (# same O/H).
Isolation site: TR: terminal rectum, PR: proximal rectum, DC: distal colon, PC: proximal colon, IL: ileum.
GLUC: glucuronidase positive (+)/negative (-); SOR-F: sorbitol-fermenting(+)/non-fermenting (-), O-pattern: O-RFLP pattern, H-pattern: H-RFLP-pattern. NA: was not culturable in LB and therefore discounted from the final analysis).
Notes
*: unfortunately, this strain was lost from the collection.
a: This strain was omitted from PCR-screening for virulence factors, although as the only representative of this O/H pattern from this animal, it would have been discounted from the analysis presented in Section 4.2.3 regardless.

Animal	Isolation	Colony	GLUC	SOR-F	H-pattern	O-pattern	Ref. No.
700217	RS	1	+	ND	H8	Or17	MCI 0628
700217	RS	2	+	ND	H+(NT)5	Or55	MCI 0629
700217	RS	3	+	ND	H8	Or17	MCI 0630
400354	RS	1	+	ND	H+(NT)5	Or55	MCI 0631
400354	RS	2	+	ND	H2	Or56	MCI 0632
400354	RS	3	+	ND	H2	Or56	MCI 0633
301437	RS	1	+	ND	H+(NT)6	O80	MCI 0634
301437	RS	2	+	ND	H+(NT)6	O80	MCI 0635
301437	RS	3	+	ND	H+(NT)6	O80	MCI 0636
301437	RS	4	+/-	ND	H16NM	Or50	MCI 0637
401473	RS	1	+	ND	H+(NT)5	Or55	MCI 0638
401473	RS	2	+	ND	H+(NT)5	Or55	MCI 0639
401473	RS	3	+	ND	H16NM	Or42	MCI 0640
200522	RS	1	+	ND	H25	Or54	MCI 0641
200522	RS	2	+	ND	H+(NT)5	Or55	MCI 0642
200522	RS	3	+	ND	H+(NT)5	Or55	MCI 0643
200522	RS	4	+/-	ND	H2	-	MCI 0644

Table A3: Summary of *E coli* isolated by rectal swab three weeks prior to necropsy sampling: (Gluc: glucuronidase positive/negative, SorF: sorbitol-fermenting/non-fermenting, O-pattern: O-RFLP pattern, H-pattern: H-RFLP-pattern).

Appendix III Molecular Serotyping Data

Supporting images for the assignation of serotype by molecular method (O-RFLP and H-RFLP; Section 4.2.1.2) are given in Figures A2-A7. H-RFLP patterns were matched to expected patterns given in Table A4.

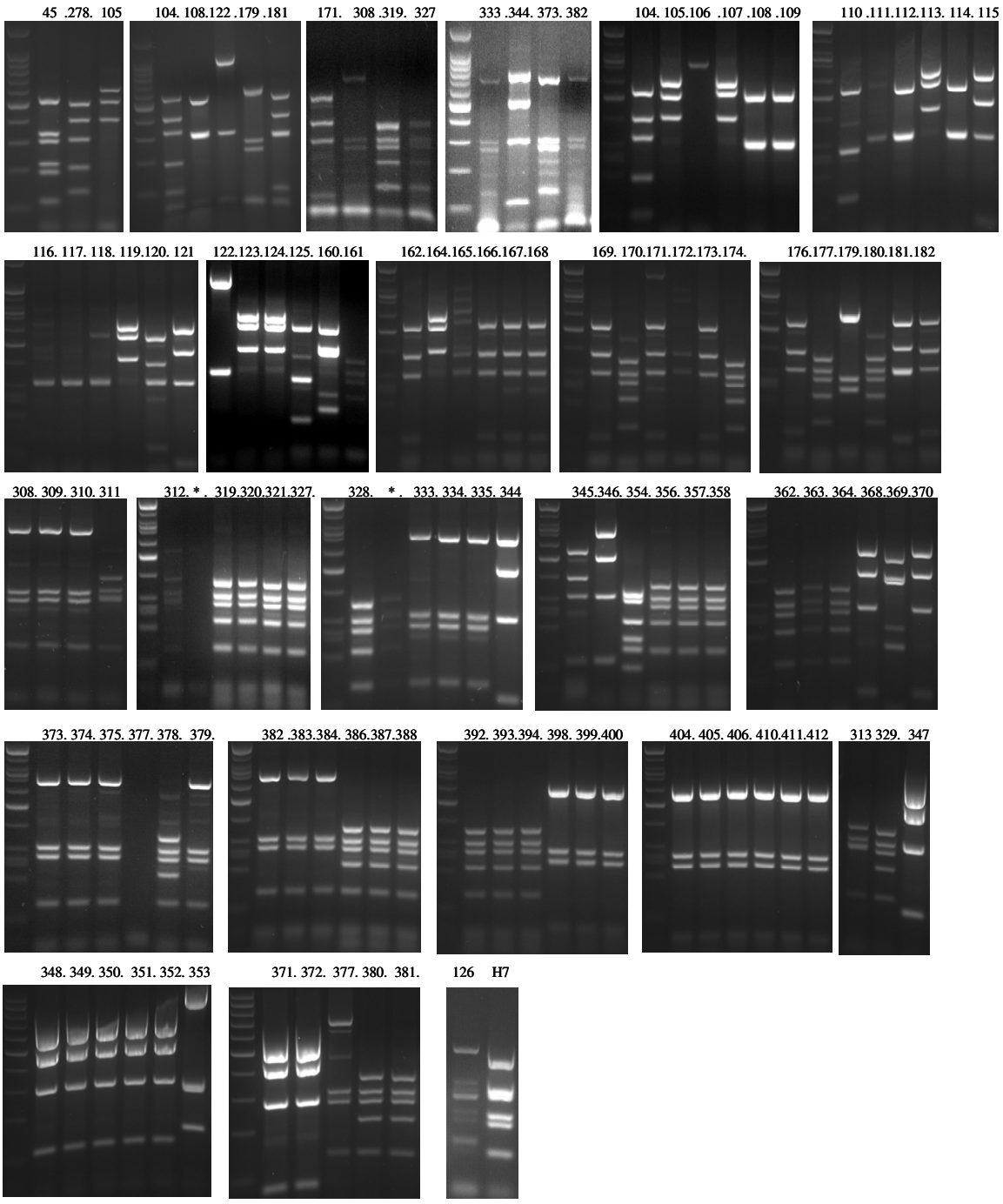


Figure A2: H-RFLP patterns of bovine isolates provided by Neil Paton and Stuart Naylor: Each number corresponds to the MCI reference numbers given in Table A1.

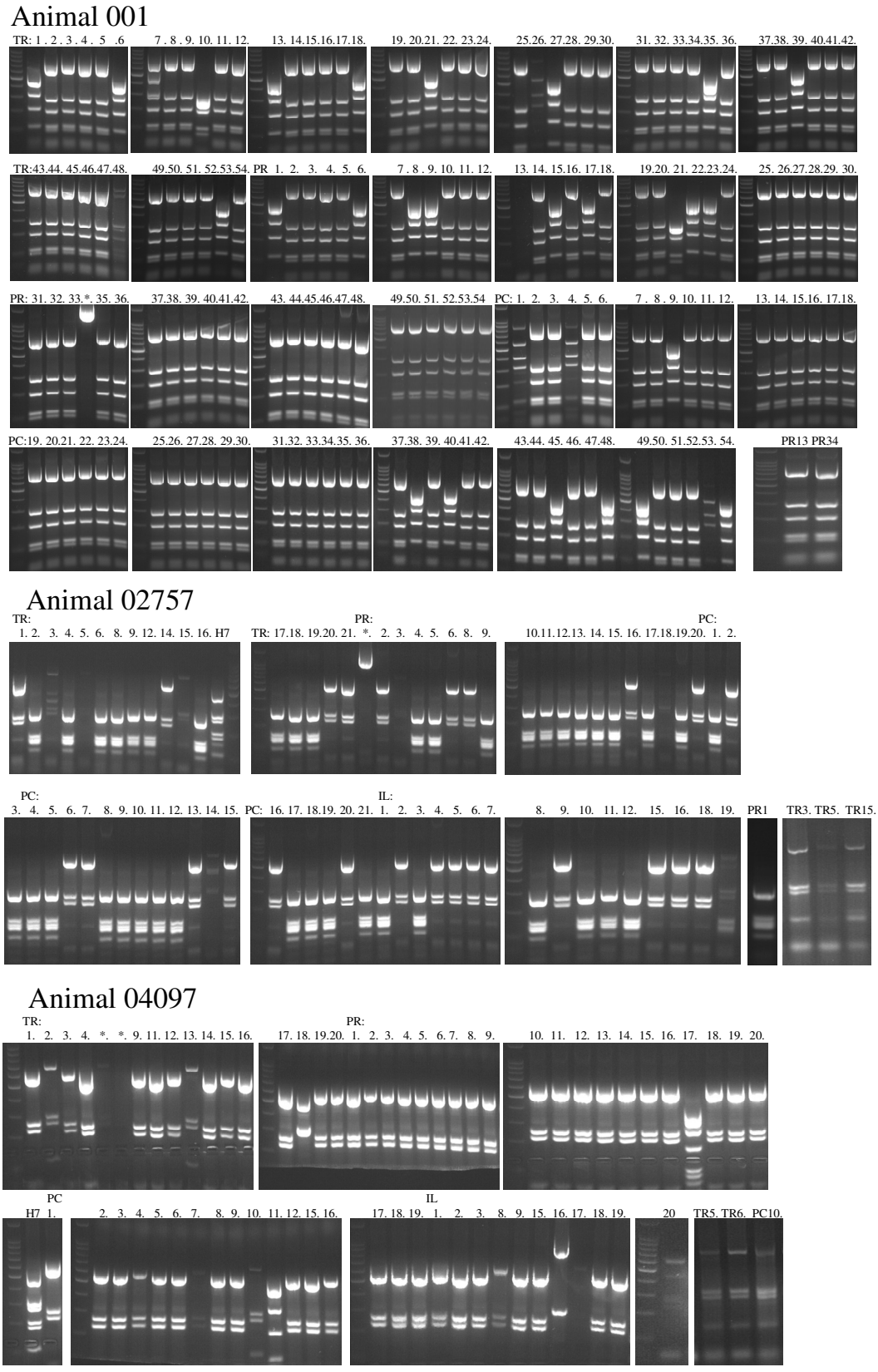


Figure A3 (part 1): H-RFLP patterns of bovine *E. coli* strains isolated during this study

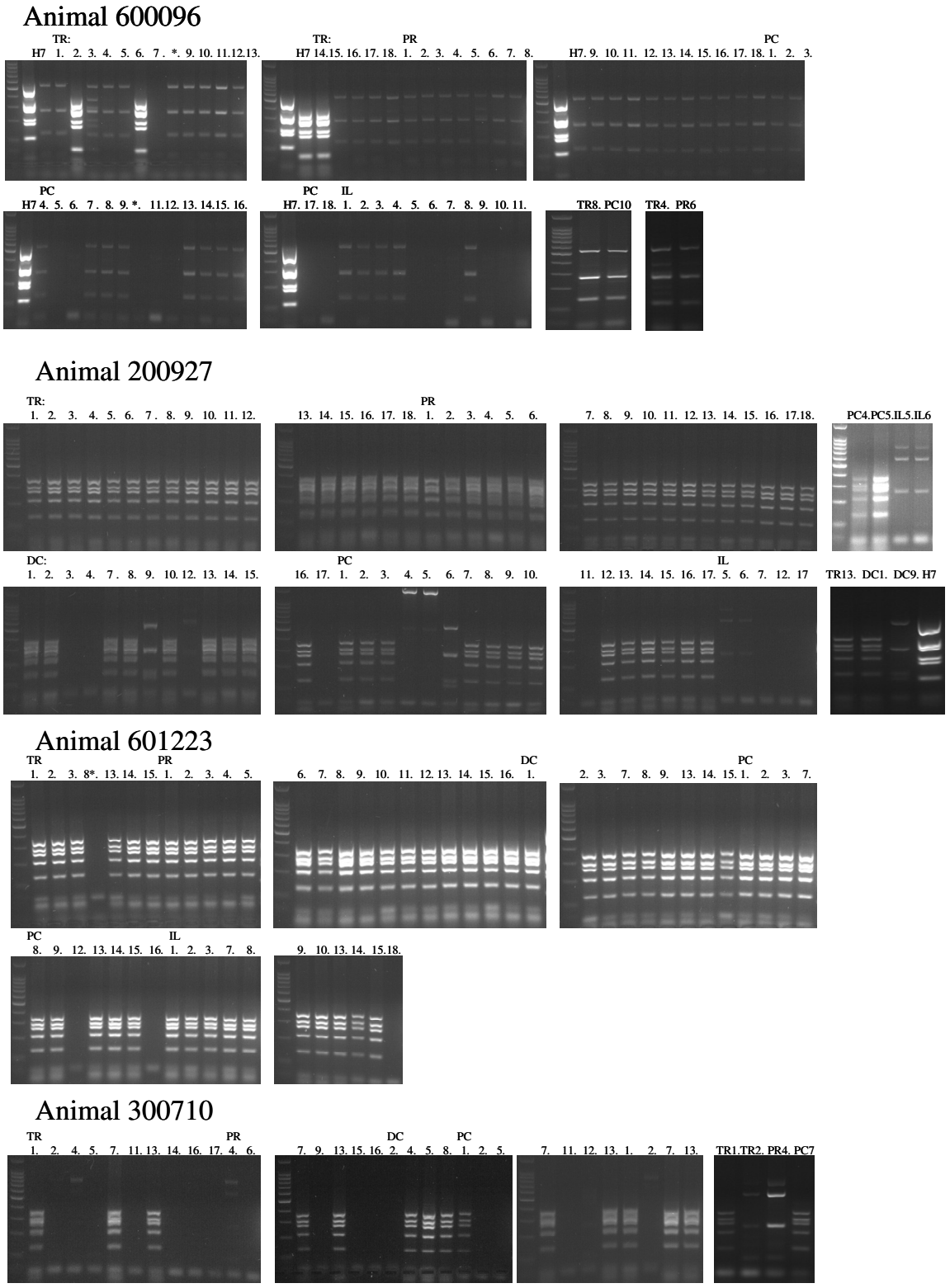


Figure A3 (part 2): H-RFLP patterns of bovine *E. coli* strains isolated during this study

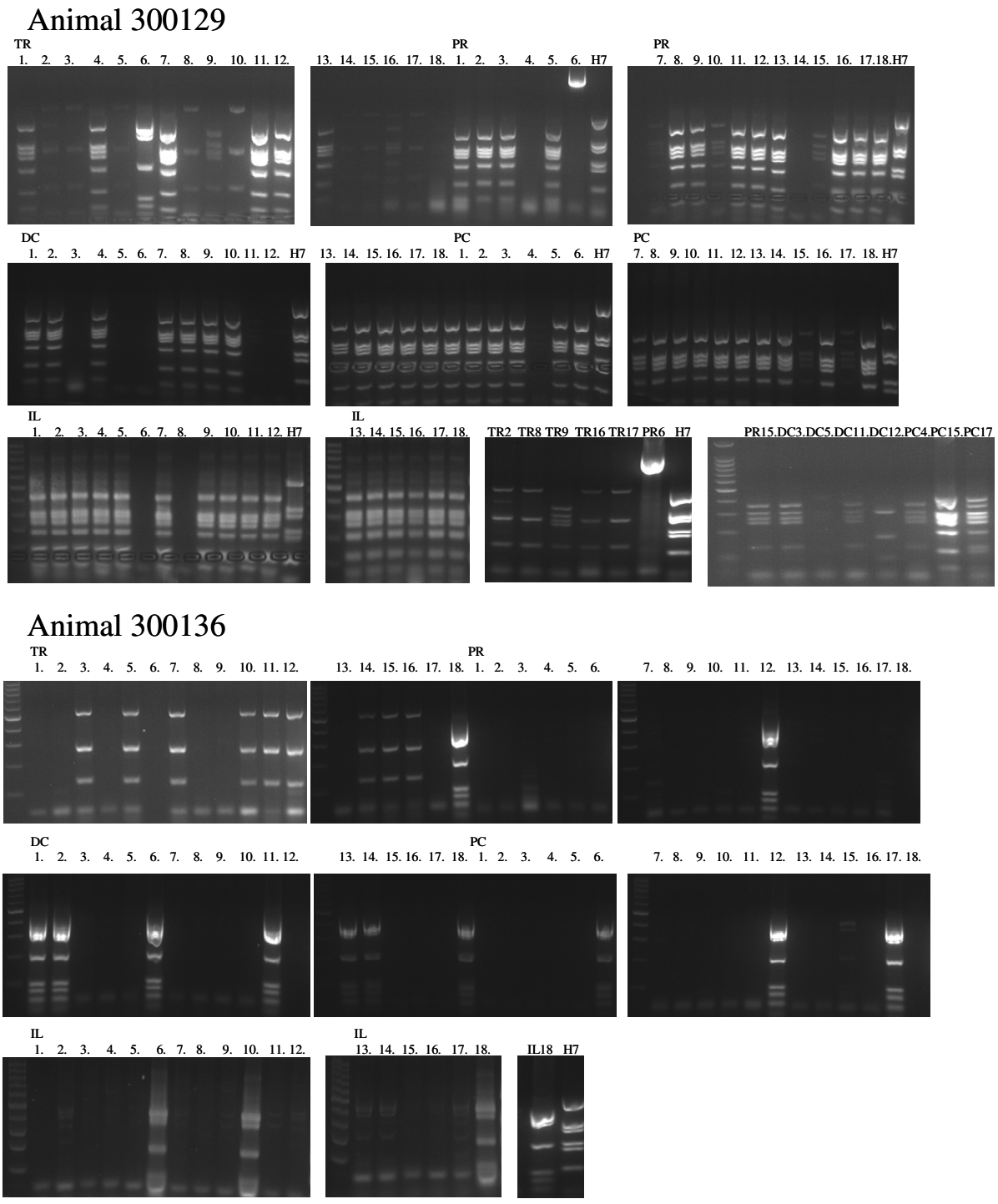


Figure A3 (part 3): H-RFLP patterns of bovine *E. coli* strains isolated during this study

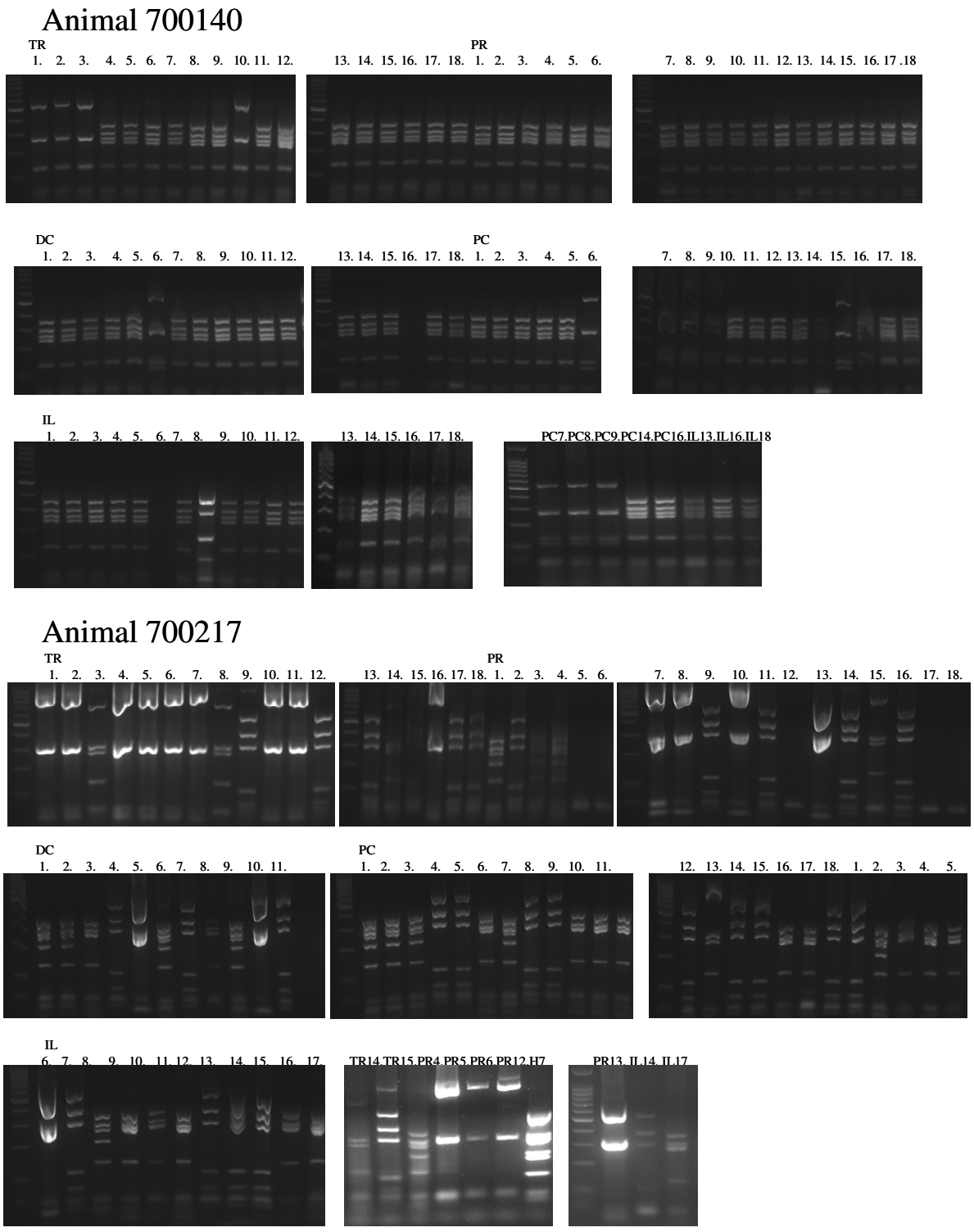


Figure A3 (part 4): H-RFLP patterns of bovine *E. coli* strains isolated during this study

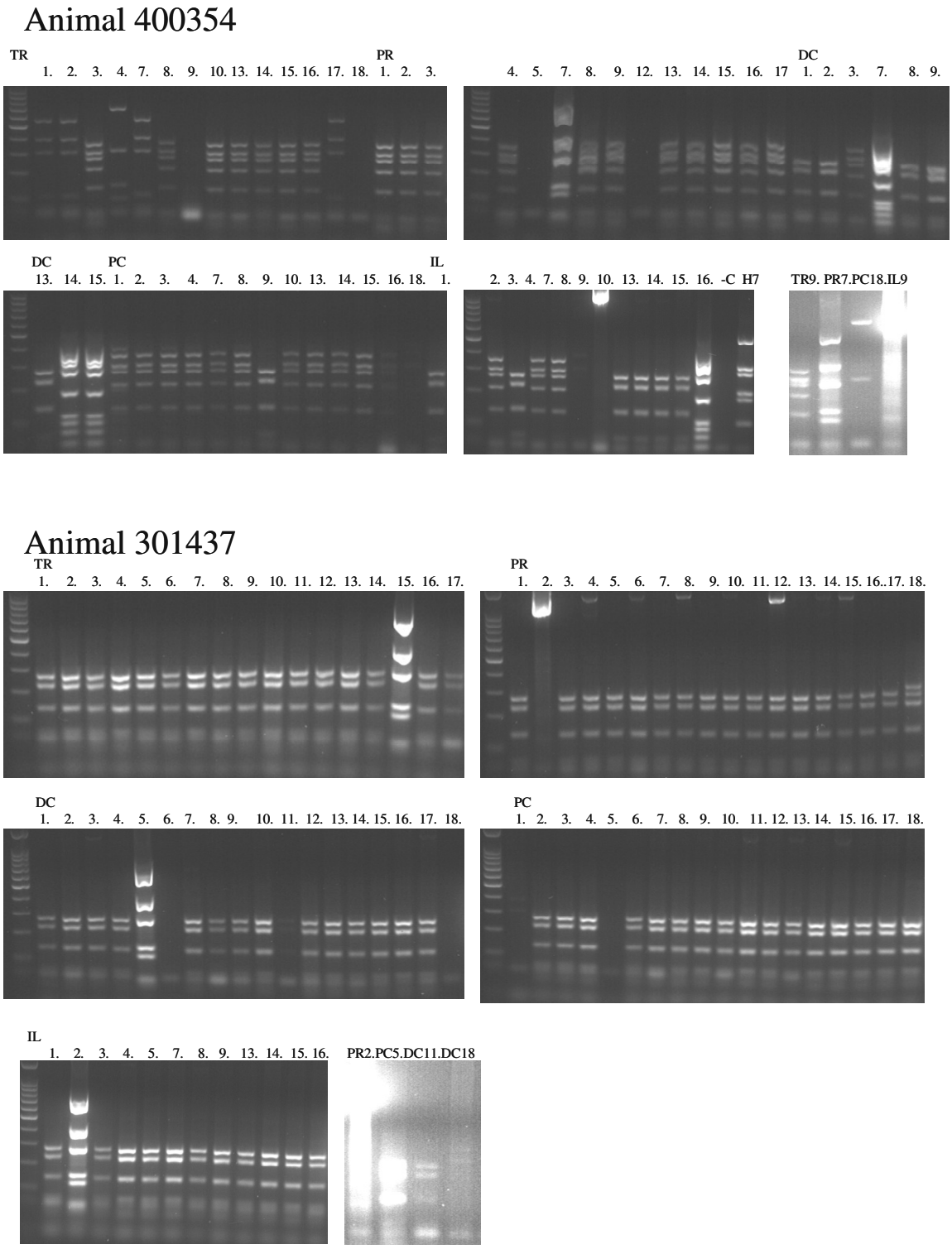


Figure A3 (part 5): H-RFLP patterns of bovine *E. coli* strains isolated during this study

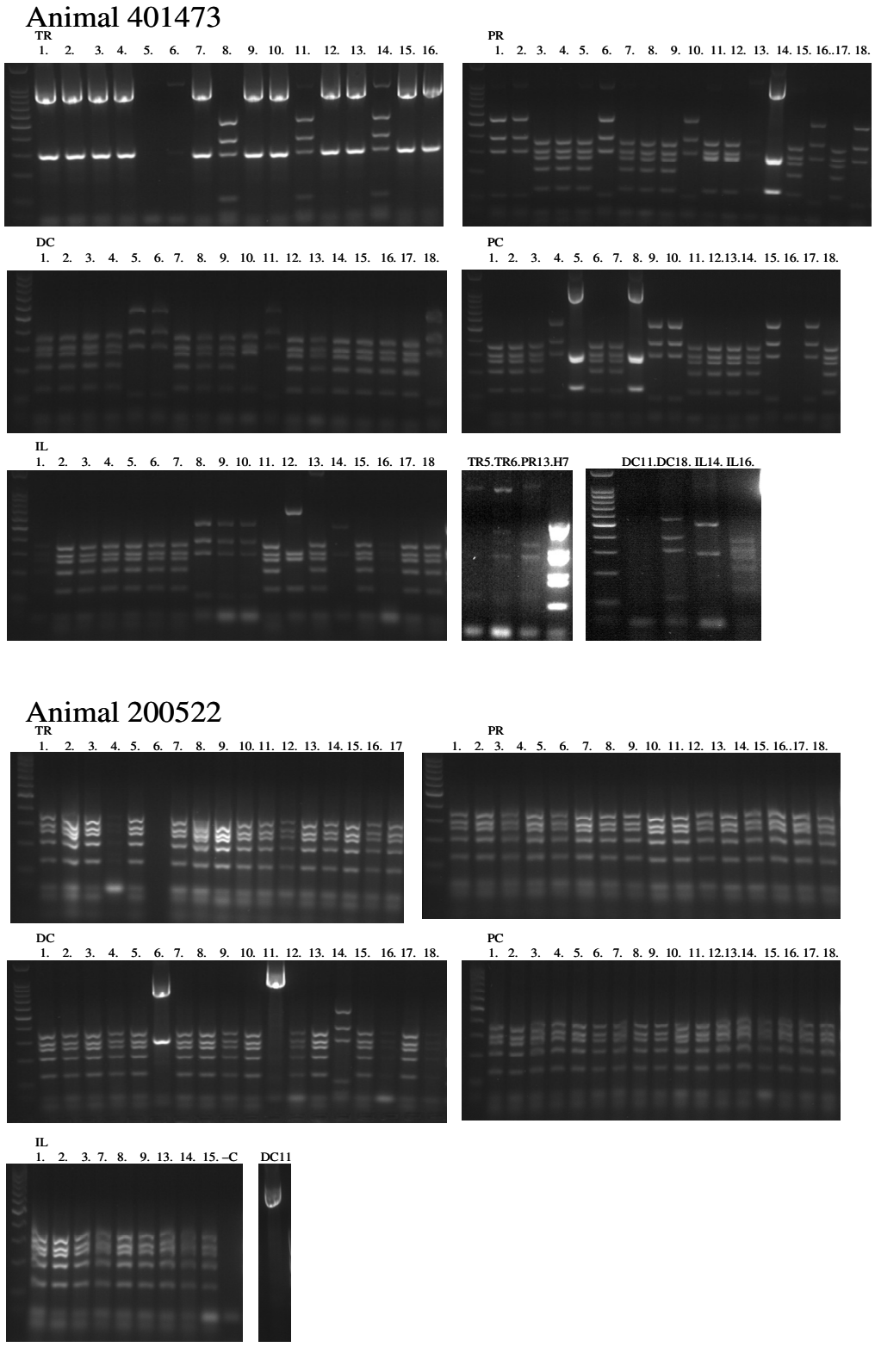


Figure A3 (part 6): H-RFLP patterns of bovine *E. coli* strains isolated during this study

Figure A3: H-RFLP patterns of bovine *E. coli* strains isolated during this study (shown in 6 parts above). Isolation site is given above the colony number, which was assigned arbitrarily to each colony recovered from each site. Isolation sites were: TR: terminal rectum, PR: proximal rectum, DC: distal colon, PC: proximal colon, IL: ileum.

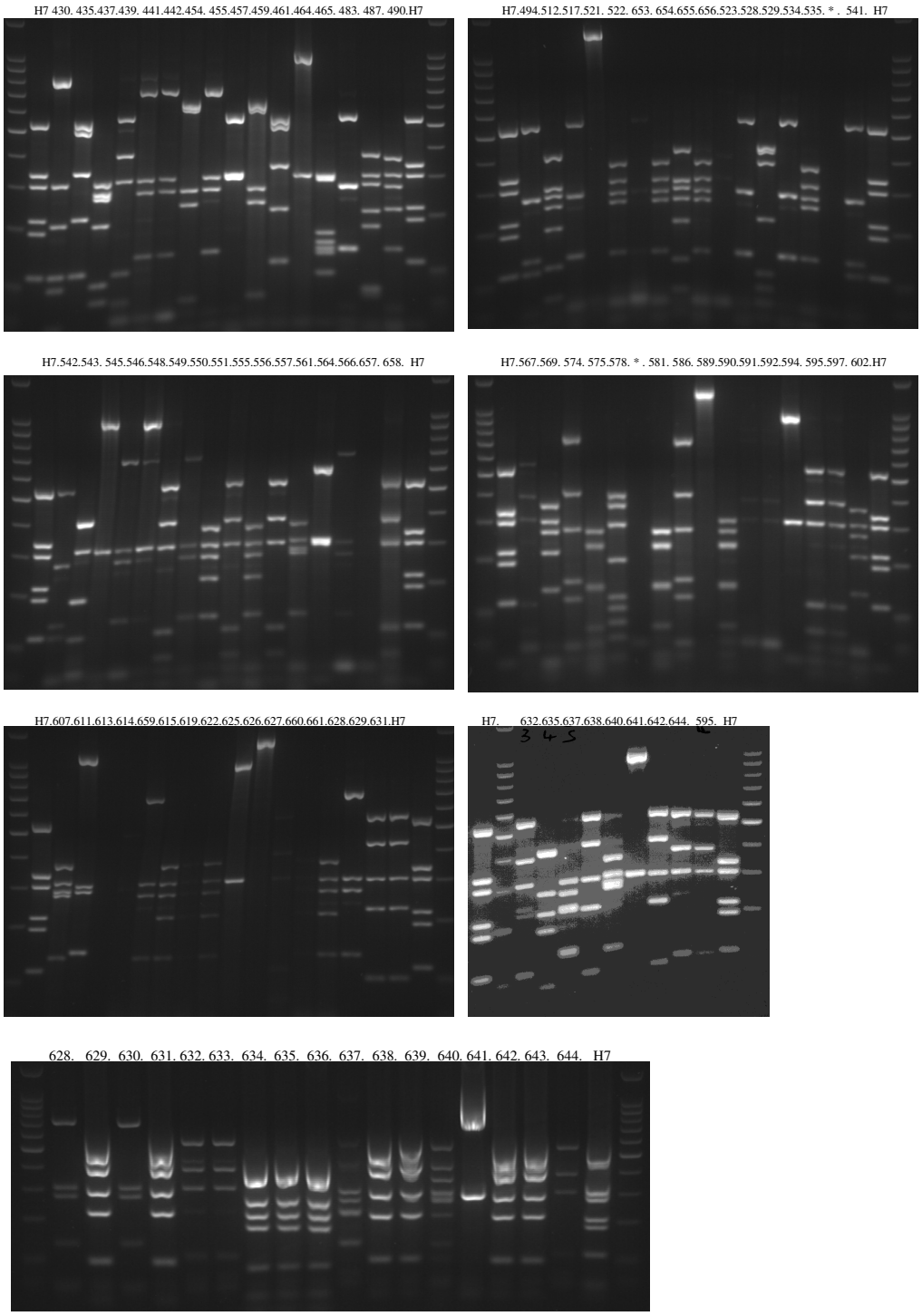


Figure A4: All H-RFLP patterns identified in bovine *E. coli* isolated during this study: visualised on 18cm agarose gels for greater resolution in order to match observed patterns to a database of expected RFLP fragment sizes (Table A4).

H-TYPE	Pattern	Expected fragment sizes (bp)							
H1	1	726	410	281	210	127	34		
H1	M	740	420	290	170	140			
H2	1	546	391	306	116	75	41	22	
H2	M	550	400	310	160	120			
H3	M	390	340	300	290	160	150		
H4	1	495	359	84	78	34			
H4	2	495	293	84	78	66	34		
H4	M	490	290						
H5	1	1311							
H5	2	1256	24	31					
H5	M	1300							
H6	1	570	410	316	209	142			
H6	M	510	410	320	220	150			
H7	1	537	341	308	222	196	120	34	
H7	2	410	341	308	222	196	127	120	34
H7	3	537	341	308	222	196	120	77	
H7	M1	410	350	310	230	210	140	130	
H7	M2	540	500	350	230	130			
H8	M	710	320	290	170	150			
H9	1	1077	305	288	153	150	34	6	
H9	M	1120	310	300	160				
H10	1	427	318	306	31	30			
H10	M	520	330	310					
H10NM	1	544	318	306	34	31	30		
H11	M	540	280	170	150	130			
H11NM	1	539	276	275	142	141	41		
H12	1	726	410	281	156	127	54	34	
H12	M	740	420	290	170	140			
H14	1	1035	557	34	27				
H14	2	625	557	410	34	27			
H14	M	610	560	420					
H15	1	437	312	306	286	213	78	34	27
H15	M	440	320	280	220				
H16	M	390	340	300	290	160	150		
H16NM	1	372	318	290	282	142	41	34	26
H18	1	741	557	195	105	34	33		
H18	M	760	560	200	110				
H19NM	1	620	615	273	238	71	34		
H19	1	629	597	238	71	34			
H19	M1	590	410	280	240	200			
H19	M2	630	590	280	240				
H20	1	377	306	285	215	183	80	78	72
H20	M	380	310	290	220	190			
H21	M1	380	320	290	280	170	150		
H21	M2	380	320	290	230	170	150		
H21	M3	380	320	290	260	170	150		
H23	1	479	288	282	275	207	133	38	34
H23	M	480	290	270	200	130			
H24	1	370	320	290	280	160	140		
H25	M	900	300						

H26/H26NM	1	841	545	138	75	41	34		
H26	M	520	320	140					
H27	1	533	276	275	142	120			
H27	M	540	280	170	150	130			
H28	1	413	410	318	190	186	114	41	
H28	M	400	310	180	150				
H29	1	337	306	225	171	146	104	34	
H29	2	380	337	306	171	104	34		
H29	M	380	330	300	170	100			
H30	1	562	310	407	298	102	34		
H30	M	550	390	300	290	90			
H31	1	553	434	410	175	62	34		
H31	M	540	420	390	220	170			
H32	1	743	513	306	117	34			
H32	M	750	520	320	120				
H33	1	656	412	88	79	52			
H33	M	640	400						
H34	1	622	506	410	66	34			
H34	M	620	510	390					
H35	1	546	391	306	116	75	41	22	
H35	M	540	390	320	300				
H36	M	680	540	280	210	140	100		
H37	1	806	306	211	120	104	53	43	34
H37	M	820	310	220	130	110			
H38/H38NM	1	306	300	171	154	141	134	104	34
Moreno	M	310	180	160	150	140	110		
H39	1	306	282	271	205	104	80	27	18
H39	M	320	280	270	210				
H40	M	290	250	160					
H41	1	621	490	261	204	70	34		
H41	M	620	490	260	200				
H42/H42NM	1	620	321	306	34				
H42	M	620	330	310					
H43	1	392	347	304	290	125	48		
H43	M	400	360	310	300	130			
H44	1	356	306	301	245	205	75	60	48
H44	M	720	600	500	330	300	210	130	
H45	1	424	410	368	214	107	86		
H45	M	420	310	270	240	190	110		
H46	1	476	306	285	237	187	104	72	34
H46	M	460	310	270	240	190	110		
H47	M	550	280	170	150				
H48	1	614	456	290	87	34	16		
H48	M	600	460	280	80				
H49	1	410	306	282	257	201	127	78	34
H49	2	675	340	308	283	257	201	127	78
H49	M	420	310	290	260	210	130		
H51	1	335	290	249	231	192	138	132	102
H51	M	350	290	250	230	200	140	130	100
H52	1	689	370	171	80	34			
H52	M	690	370	170					
H55	M	1300							

H56	1	901	306	104
H56	M	910	300	100

Table A4: Expected H-RFLP fragment sizes: predicted sizes (in basepairs) of theoretical *fliC* amplicon digested with *Rsa*I restriction enzyme are shown. Those predicted *in silico* using sequences available on the NCBI nucleotide database are assigned a number, whereas those given in Ramos Moreno *et al.* (2006) are denoted "M".

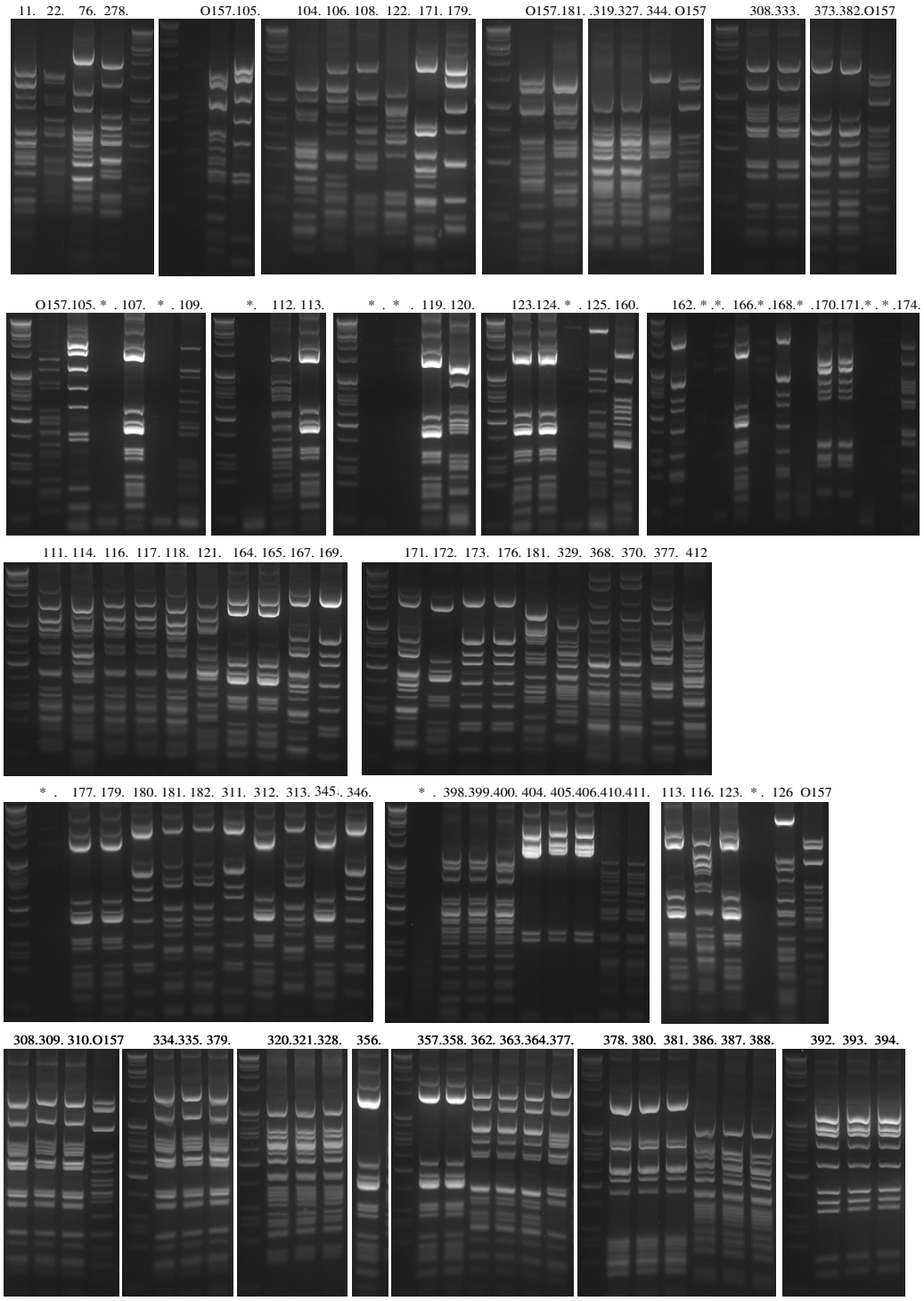


Figure A5: O-RFLP patterns of bovine isolates provided by Neil Paton and Stuart Naylor: Each number corresponds to the MCI reference numbers given in Table A1.

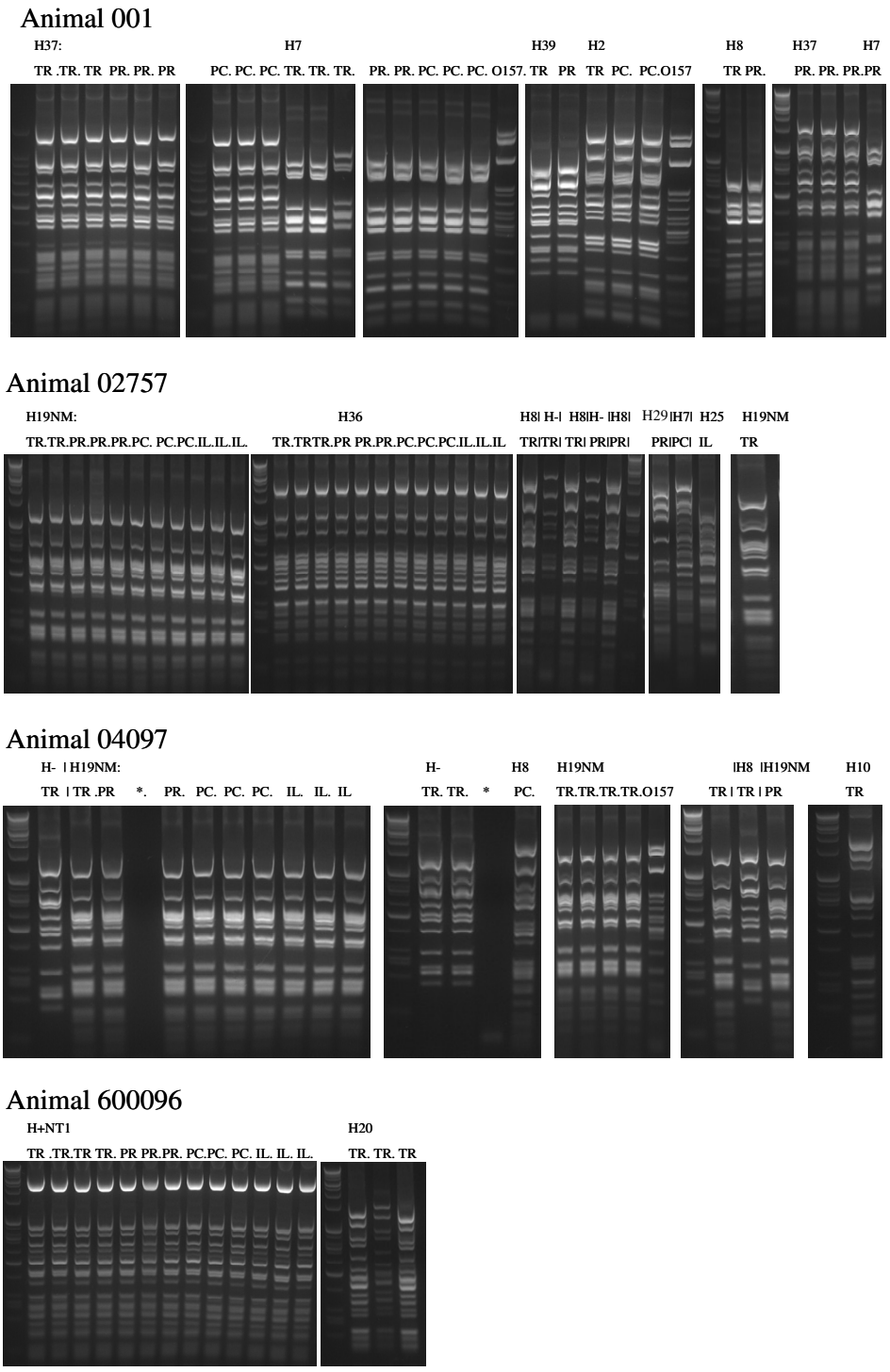


Figure A6 (part 1): O-RFLP patterns of bovine *E. coli* strains isolated during this study

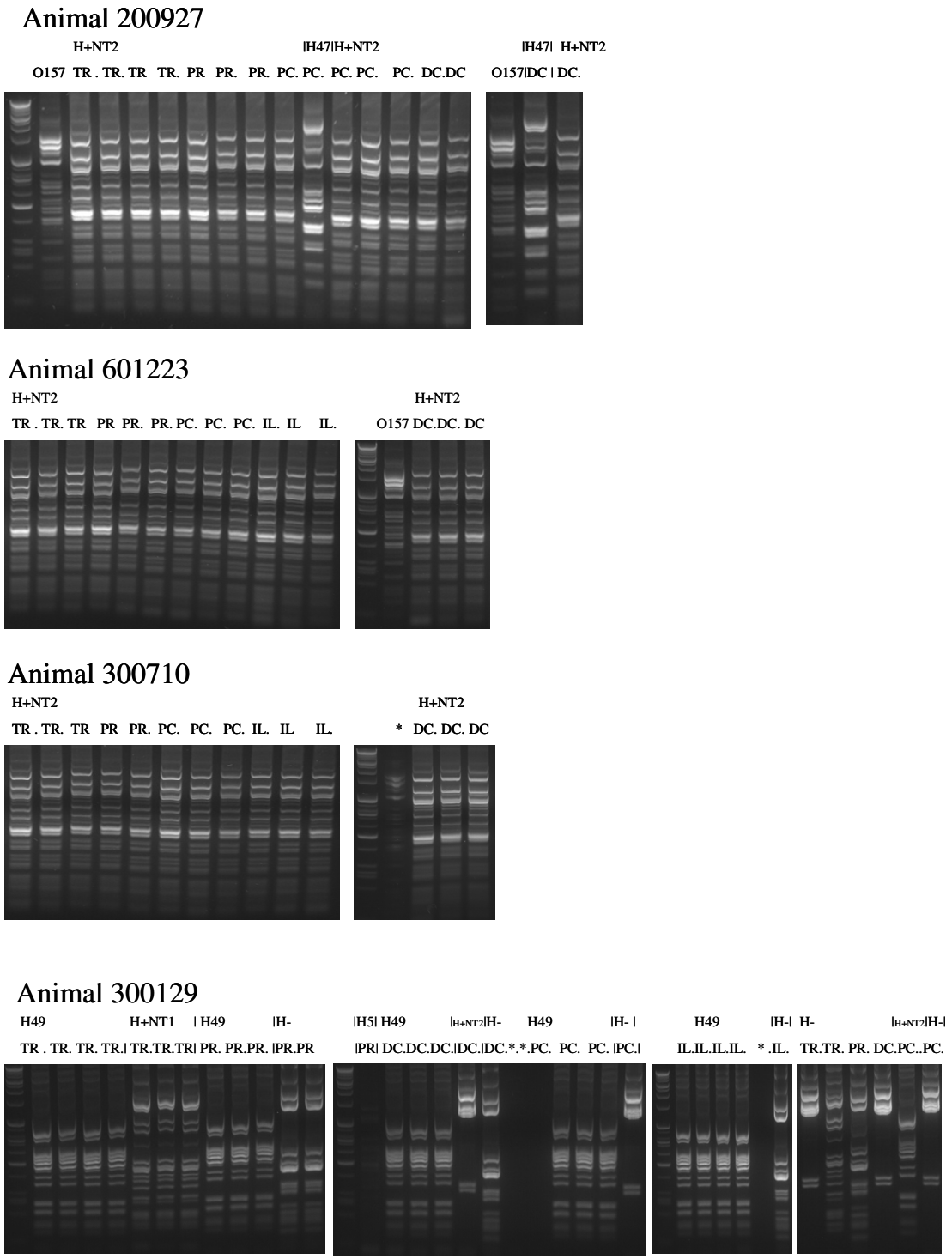


Figure A6 (part 2): O-RFLP patterns of bovine *E. coli* strains isolated during this study

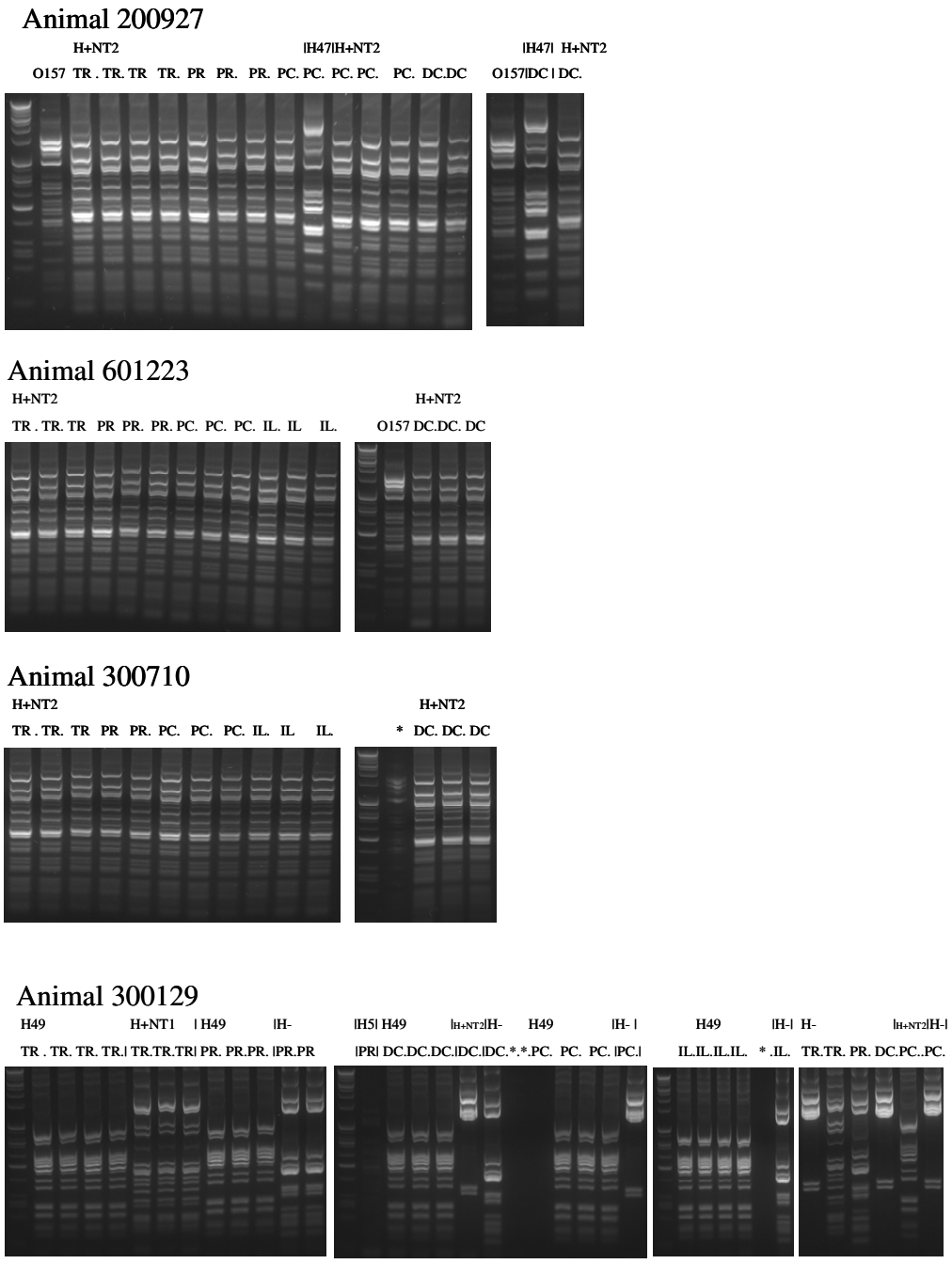


Figure A6 (part 3): O-RFLP patterns of bovine *E. coli* strains isolated during this study

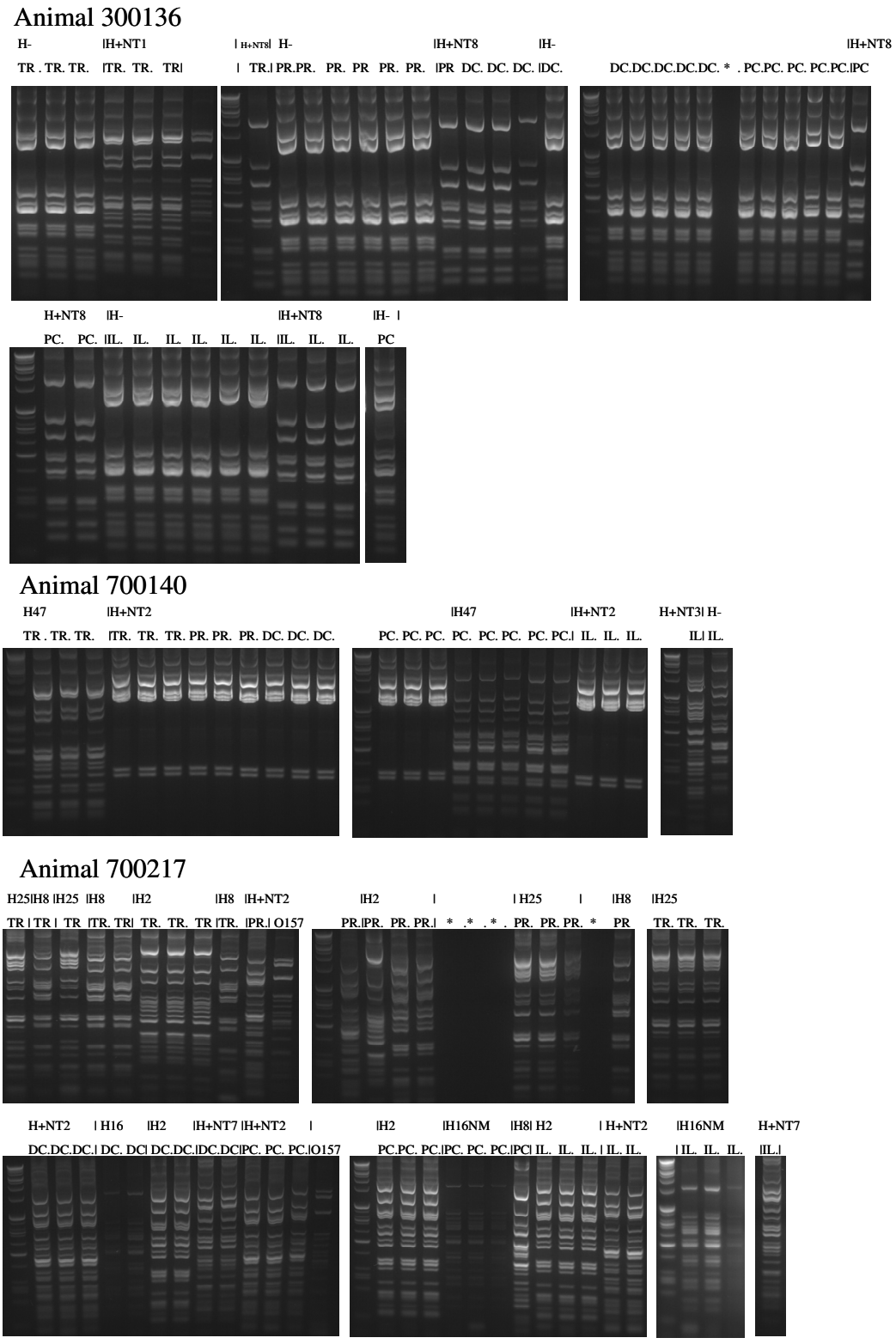


Figure A6 (part 4): O-RFLP patterns of bovine *E. coli* strains isolated during this study

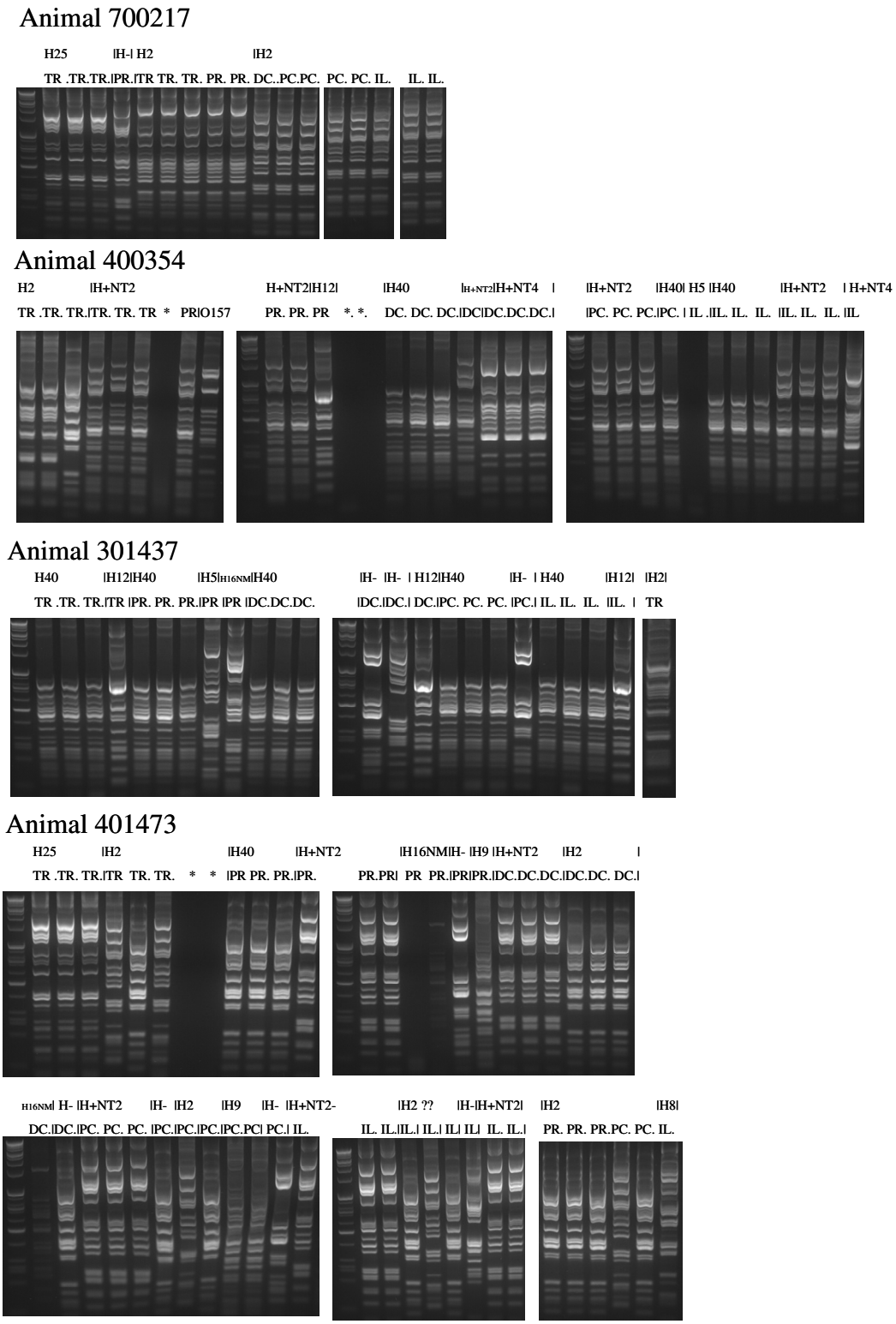


Figure A6 (part 5): O-RFLP patterns of bovine *E. coli* strains isolated during this study

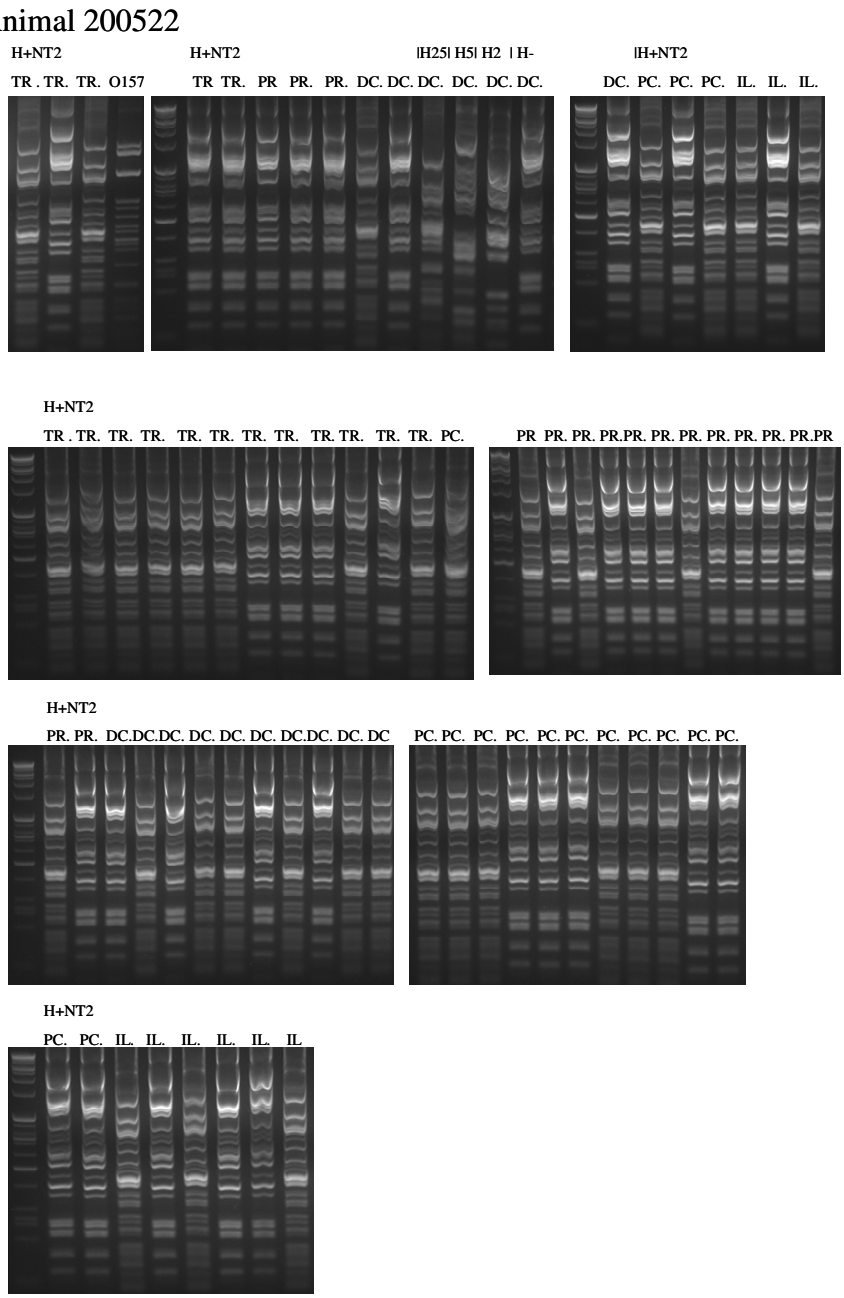


Figure A6 (part 6): O-RFLP patterns of bovine *E. coli* strains isolated during this study

Figure A6: O-RFLP patterns of bovine *E. coli* strains isolated during this study: For each animal from which *E. coli* was isolated, O-RFLP was conducted according to Section 2.6.4.1 and Coimbra *et al* (2000). Restriction fragments were visualised by agarose gel electrophoresis and are shown in 6 parts in previous pages. H-RFLP pattern and then the site from which the strain was isolated are given above each lane.

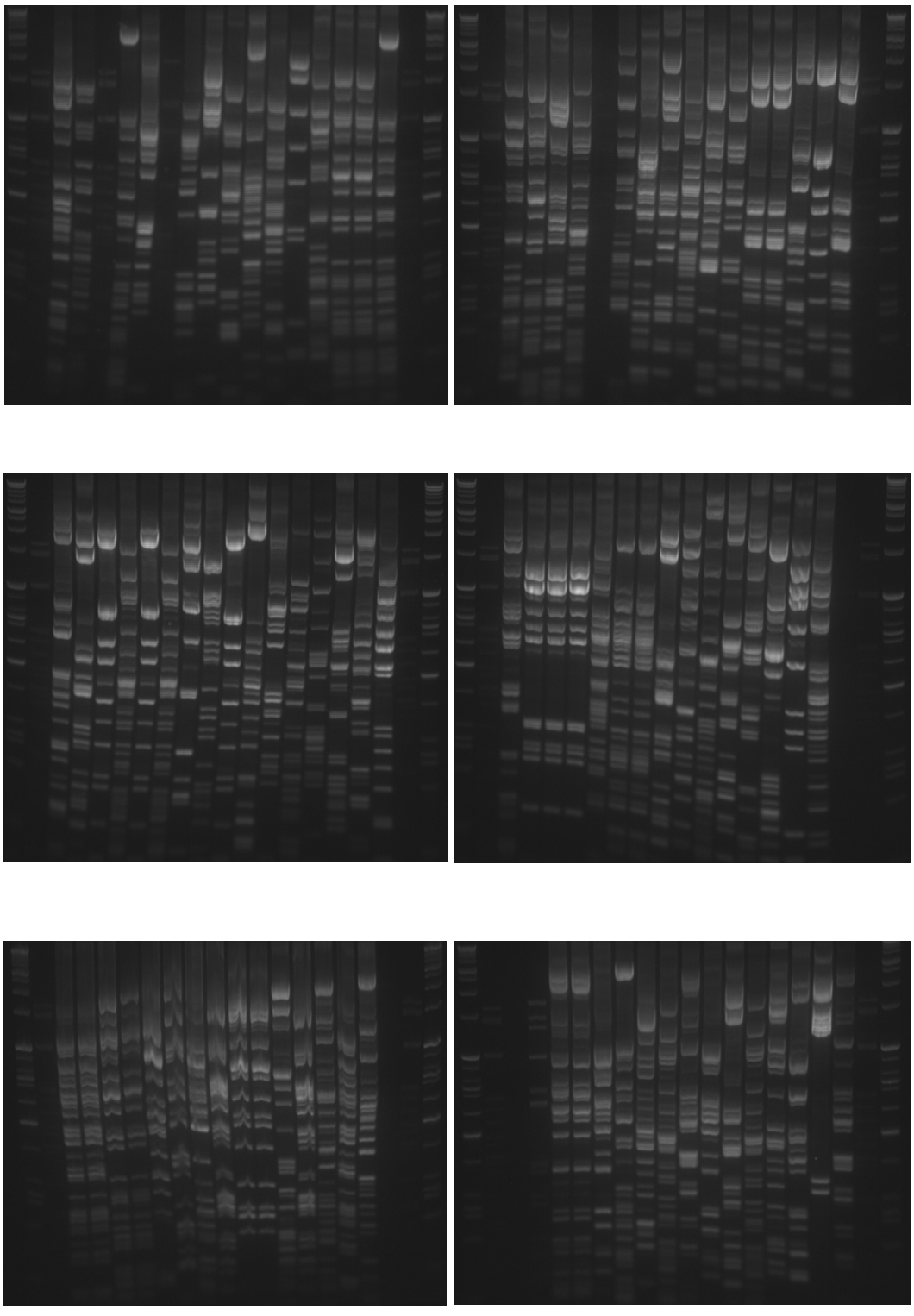


Figure A7 (part 1): All O-RFLP patterns identified during this study.

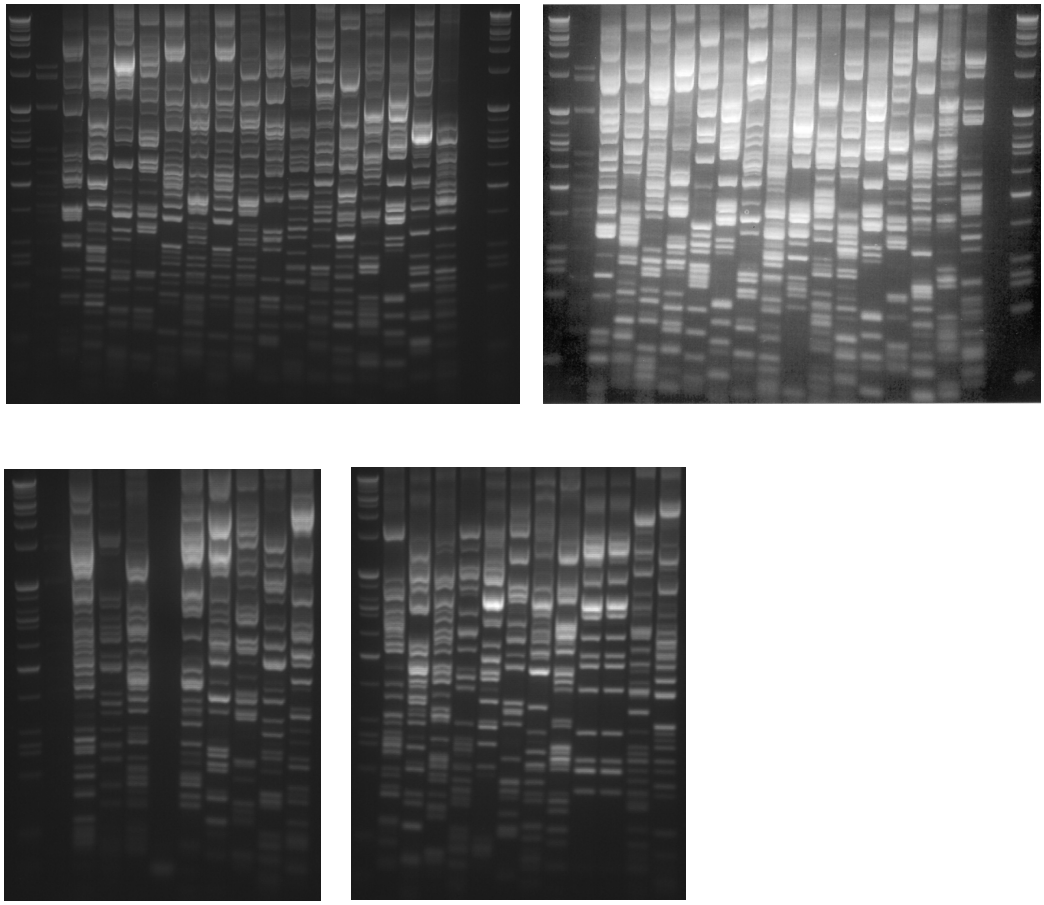


Figure A7: (part 2): All O-RFLP patterns identified during this study.

Figure A7: All O-RFLP patterns identified during this study: All O-RFLP patterns identified in bovine *E. coli* isolated during this study, visualised on 18cm agarose gels for greater resolution and to enable pattern-matching between strains. Numbers above each lane correspond to MCI reference numbers. O-RFLP patterns were assigned arbitrary numbers (unless patterns matched those of *E. coli* of known serotype, given in Table 2.1).

Appendix IV PCR-screening of bovine *E. coli* isolates

Images showing the results of the PCR-screening of selected bovine *E. coli* are included in Figures A8 and A9. Strains are labelled with MCI number (corresponding to Tables A1-A3) in the top row of each figure, with the target gene (Section 4.2.1.3) given to the right. These results are summarised in Tables 4.2 and 4.5.

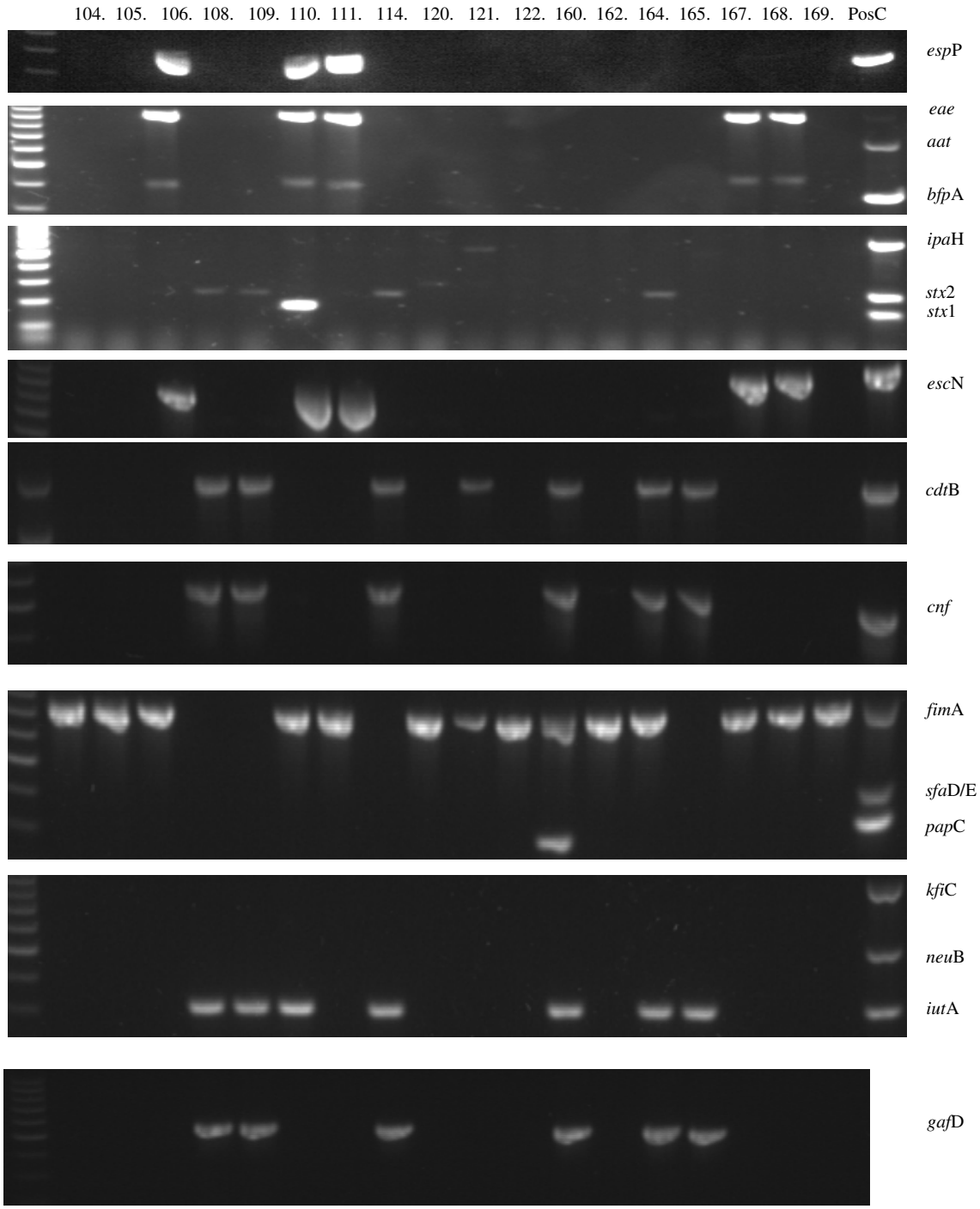


Figure A8 (part 1): PCR screening of selected bovine *E. coli* isolates for selected genes

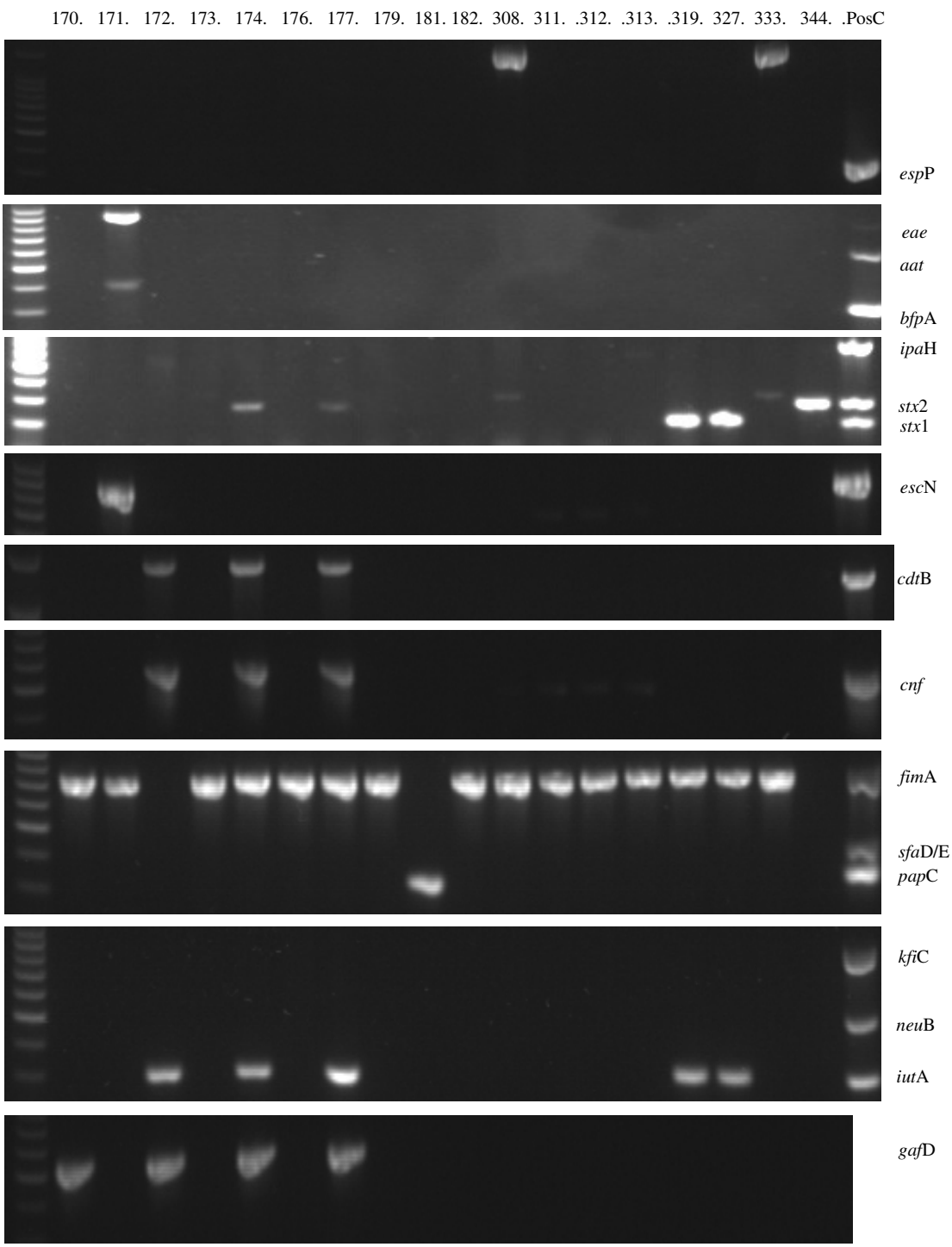


Figure A8 (part 2): PCR screening of selected bovine *E. coli* isolates for selected genes

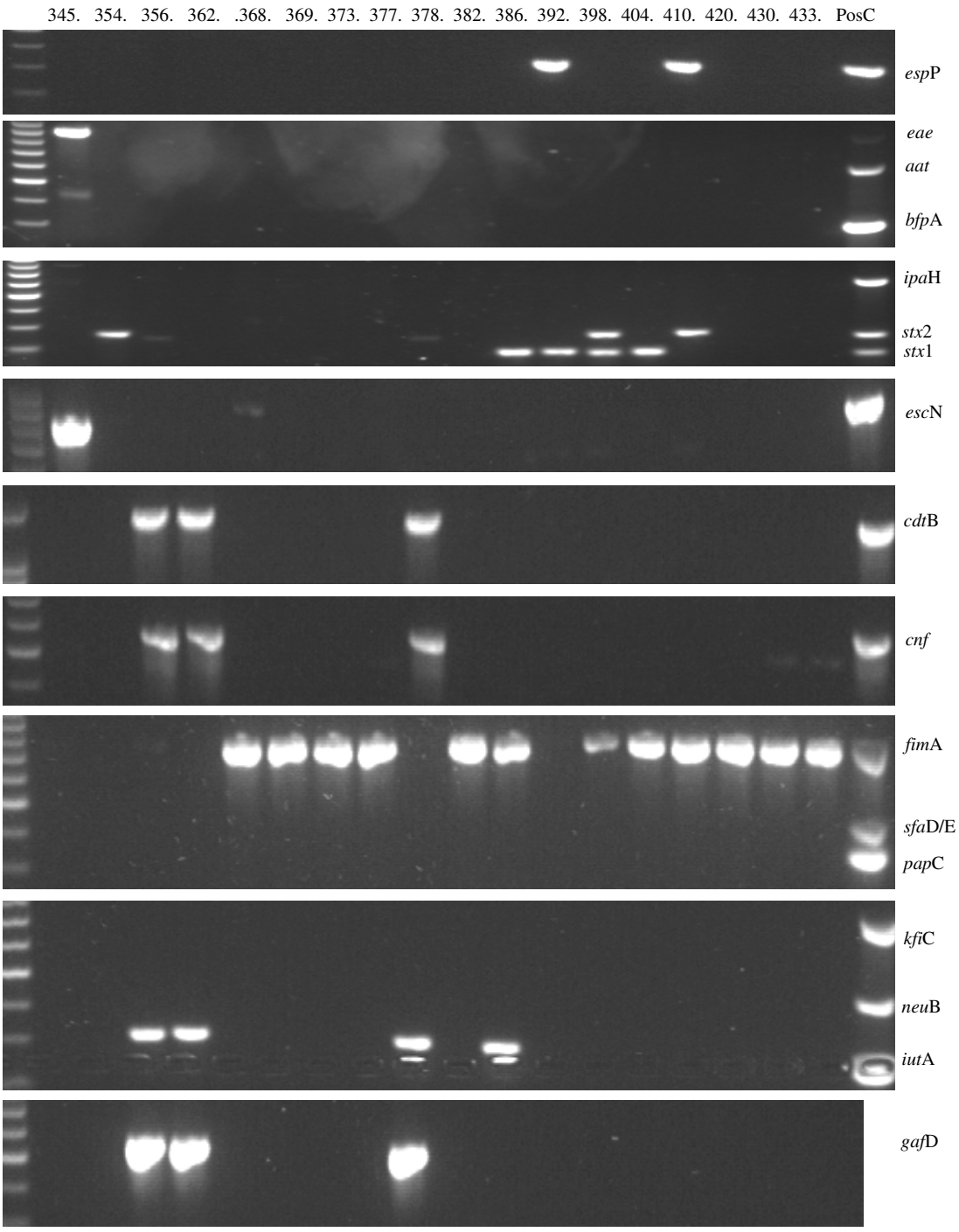


Figure A8 (part 3): PCR screening of selected bovine *E. coli* isolates for selected genes

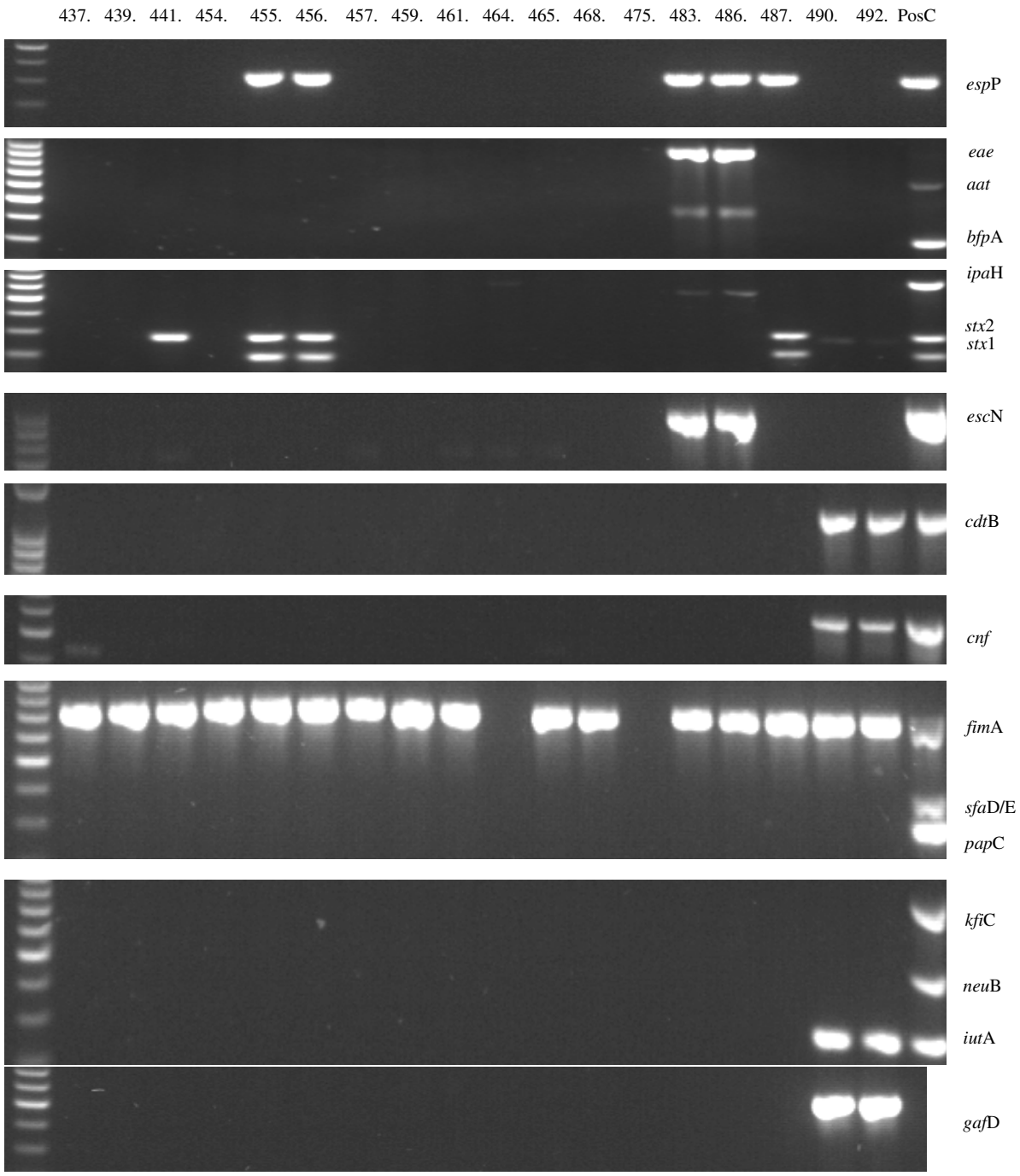


Figure A8 (part 4): PCR screening of selected bovine *E. coli* isolates for selected genes

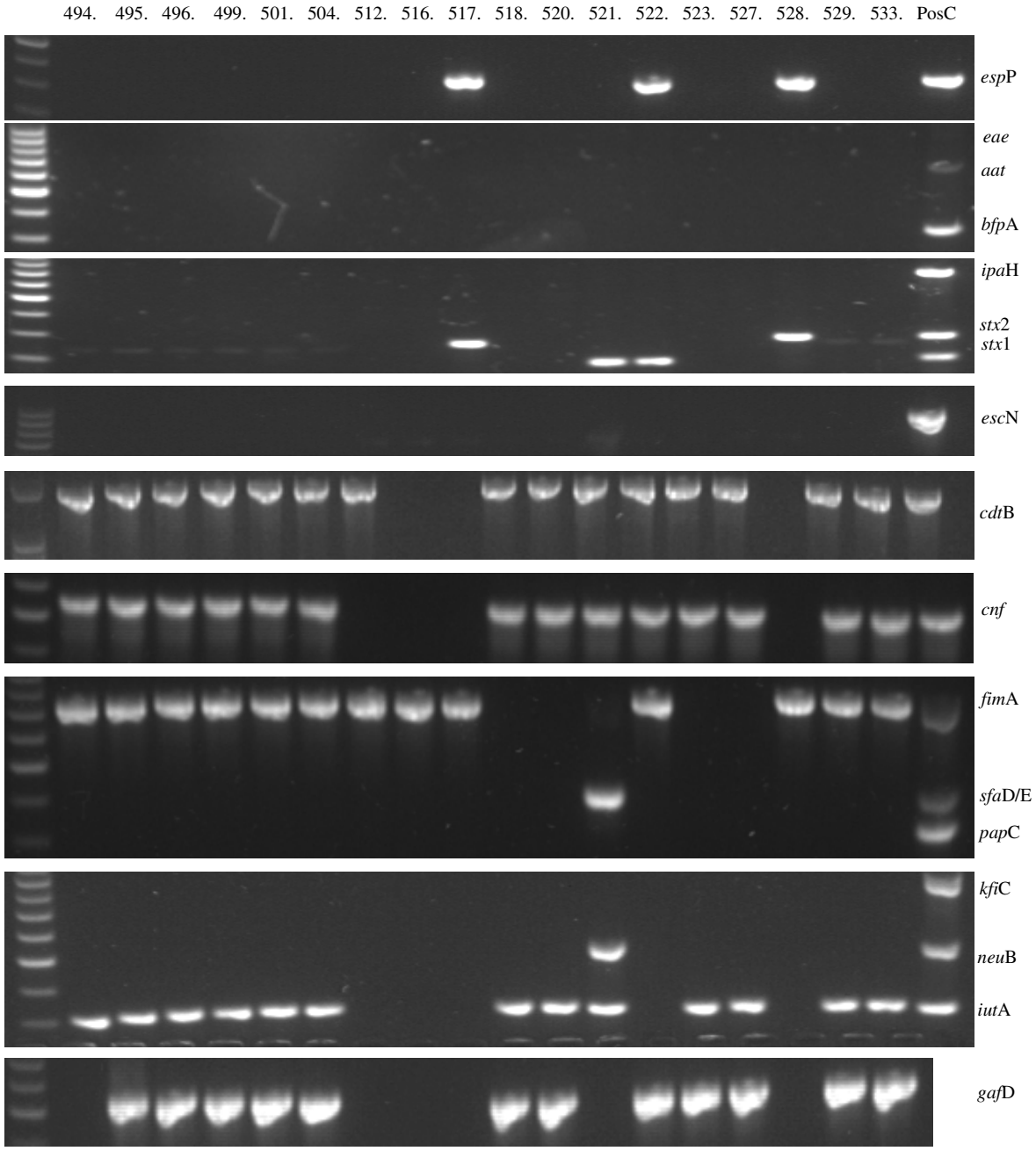


Figure A8 (part 5): PCR screening of selected bovine *E. coli* isolates for selected genes

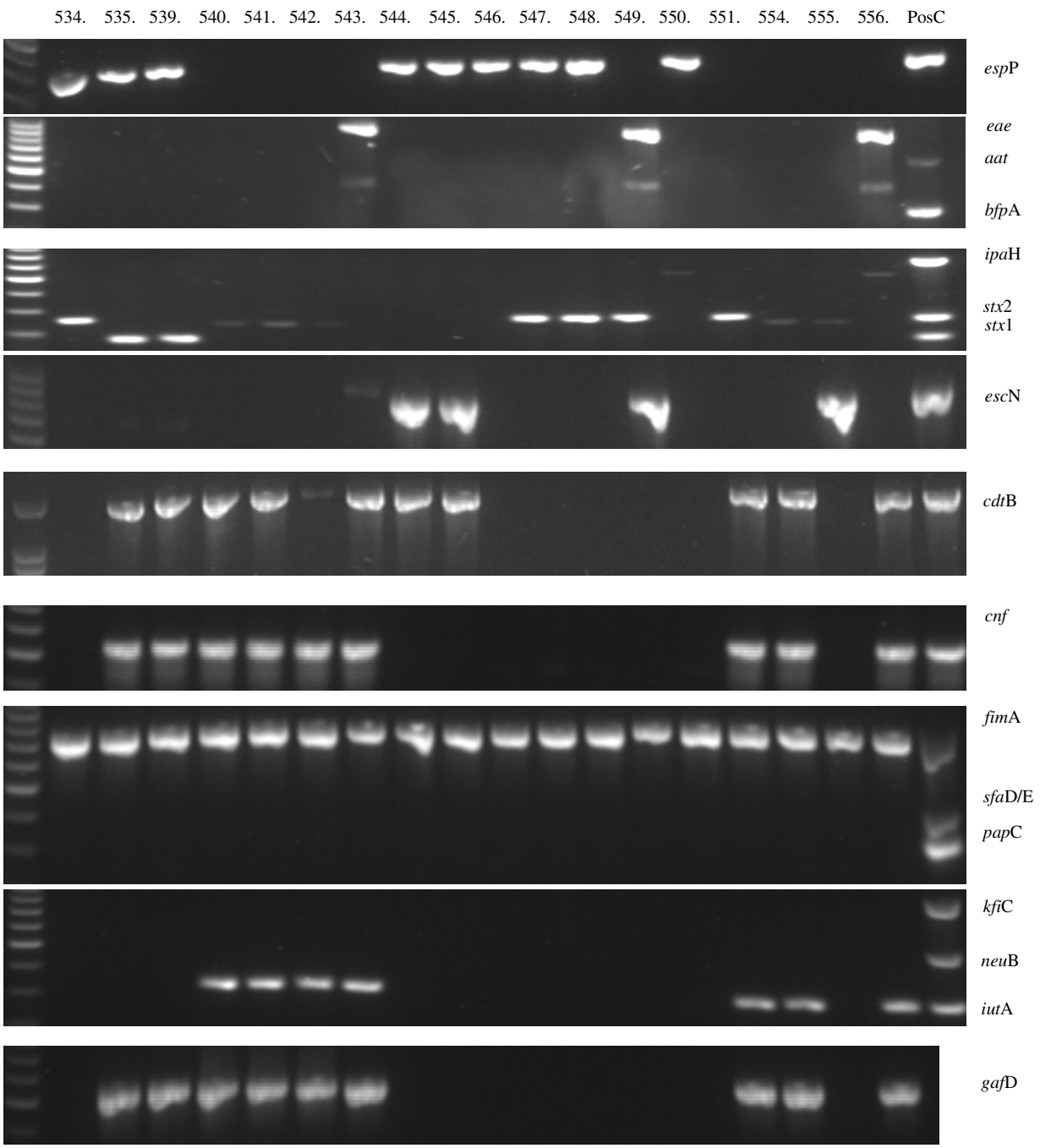


Figure A8 (part 6): PCR screening of selected bovine *E. coli* isolates for selected genes

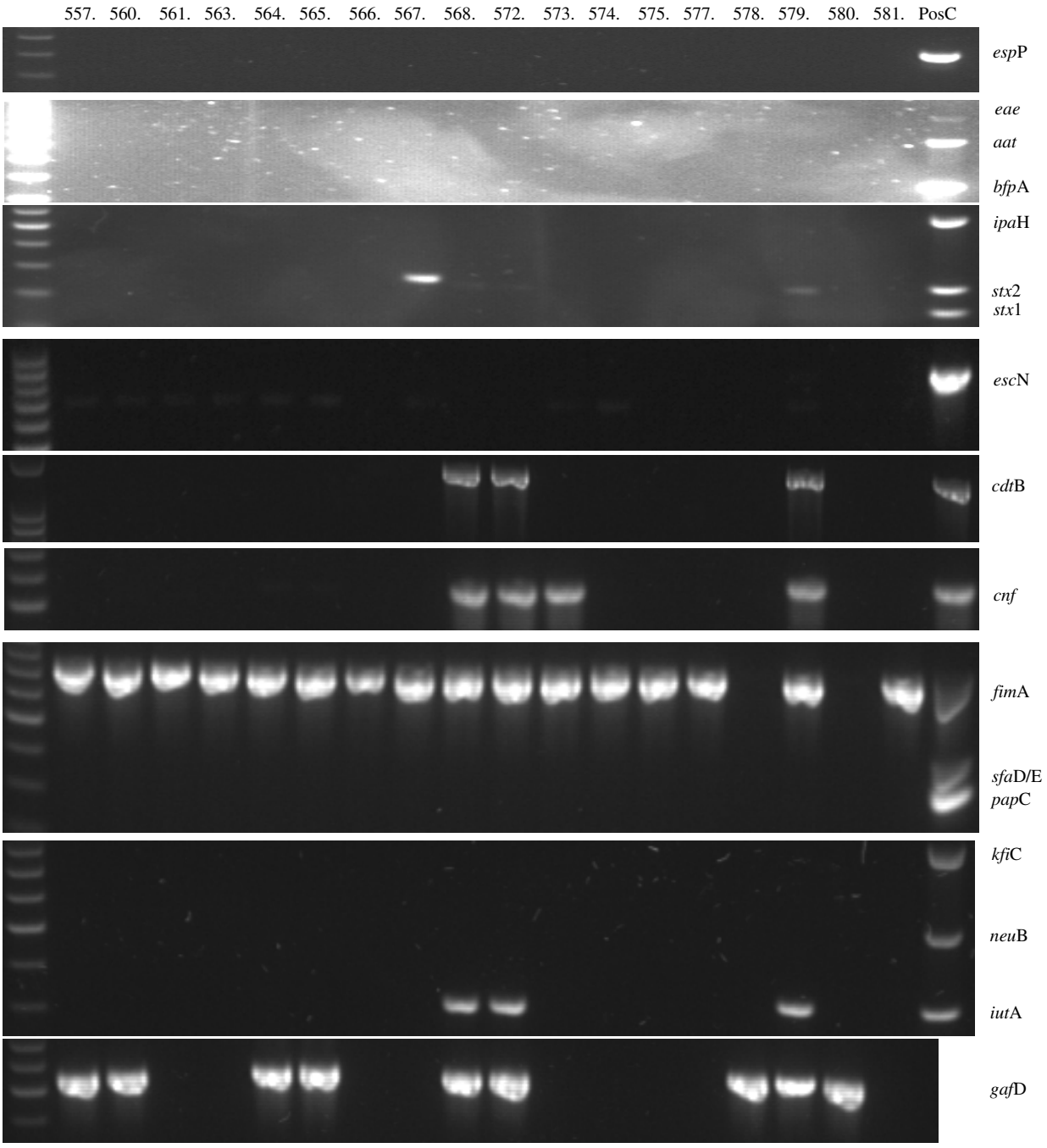


Figure A8 (part 7): PCR screening of selected bovine *E. coli* isolates for selected genes

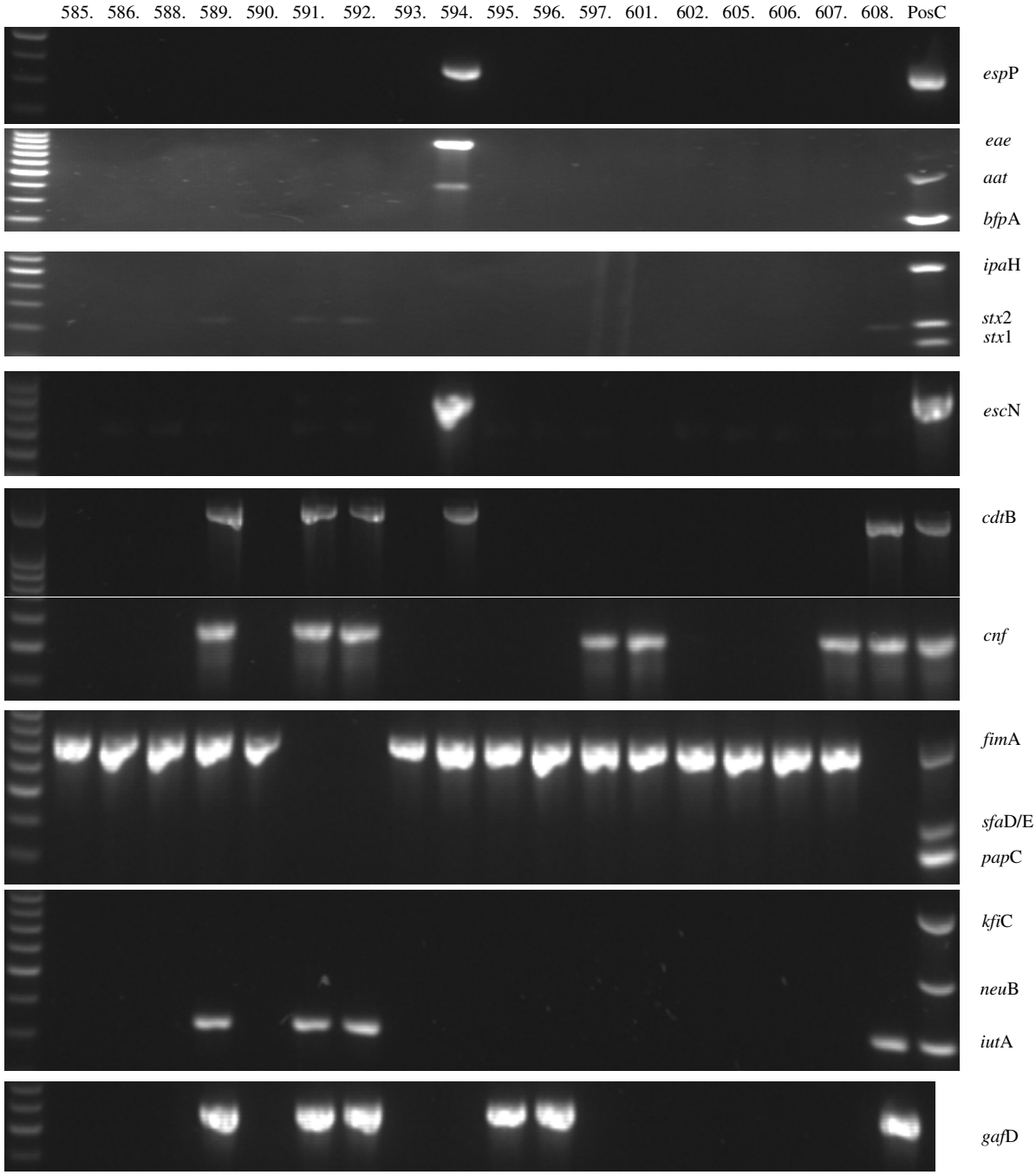


Figure A8 (part 8): PCR screening of selected bovine *E. coli* isolates for selected genes

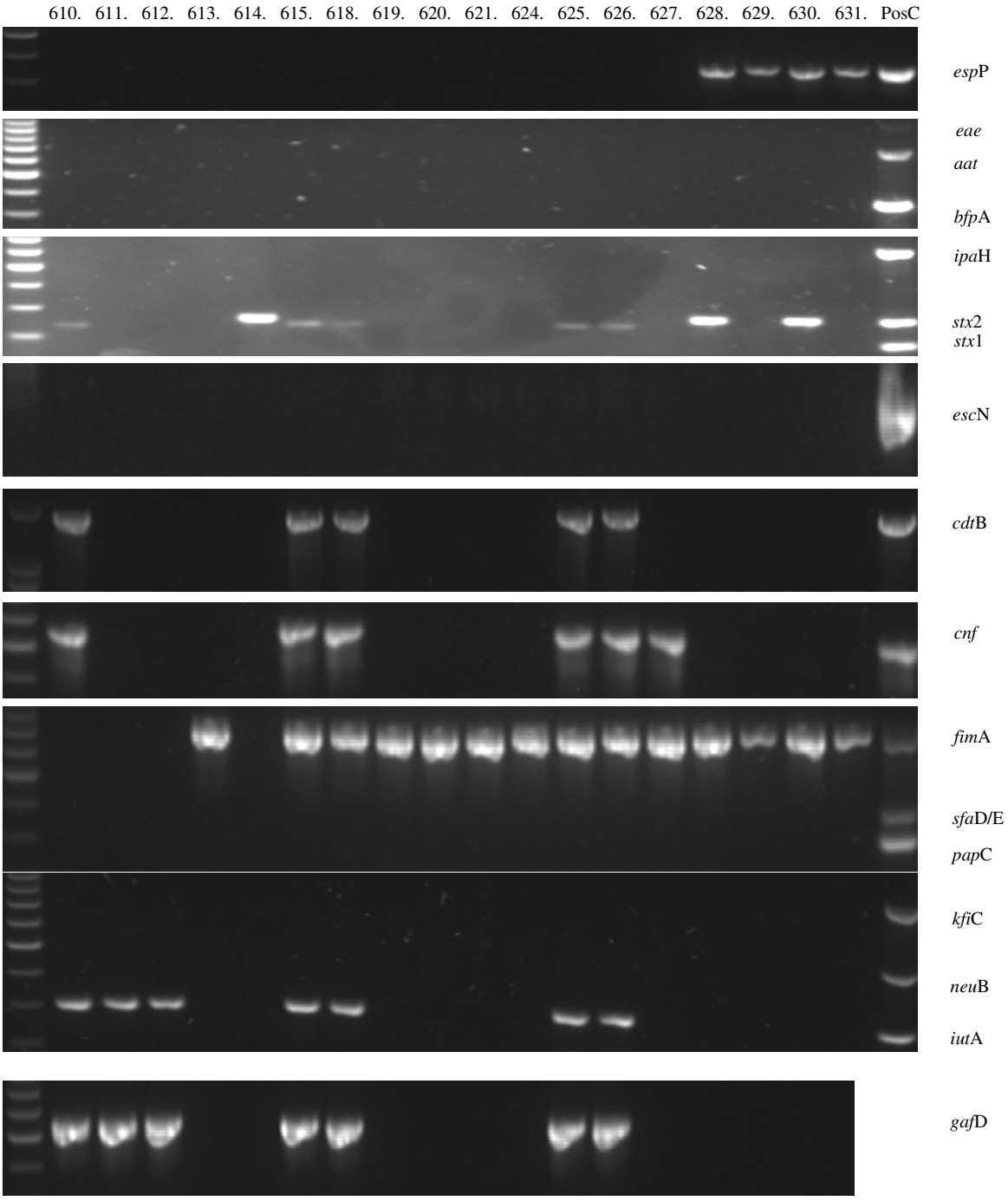


Figure A8 (part 9): PCR screening of selected bovine *E. coli* isolates for selected genes

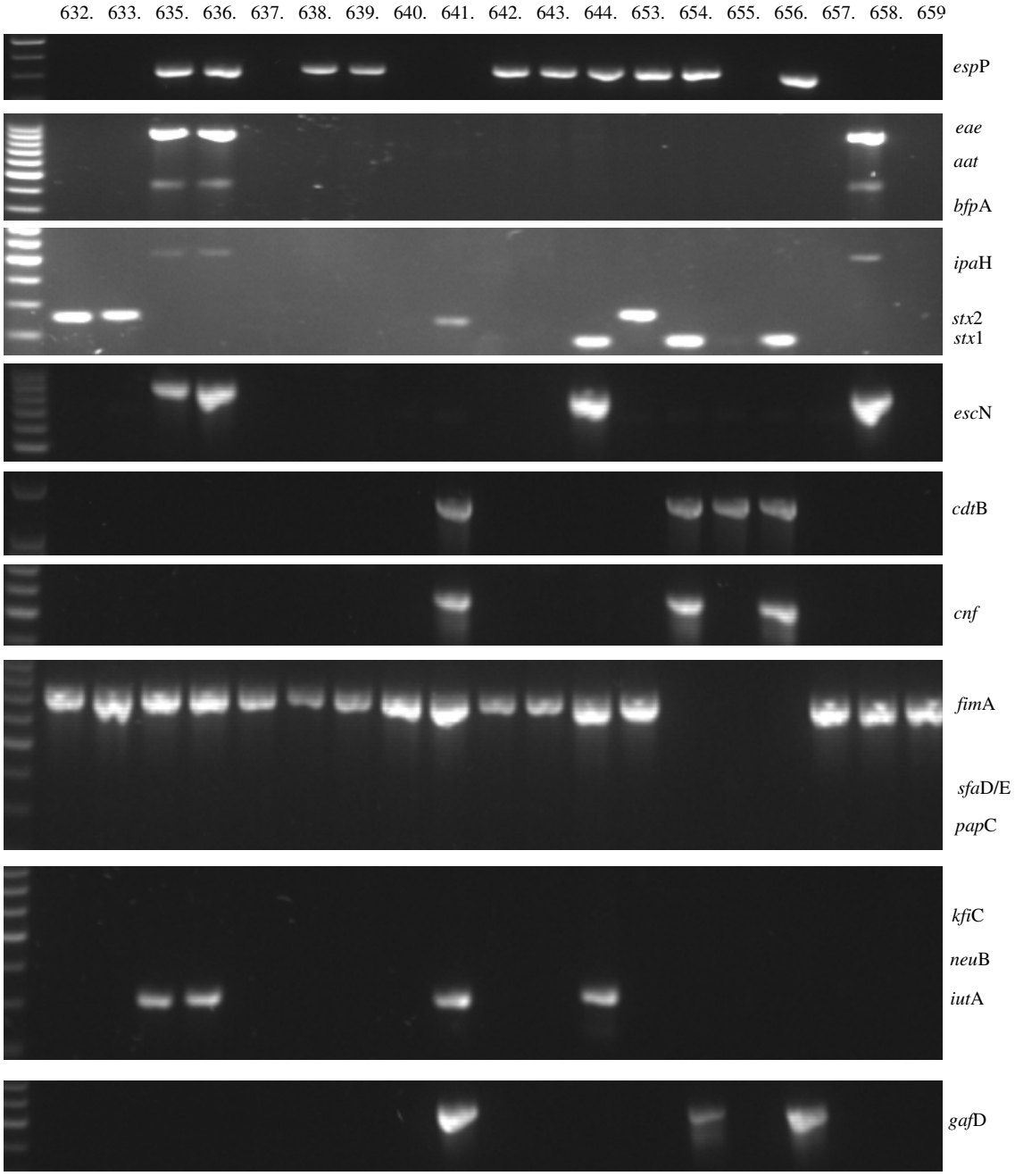


Figure A8 (part 10): PCR screening of selected bovine *E. coli* isolates for selected genes

Figure A8: PCR screening of selected bovine *E. coli* isolates for selected genes: Results of PCR-screening of bovine *E. coli* isolates: MCI number (corresponding to Table A1) is given in the top row of each figure, with the target gene (Section 4.2.1.3) given to the right. These results summarised in Tables 4.2 and 4.5.

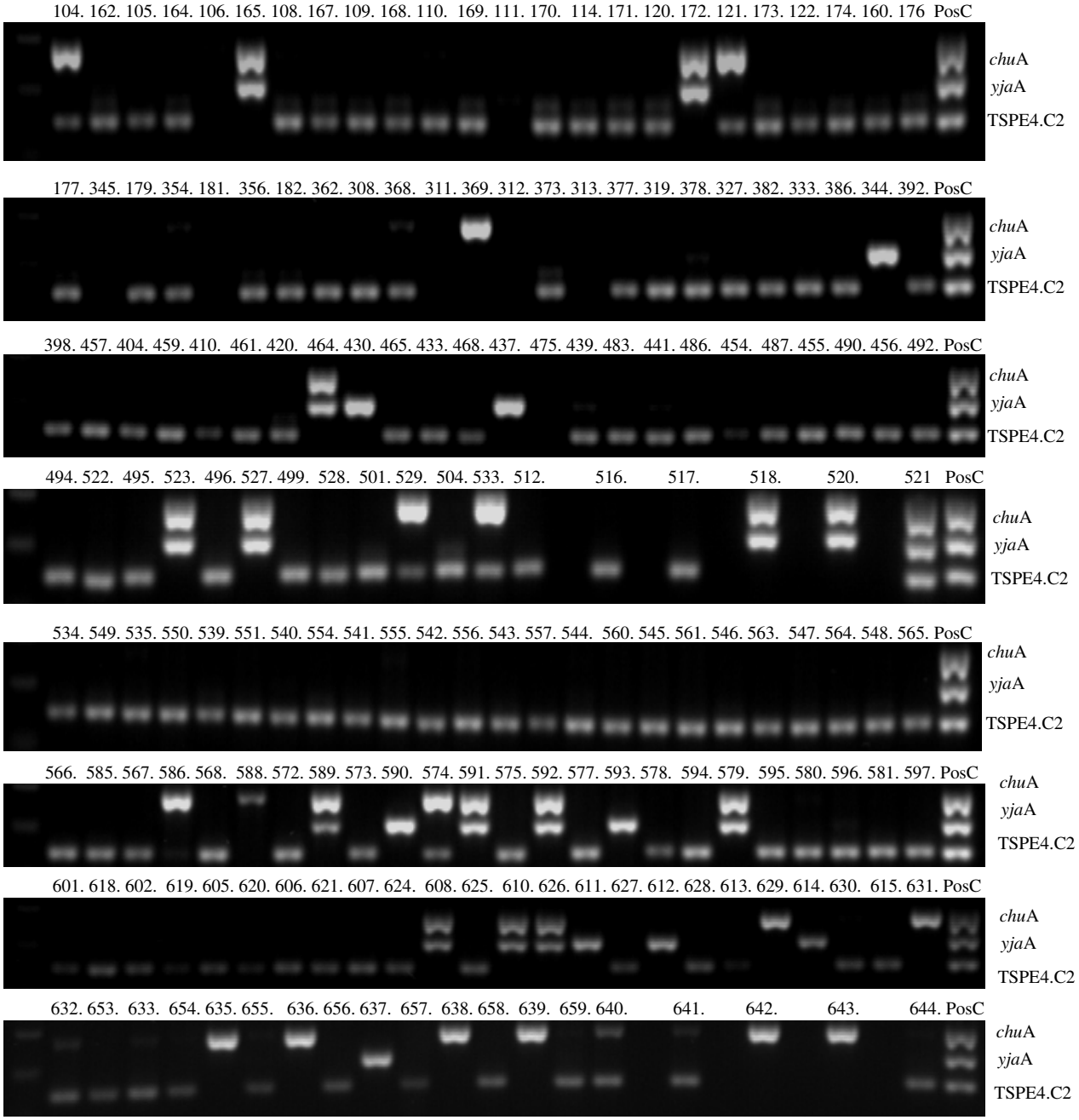


Figure A9: Results of PCR to determine phylogenetic group of bovine *E. coli* isolates: PCR to detect three genetic elements: *chuA*, *yjaA* and TSPE4.C2 was performed according to Section 2.6.3 and Clermont *et al*. (2000). Isolates encoding *chuA* were assigned to phylogenetic groups B2 (if positive for *yjaA*) or D (*yjaA*-negative). *chuA* negative strains were assigned to groups B1 (TSPE4.C2-positive) or A (TSPE4.C2-negative)

Appendix V Analysis of *E. coli* isolates by virulence gene nanoarray

Composition of the virulence gene nanoarray (Section 4.2.1.4; described in detail by Anjum *et al*. 2007) and the results of the analysis of selected strains are given in Table A5.

		Strain				
Target	**Position**	**MCI 0105**	**MCI 0106**	**MCI 0108**	**MCI 0110**	**MCI 0319**
rrl_0101_0177_10	1	+	+	+	+	-
rrl_0101_0177_20	2	+	+	+	+	+
rrl_0260_0330_10	3	+	+	+	+	+
rrl_0260_0330_20	4	+	+	+	+	+
rrl_0260_0330_30	5	+	+	+	+	+
rrl_0520_0580_10	6	+	+	+	+	+
rrl_0520_0580_20	7	+	-	A	-	-
rrl_1480_1560_coli_10	8	+	+	+	+	+
rrl_1480_1560_coli_20	9	+	+	+	+	+
rrl_1480_1560_coli_30	10	+	+	+	-	A
rrl_1480_1560_shig_40	11	A	+	+	-	-
rrl_1690_1770_coli_10	12	+	+	+	-	-
rrl_1690_1770_shig_20	13	+	+	+	-	-
rrl_1690_1770_freu_30	14	+	+	+	-	-
K88ab_10	15	-	-	-	-	-
astA_consens_10	16	-	A	-	A	-
*bfpA*_10	17	-	-	-	-	-
cba_10	18	-	-	-	+	-
ccl_10	19	-	-	-	-	-
*cdtB*_40	20	-	-	+	-	-
*cdtB*_50	21	-	-	+	-	-
*cdtB*_60	22	-	-	-	-	-
celb_10	23	-	-	-	-	+
cfa_c_10	24	-	-	-	-	-
cma_20	25	-	-	-	-	-
cnf1_20	26	-	-	+	-	-
cofA_10	27	-	-	-	-	-
eae_consensus_10	28	-	+	-	+	-
eae_consensus_20	29	-	+	-	+	-
eae_consensus_30	30	-	+	-	+	-
eae_consensus_40	31	-	+	-	A	-
espB_O157_20	32	-	-	-	+	-
espB_O26_40	33	-	-	-	+	-
f17-A_40	34	-	-	A	-	A
f17-A_50	35	-	-	-	-	-
f17-A_60	36	-	-	-	-	-
f17-G_20	37	-	-	+	-	-
fanA_10	38	-	-	-	-	-
fasA_10	39	-	-	-	-	-
fedA_10	40	-	-	-	-	A
fedF_10	41	-	-	-	-	-
fim41a_10	42	-	-	-	A	-
gad_10	43	+	A	+	A	+
hlyA_20	44	-	+	-	+	+

hlyE_10	45	-	-	-	-	-
ipaD_10	46	-	-	-	-	-
*ipaH*9.8_20	47	-	-	-	-	-
ireA_20	48	-	-	A	-	-
iroN_10	49	-	-	-	-	-
iss_10	50	-	-	+	+	+
lngA_20	51	-	-	-	-	-
ltcA_20	52	-	-	-	-	-
mchB_10	53	-	-	-	-	+
mchC_20	54	-	-	-	-	+
mchF_10	55	-	-	-	-	+
mcmA_10	56	-	-	-	-	-
nfaE_10	57	-	-	-	-	-
perA_10	58	-	-	-	-	-
perA_20	59	-	-	-	-	-
pet_20	60	-	-	A	-	-
prfB_30	61	-	-	-	-	-
senB_20	62	-	-	-	-	+
sfaS_10	63	-	-	-	-	-
sta1_110	64	-	-	-	-	-
sta2_210	65	-	-	A	-	-
stb_10	66	-	-	-	-	-
stx1A_10	67	-	-	-	+	+
stx2A_10	68	-	-	-	-	-
virF_20	69	-	-	-	-	-

Table A5: Composition and results of the virulence gene nanoarray: Names of each oligonucleotide are given in the first column, with the results for each strain given in subsequent columns (+: present, A: ambiguous, -: absent) For a full list of oligonucleotides and sequences, refer to Anjum *et al*. (2007). Position of each oligonucleotide in the array is shown in Figure 4.8.

Appendix VI: DNA sequencing

The following sequences (corresponding to Tables 6.3, 6.4 and 6.5) represent those which yielded BLAST hits of lower than 90% identity meaning that they may represent previously undescribed gene sequences.

>w2E9

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ACGCAGTTTGATGAGTTTCGTAAAAATCTGACAATTCTTGCCGGGTTTTATGATGTGGTCACAACCATAAGAGGCGAGGTGAGATATCGGGCAA
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AAACAGCCCTGAACTGAATAACCGCCTGCTGTCGTTTTTCTGAGGTGATGATGCAGCAACGTAAACCTAAAAAATGCAAAGTGTGCGGAACCCC
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GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAA
AGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCAT
TAATGAATCGGGCCAACGCGCGGGGAGAGGCGGT

>w2G10

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GCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCA

>w3A11

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TACAACGAGGAGCAGCTCGCATCCATCATGGGGAGTTCGGATCTGGTTGGCGATATCGTTCGCAACTGGCTTGAGAATGGCGAGGATTTGCCGA
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>w3C4

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TTAGCTAATGAATCGCATCCGATTGATGGCCTGTCCGGTGAATATGCAGAGAAATCCCTTGAAGAATGGGCGGAACGGCTTCGCAAAGGAGGCA
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>w1B4
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