Investigation of virulent and avirulent *Brachyspira hyodysenteriae* isolates

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the Faculty of Veterinary Medicine, University of Glasgow

2013

“Ich habe fertig ! ”

Giovanni Trapattoni

Abstract

Brachyspira hyodysenteriae is an anaerobic intestinal spirochaete and the aetiological agent of swine dysentery (SD). Throughout the UK and Europe, pathogenic and potential non-pathogenic isolates of *B. hyodysenteriae* have been recovered from pig herds, creating major obstacles for the detection and control of this economically important pathogen. Therefore, the main aim of this research was to compare one representative of virulent (P8544) and one representative of avirulent (P7455) strains of *B. hyodysenteriae* using genomic and proteomics approaches with a view to identify distinctive genes or proteins.

The *B. hyodysenteriae* draft genomes of P8544 and P7455 consisted of a circular 3.0 Mb chromosome and a 31,469-34,822 bp circular plasmid that is also present in the only published *B. hyodysenteriae* genome, WA1. A considerable number of genes (~27-35) were identified in both the virulent and avirulent strains that shared high sequence homology with genes found in other spirochaetes, such as *B. murdochii* and *B. intermedia*, as well as in other species of bacteria; these may have been acquired via horizontal gene transfer. Comparative genomics of the two pathogenic genomes P8544 and WA1 versus the non-pathogenic genome P7455 revealed that the gene encoding for the methyltransferase type 11 (Bhyoa7455_20) was identified as being unique to the P7455 plasmid sequence and was successfully PCR amplified in a greater number of avirulent than virulent strains. However, as this was only just statistically significant (P=0.049), screening of a much larger strain set would clearly be required to support this gene as a suitable genetic marker to distinguish virulent and avirulent *B. hyodysenteriae* strains.

Bacterial acquisition of iron *in-vivo* is crucial for successful colonisation and persistence in the host. A further aim of this study was to compare the growth phenotype of *B. hyodysenteriae* isolates P8544 and P7455 grown under iron-limiting conditions; such as would be found *in-vivo* in the large intestine of the host. Analysis of P8544 and P7455 growth rate in iron-sequestered media (containing 0.1 mM of the iron-chelator dipyridyl) demonstrated that both these isolates could replicate in this media although with an extended lag-phase of approximately 32-34 hrs; growth rate was on par with the iron-replete conditions. qRT-PCR analysis of eight putative iron-acquisition genes under iron-sequestered and iron-replete conditions revealed a difference in transcription for a number

of ABC-transporter genes in P8544 and P7455, however, none of these were classified as statistically significant.

Non-quantitative shotgun proteomic based approach was used to analyse outer-membrane protein (OMPs) expression of P8544 and P7455 under low-iron and iron-replete growth conditions and revealed alteration in the OM expression profiles between the isolates and conditions using KEGG analysis. The majority of expressed proteins under iron-replete conditions were categorized in membrane transport (11%) and carbohydrate metabolism (7%). Under iron-restriction the OM profile changed most obviously in a decreased percentage of proteins particularly assigned in the categories energy metabolism and membrane transport. The percentage of proteins assigned no predicted function increased by 19% under iron-limited conditions highlighting the fact that biological functions of the majority of these expressed proteins in such an environment remains to be determined.

Two-dimensional gel-electrophoresis (2-DGE) of whole cell fraction indicated that the alkyl-hydrogen peroxide reductase protein (*AhpC*) in P7455 and the non-haem iron-containing ferritin (Bhyov8544_1528) in P8544 were significantly ($P<0.05$; 1.5-fold) more expressed under iron-restricted conditions than under iron-replete conditions. These data confirmed the importance of iron to virulent and avirulent *B. hyodysenteriae*. The so far identified significantly expressed proteins may serve as a potential biomarker for global diagnostic purposes for *B. hyodysenteriae* infections rather than a tool for differentiation for virulent and avirulent isolates. However, further work is required to prove if these candidates are expressed *in-vivo* and conserved in a wider panel of field isolates.

In conclusion, this research has contributed to the scientific knowledge regarding *B. hyodysenteriae* stress responses induced by iron-starvation and has provided further insight into the genetic and proteomic make up of this spirochaete. This work should also aid future investigations concerning the biology and pathogenicity of this important and grossly understudied swine pathogen.

Declaration

The work reported in this thesis was carried out under the supervision of Dr. Michael C. Fontaine at the Moredun Research Institute, Professor David G.E. Smith at the Moredun Research Institute and the Faculty of Veterinary Medicine, University of Glasgow and Professor Jill Thomson at the Scottish Agriculture College. All results presented, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

Signed Date

Danksagung

Eine wissenschaftliche Arbeit ist nie das Werk einer einzeln Person, daher möchste ich mich allen Beteiligten bedanken die zu diesem Werk beigetragen haben. Als erstes möchte ich mich bei meinen Betreuern Michael Fontaine, David G.E Smith and Jill Thomson für ihre Zusammenarbeit und Unterstützung während des Projekt bedanken sowie bei BPEX, für die finanzielle Unterstützung und Möglichkeit dieser Arbeit.

Weiterhin möchte ich meinen ehemaligen und maligen Labor-und Bürokollegen Anita, Scott, Allan, Eleanor, Caray, Leena und Tammy danken, für die anregenden akademischen Diskussionen, ihre Hilfsbereitschaft und für eine heitere Arbeitsatmosphäre.

Ebenso möchte ich mich bei der Proteomics Abteilung bedanken, vorallem bei Neil für seine Unterstützung sowie bei Erin und Kevin als auch bei Richard Burchmore und Alan Scott von der Universität Glasgow für ihre technische Hilfestellung der 2D gele. Weitere Dank gilt ebenso Alex and Raja und dessen Einführung in die Welt der Bioinformatik.

Nicht zu vergessen sind meine Kommilitonen Christian, Riccardo and David die immer für ein Bierchen nach einem schlechten Tag im Labor zu haben waren.

Eine herausragende Stellung in jeglicher Hinsicht nehmen meine Freunden und meine Familie, ohne deren mentale Unterstützung die Vollendung dieser Arbeit nicht möglich gewesen wäre. Hierbei sind vorallem meine Mutter, Vanessa, Kane und Marion zu erwähnen. Vielen Dank für all die ellenlangen und ermutigenden Telefongespräche, vorallem in den dunkelsten Tiefphasen dieser Arbeit. Besonderen Dank gilt ebenso Flo and John für ihre Freundschaft und ihrer Unterstützung, besonders in den letzten Monaten meiner Doktorarbeit.

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Abbreviations

A	Avirulent
Acc. #	NCBI Accession number
ACT	Artemis Comparison Tool
AGB	Antibiotic growth promoter
ANOVA	Analysis of variance
Approx.	Approximately
arp	Ankyrin-like protein
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B	Bovine
BA	Blood agar
BAM	Blood agar medium
BASys	Bacterial Annotation system
BHI	Brain Heart Infusion
Bhyo	*Brachyspira hyodysenteriae*
BIT	Brachyspira iron-transport system
BLAST	Basic Local Alignment Search Tool
bLf	bovine lactoferrin
bp	Base pair
BRIG	BLAST Ring Image Generator
BSA	Bovine Serum Albumin
C	Colistin
ca.	*circa* (around)
$CaCl_2$	Calcium chloride
CAS	Chrome azurol S
CDM	Chemically-defined Medium
CDS	Coding sequence
cDNA	Complementary deoxyribonucleic acid
cfu	colony forming units
cm	Centimetre
COG	Cluster of orthologus groups
Ct	Cycle threshold
Da	Dalton (Atomic mass unit)
dH_20	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
E	Efficiency
EDDA	Ethylenediamine-N,N'-diacetic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
E_T	Efficiency of the target gene

E_R	Efficiency of the reference gene
EU	European Union
FAT	Fluorescent antibody test
FCS	Foetal calf serum
Fe^{2+}	ferric iron
Fe^{3+}	ferrous iron
FeoB	ferrous protein B
g	Gram
gbk	genbank file
gDNA	Genomic deoxyribonucleic acid
h	Hour(s)
HCl	Hydrochloric acid
HDTMA	Hexadecyltrimethyl ammonium bromide
HGM	Hog gastrine muscin
HIS	Human intestinal spirochaetosis
HKG	Housekeeping gene
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
Hz	Hertz (SI unit of frequency)
i.e.	*id est* (that is)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IS	Intestinal spirochaetosis
kb	Kilo base
kDa	Kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG orthologus
kPa	Kilo Pascal
kV	Kilo volt
l	Litre
Lbp	Lactoferrin-binding protein
LC-ESI-MS/MS	liquid chromatography-electrospray ionization-mass/mass spectrometry
Lf	Lactoferrin
Ltd	Private Limited Company
M	Molar
M	Expression stability
m	Minute(s)
mA	Milliamp
mAb	Monoclonal antibody
MALDI-ToF	Matrix assisted Laser Desorption Ionisation Time of Flight
MCP	Methyl-accepting chemotaxis protein
MIC	Minimal inhibition concentration
mg	Milligram

Mg	Magnesium
$MgCl_2$	Magnesium chloride
MglB	galactose/glucose binding protein
ml	Millilitre
MLST	Multi-Locus Sequence Typing
mM	Millimolar
Mn	Manganese
mRNA	Messenger ribonucleic acid
ms	Microseconds
MS	Mass Spectrometry
m/z	Mass to charge ratio
NaOH	Sodium Hydroxide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NCBI	National Centre for Biotechnology Information
ND	Not done
ng	Nanogram
nm	Nanometre
NRF	Novel region finder
NCTC	National Collection of Type Cultures
O	Ovine
OD	Optical density
OMP	Outer-membrane protein
Opp	Oligopeptide transport protein
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Tris phosphate buffered saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
pmol	Picomole
psi	Pounds per square inch
PTM	Post-translational modification
R	Rifampicin
RFLP-PCR	Restriction-Fragment-Length-Polymorphism
RI	Rieti
RNA	Ribonucleic acid
RNase	Ribonuclease
RM	Roma
rpm	Revolutions per minute
RPMI	Media developed at Roswell Park Memorial Institute
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
RT	Room temperature
RT	Reverse transcription

qRT-PCR	Quantitative reverse transcription PCR
s	Second(s)
S	Spectinomycin
SAC	Scottish Agriculture College
SD	Standard deviation
SD	Swine dysentery
SDS	Sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism
SOD	Superoxide-dismutase
SOSPA	Sawn-off shotgun proteomics analysis
SPANC	self preservation and nutritional competence
TAE	Tris/acetate/EDTA
TBS	Tris-Buffered Saline
Tris-HCl	Tris-Hydrochloride
TS	Training set
U	Units
UDP	Uridine diphosphate
UK	United Kingdom
US	United States
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
V	Volt
v	Volume
V	Virulent
VSH	Virus of *Serpulina hyodysenteriae*
VL-S	Virulence life-style
Vsp	Variable surface protein
WM	Weight matrix
WBHIS	Weakly-β-haemolytic intestinal spirochaetes
µF	Microfarad (SI unit capacitance)
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
Δ	Delta
$\times g$	Times gravity
2-DGE	2D-gel electrophoresis
^{o}C	Temperature in degrees centigrade

Chapter 1: General Introduction

1.1 Spirochaetes

The order of *Spirochaetales* includes the two families *Spirochaetaceace* and *Leptospiraceace*. The family *Spirochaetaceace* contains six genera: *Treponema, Brevinema, Borrelia, Brachyspira, Spirochaetae* and *Christispira.* Spirochaetes have a tremendous impact on health in humans and animals but are still poorly understood. Therefore, advanced research has been conducted with focus on intestinal spirochaetes in genome sequencing and pylogenetic studies to improve our understanding of the pathogenicity of this family (Bellgard *et al.*, 2009; Fraser *et al.*, 1997; Petrosova *et al.*, 2012).

Spirochaetes are a group of Gram-negative, oxygen-tolerant and chemo heterotrophic bacteria which represent a monophyletic lineage and a major early branching in eubacterial evolution (Paster *et al.*, 1991). They are slender organisms with a flexible and helical shape and possess a cellular ultra structure that features internal periplasmic flagella (endoflagella), distinguishing them from other bacteria (Paster & Dewhirst, 2000). The periplasmic flagella form the axial filament and provide spirochaetes with their characteristic motility, including rotation around their long axis and a boring corkscrew rotation (**Figure 1.1**).

As a family, its members share a distinct morphology, but are quite diverse in respect of colonizing ecological niches and thus differ in their level of pathogenicity and habitat. Further variation is seen in their oxygen-tolerance depending on their physiological characteristics. Thus, spirochaetes can be classified as oxygen tolerant anaerobes (*Brachyspira* spp.), obligate anaerobes (e.g. *Treponema* spp.), aerobes (*Leptospira* spp.), microaerophilic (*Borrelia* spp.) and free-living organisms (e.g. *Spirochaeta aurantia*) (Canale-Parola *et al.*, 1967).

The genus *Treponema* includes bacteria causing the sexually transmitted disease syphilis and yaws in humans by *Treponema pallidum (*Giacani *et al.*, 2012; Petrosova *et al.*, 2012). Some *Treponema* species, such as *Treponema denticola* also cause necrotizing, ulcerative gingivitis and other gum diseases (Miller *et al.*, 2012). Although many bacteria of this genus are both pathogenic and parasitic, some aid food digestion in cattle and are therefore known as non-pathogenic including *Treponema bryantii* (Stanton & Canale-Parola, 1980)

and *Treponema saccharophilum* (Paster & Canale-Parole, 1985). The species *Treponema succinifaciens* is mainly found in the colon of pigs and is regarded as non-pathogenic (Harris & Kinyon, 1974).

Among livestock and especially in pigs, *Brachyspira hyodysenteriae* is known to cause swine dysentery (**1.7**) in pigs. The disease leads to major financial losses in the pig industry. The impact of *B. hyodysenteriae* and its virulence potential will be further discussed throughout this thesis in detail.

The *Leptospira* genus includes bacteria which cause Leptospirosis which is one the most common zoonotic disease worldwide (Ko *et al.*, 2009). The genus consists of pathogenic as well as saprophytic species including *L. biflexia* which are free-living organisms, found in water and soil, and unlike pathogenic species, are not able to cause infection in animal hosts (Ko *et al.*, 2009).

Borrelia is a parasitic genus of spirochaetes. These bacteria spread to humans and animals through the bites of infected ticks and lice and are mainly known for causing relapsing fevers by *B hermsii* (Lopez *et al.*, 2011) and *B turicatae* (Toledo *et al.*, 2010) as well as Lyme disease by *B. burgdorferi* (Barbour & Hayes, 1986; Rudenko *et al.*, 2012)

Moreover, there are some spirochaetes that have been identified but it has not been possible to culture and maintain them *in vitro* e.g. the genus *Christispira*. *Christispira* is found exclusively within mollusks (Paster *et al.*, 1996).

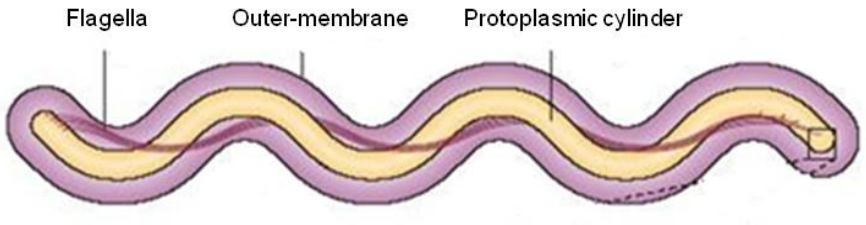


Figure 1.1: Schematic representation of the morphology of *B. burgdorferi* and location of the periplasmic flagella. The image was modified from the figure in (Rosa *et al.*, 2005).

1.2 The porcine intestinal microflora

The intestinal microflora is the aggregation of various non-pathogenic microorganisms in the gastrointestinal tract of mammals. This complex ecosystem is an active and dynamic metabolic unit which plays a key role in the immunity of the host (Pabst *et al.*, 1988)

including host-defence by suppressing colonization of incoming bacteria as well as gut maturation and digestion (Tsai *et al.*, 2012).

The gastrointestinal microbiome has been intensively studied to understand the relationship between the gut microbiota, health and disease in mammal's (Jacobson *et al.*, 2004; Nelson *et al.*, 2012). Compared to the human microbiota, limited information about the porcine gut is available with 80% of the bacteria being unknown species (Leser *et al.*, 2002). Studies of the gastrointestinal tract of pigs were mainly done by phylogenetic analysis using 16S rRNA revealing high microbial diversity. However, metagenomics is becoming a more widely used tool to study the ecology and evolution of bacterial communities (Hugenholtz & Tyson, 2008) and has already been shown to be more sensitive in the detection of low-abundant bacteria species than culture-dependent methods or the 16S analysis (Lamendella *et al.*, 2011). Metagenomics is the genomic analysis by directly accessing the genetic contents of entire communities of organisms (Thomas *et al.*, 2012). The first metagenomics studies of healthy swine faecal samples carried out by Lamendella *et al.* (2011) revealed that the six most abundant bacterial genera present were as follows: *Clostridiales*, *Firmicutes*, *Bacteroidales*, *Spirochaetes*, unclassified gammaproteobacteria and *Lactobacillales*. Other studies reported that 92% of isolated bacteria from pig faecal samples belonged to the phyla *Firmicutes* and *Bacteroidetes* regardless of age (Kim *et al.*, 2012). It was also reported that a significantly higher number of spirochaetes were identified than in any other hosts. Spirochaetes, particularly *Brachyspira* spp. are mainly associated with enteric diseases such as swine dysentery (SD) in the porcine gut which will be further discussed throughout this thesis. However, *Treponema* and *Anaerovibrio* were exclusively detected in the faeces of pigs and were not found in any other tested host species (Lamendella *et al.*, 2011).

Recent investigations have shown that the gut microbiota structure is shaped and changes with age (Kim *et al.*, 2011), antibiotic usage (Kim *et al.*, 2012) and diet (Liu *et al.*, 2012a).

Metagenomics of the porcine gut revealed the presence and high diversity of antibiotic resistance mechanisms (Lamendella *et al.*, 2011). Since the 1950s, antibiotics have been used in the swine industry to control and prevent diseases and growth promotion (Dibner & Richards, 2005; Niewold, 2007); leading to the emergence of antimicrobial resistance (e.g. resistance to tetracycline may have occurred or been exacerbated due to this antibiotic

being commonly supplemented into the feed of production animals. Nevertheless, a considerable percentage of metagenomics reads associated with fluoroquinolones resistance has been detected despite no farm history of treatment with this type of antibiotic indicating horizontal gene transfer between bacteria of the intestinal microflora. Fluoroquinolones have been widely used in veterinary practice in order to prevent and treat infections with e.g. *Salmonella* and *E. coli* in livestock which would explain the general increase in fluoroquinolones resistance reported in several in pathogenic bacteria (Jong . *et al.*, 2012).The acquisition of genes related to antibiotic resistance might not be surprising as 26 different transposase families were identified in the metagenome of pigs, suggesting that gene transfer among bacterial species in the pig gut has the potential to contribute to the persistence and evolution of multi-drug resistance in these enteric bacteria.

1.3 Intestinal spirochaetes

Intestinal spirochaetes are classified as anaerobic but oxygen tolerant bacteria which cause intestinal spirochaetosis (IS) in various mammals. Intestinal spirochaetes have been identified within the genus *Treponema* and the genus *Brachyspira*. Infection of the human gastrointestinal tract as well as diverse animals including dogs (Turek & Meyer, 1978) has been known for a long time. In humans, IS differs between geographical regions and it seems to occur with greater frequency in homosexual males. The two main recognized human species are *Brachyspira aalborgi* and *Brachyspira piloscoli*. Their roles will be further described in sections **1.5.1** and **1.5.3**.

However, the clinical importance of IS has been controversially discussed. It has been suggested that spirochaetes are commensals and form a part of the enteric microflora due to their findings in faeces of apparently healthy humans, birds and dogs. Moreover, pathogenic and non-pathogenic species of spirochaetes seem to inhabitant the large intestine of chickens and pigs. The non-pathogenic *Treponema succinifaciens* seems to colonize the colon of pigs without associations with gastrointestinal symptoms (Harris & Kinyon, 1974). Commsensal intestinal spirochaetes may contribute to the lifestyle of the host. The non-pathogenic *Treponema bryantii* (Stanton & Canale-Parola, 1980) and *Treponema saccharophilum* (Paster & Canale-Parola, 1985) were found to colonise the rumen of bovine and were involved in pectin, xylan and starch degradation which supports the digestive system. Intestinal spirochatosis is mainly associated with pigs and particularly

with bacteria of the genus *Brachyspira*. Nevertheless, further studies have proven the invasive tendency and pathogenicity of *Brachyspira* spp. in warm-blooded species other than pigs. The ability of *Brachyspira* spp. to cause IS in various animals and humans will be further described in section **1.5**.

1.4 The genus *Brachyspira* spp.

The genus *Brachyspira* belongs to the order Spirochaetales and was previously known as *Treponema* (Harris *et al.*, 1972), then reclassified as *Serpulina* (Stanton, 1992) and subsequently renamed as *Brachyspira* (Ochiai *et al.*, 1997).

The *Brachyspira* spp. are readily differentiated from those in other spirochaetes genera based on their comparison of their highly conserved 16S rRNA sequence (Paster & Dewhirst, 2000). Depending on the species, the cell size and number of periplasmic flagella vary within this genus (**Table 1.1**). *Brachyspira* spp. optimally grows at a temperature range of 37 to 42°C (Hampson & Stanton, 1997). Compared to other anaerobic species *Brachyspira* spp. is aerotolerant due to high NADH oxidase activity (this feature is discussed in section **1.11.4**), and it has been demonstrated that up to 1% oxygen (Vol %) can stimulate growth (Stanton & Lebo, 1988). Another typical characteristic of *Brachyspira* spp. is haemolysin production, and the genus includes strongly and weakly haemolytic species. Beta-haemolysis is a complete lysis of red blood cells. *Brachyspira hyodysenteriae* is the only strongly β-haemolytic species whereas all other species are weakly β-haemolytic intestinal spirochaetes (WBHIS). *Brachyspira* spp. are regarded as fastidious, difficult to isolate and slow growing (Duhamel & Joens, 1994).

There have been many years of research carried out on the gastrointestinal spirochaetes of the genus *Brachyspira* encompassing both pathogenic and commensal species. Several species have been associated with enteric diseases in humans and a wide variety of animal species. At present there are seven officially named species in the genus: *B. hyodysenteriae*, *B. piloscoli*, *B. intermedia*, *B. innocens*, *B. murdochii*, *B. alvinpulli* and *B. aalborgi*. Additionally, several new species have been proposed, including *B. suanatina* (Råsbäck *et al.*, 2007), *B. corvi* (Jansson *et al.*, 2008), *B. canis* (Hidalgo *et al.*, 2010), *B.*sp SASK30446 (Harding *et al.*, 2010) and *B. hampsonii* (Chander *et al.*, 2012)

Phylogenetic studies comparing the 16S rRNA sequence revealed that *B. hyodysenteriae* and *B. intermedia* as well as *B. innocens* and *B. murdochii* are closely-related, and share many phenotypic characteristics, whilst *B. piloscoli* and *B. aalborgi* are more distantly related to all the other *Brachyspira* spp.

Table 1.1: Morphological characteristics of intestinal spirochaetes.

Species	Length [µm]	Width [µm]	Number of flagella	Reference
B. hyodysenteriae	5.6-15.9	0.29-0.45	7-14	(Trott *et al.*, 1996)
B. innocens	5.3-14.1	0.25-0.40	7-14	(Trott *et al.*, 1996)
B. piloscoli	5.2-11	0.19-0.30	4-7	(Trott *et al.*, 1996)
B. intermedia	7.5-10	0.35-0.45	12-14	(Stanton *et al.*, 1997)
B. murdochii	5-8	0.34-0.4	11-13	(Stanton *et al.*, 1997)
B. aalborgi	2-6	0.2	4	(Hovind-Hougen *et al.*, 1982)

1.5 Pathogenicity of *Brachyspira* spp.

Anaerobic spirochaetes of the genus *Brachyspira* have long been recognized as important pathogens that colonize the intestinal tract of various mammalian and avian hosts (**Table 1.2**). The two main and best studied pathogens of this genus are *B. hyodysenteriae* and *B. piloscoli*.

1.5.1 *Brachyspira* infections in the porcine host

The species *B. hyodysenteriae* (**1.6**) and *B. piloscoli* are mainly recognized in the intestinal tract in pigs. *Brachyspira piloscoli* is the aetiological agent of IS and has been, compared to *B. hyodysenteriae*, isolated from a wide range of hosts including humans, birds, dogs and pigs (Hampson *et al.*, 2006; Jamshidi & Hampson, 2003; Jensen *et al.*, 2004; Munshi *et al.*, 2004). *Brachyspira piloscoli* infection leads to important production-limiting disease of pigs and chickens. The majority of *B. piloscoli* cells are 5.2 µm in length and 0.19-0.30 µm in width (**Table 1.1**) and thus one of the thinnest and smallest cells. Compared to the other characterised porcine *Brachyspira* spp., *B. piloscoli* grows relative quickly with a doubling time of 1-2 hrs in BHI broth at 38°C (Trott *et al.*, 1996), which is shorter than

that of *B. hyodysenteriae* (3-5 hrs). Moreover, *B. piloscoli* has an oxygen tolerance of 7% whereas that of *B. hyodysenteriae* is 5% (Trott *et al.*, 1996).

Brachyspira murdochii were regarded to be non-pathogenic. Nevertheless, recent studies have also shown that high numbers of *B. murdochii* are mildly pathogenic for pigs (Jensen *et al.*, 2010). Therefore, it has been suggested that *B. murdochii* is involved in mild colitis in pigs (Lee *et al.*, 2004).

Brachyspira suanatina has been identified in pigs and ducks with diarrhoea (Rasbäck *et al.*, 2007).

So far, *B. innocens* is commonly found in the colon of healthy pigs (Lee *et al.*, 1993) and considered to be non-pathogenic as experimental studies showed *B. innocens* is unable to cause disease in chickens (Trott *et al.*, 1995). However, due to observed decreased egg production in free range layer flocks associated with *B. innocens* (Burch, 2010), it can be concluded that under undefined conditions all *Brachyspira* spp. may be able to cause disease (Weissenbock *et al.*, 2005).

Table 1.2: Summary of the recognized species of *Brachyspira* spp. and their habitat.

Species	Main recognized hosts	Location	Pathogenicity
B. hyodysenteriae	Pig	large intestine	pathogenic
B. piloscoli	pig, chicken, dog, human, horse	large intestine	pathogenic
B. innocens	Pig	large intestine	non-pathogenic
B. alvinpulli	Chicken	Caecum	pathogenic
B. murdochii	pig, rats	large intestine	mild pathogenic
B. aalborgi	Human	large intestine	pathogenic
B. intermedia	pigs, chicken	large intestine	pathogenic
B. canis	Dog	large intestine	maybe non-pathogenic
B. corvi	corvi birds	large intestine	maybe non-pathogenic
B. suanatina	pigs, ducks	large intestine	pathogenic
B. hampsonii	Pig	large intestine	pathogenic
B. sp. SASK30446	Pig	large intestine	pathogenic

The table was modified from (Stanton, 1997)

1.5.2 *Brachyspira* infections in avian host

Brachyspira alvinpulli has been shown to be pathogenic in poultry, mainly in chickens (Stanton *et al.*, 1998), causing avian intestinal spirochaetosis (AIS). In chickens, AIS leads to reduced growth rate and egg production as well as chronic diarrhoea. Additionally another species termed *B. corvi* has been isolated from three corvi bird species collected from four separate geographical locations in Sweden. However, no association with enteric disease could be made after examination of the birds suggesting that this species might be non-pathogenic for birds (Jansson *et al.*, 2008).

Brachyspira intermedia is commonly isolated from older layer flocks with chronic diarrhoea in Europe, Australia and USA (Myers *et al.*, 2009) and have also been found in pigs (Backhans *et al.*, 2011). Multi locus sequencing typing (MLST) studies of 77 *B. intermedia* strains revealed high strain diversity among the spirochaete suggesting that some isolates may represent multiple distinct species (Phillips *et al.*, 2009). These findings were supported by a study of Burrough *et al.* (2012) detecting pathogenic and strong β-haemolytic strains of *B. intermedia* which formed a different cluster of all the previously published sequences of the *nox* gene.

1.5.3 *Brachyspira* infections in human

At 2-6 µm in length and 0.2 µm in width, *B. aalborgi* is the smallest species among *Brachyspira* spp. (**Table 1.1**). To-date *B. aalborgi* has only been isolated from humans and non-human primates (Munshi *et al.*, 2003; Munshi *et al.*, 2004). In humans, *B. aalborgi* and *B. piloscoli* are recognized as the aetiological agents of human intestinal spirochaetosis (HIS). A few cases have been reported of spirochetaemia in mainly critically ill and immunocompromised patients suffering from diarrhoea, polyps and HIV in Japan. Moreover, humans, especially those living in developing countries like Indonesia and Thailand, are with *Brachyspira* infection (Peruzzi *et al.*, 2007).

1.5.4 *Brachyspira* infections in other hosts

Recently, potentially new strongly β-haemolytic *Brachyspira* spp. have been identified and isolated from pigs suffering from SD, provisionally named *B. sp* SASK30446 and *B. hampsonii*. The pathogenicity of *B. sp* SASK30446 could be proven in a mouse model in which the isolate showed the greatest degree of cecal inflammation compared to the other tested *Brachyspira* spp. (Burrough *et al.*, 2012).

Brachyspira hampsonii was detected and recovered from clinical cases of SD in five states in the US. Phylogenetic and MLST analysis of the 16S RNA and the *nox* gene revealed that the species is genetically distinct from all the other known *Brachyspira* spp.suggesting that *B. hampsonii* is a new virulent species (Chander *et al.*, 2012). The pathogenicity of *B. canis* remains unclear since most of the four strains characterized so far were isolated from healthy dogs (Duhamel *et al.*, 1998).

The pathogen *Brachyspira hyodysenteriae* is the main focus in thesis and some previously mentioned main characteristic features including pathogenicity, main habitat and diagnosis will be described in more detail in the following sections.

1.6 *Brachyspira hyodysenteriae*

Brachyspira hyodysenteriae (**Figure 1.2**) is considered to be the primary etiological agent of SD (Taylor & Alexander, 1971). Although *B. hyodysenteriae* has been found in the large intestine of rats and mice (Joens & Kinyon, 1982) this bacterium predominantly afflicts pigs and is therefore more host specific then *B. piloscoli*. *Brachyspira hyodysenteriae* is the most pathogenic *Brachyspira* species in pigs, inducing severe diarrhoea, whereas *B. piloscoli* is associated with a milder form of colitis in swine. Compared to the other known *Brachyspira* spp., *B. hyodysenteriae* is the largest pathogen at 0.6-15.9 µm in length by 0.29-0.45 µm in width (**Table 1.1**), and tends to grow below the agar surface (Jansson *et al.*, 2004). Until recently *B. hyodysenteriae* was the only species which was known to be strongly β-haemolytic. However, novel strongly haemolytic *Brachyspira* spp. beside *B. hyodysenteriae* has been recognized as mentioned earlier.

Recently, the first genome of the pathogenic *B. hyodysenteriae* strain WA1 was published, allowing advanced research to study adaption as well as virulence factors in this spirochaete and vaccine development (Bellgard *et al.*, 2009). The genome of *B. hyodysenteriae* will be further discussed in **Chapter 4**.

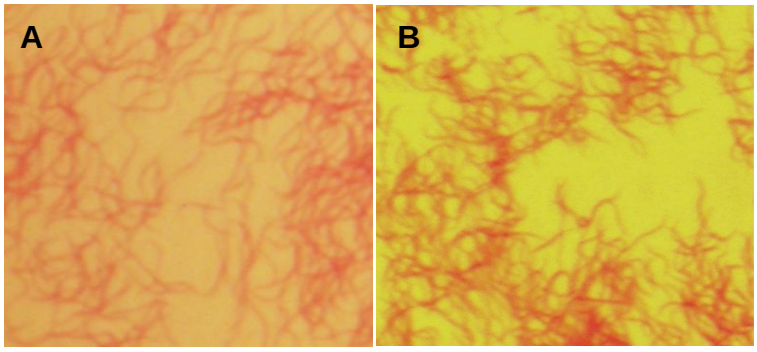


Figure 1.2: *Brachyspira hyodysenteriae* cells. Cells of *B. hyodysenteriae* field isolates P8544 (A) and P7455 (B) were grown in BHI media supplemented with 5% FCS under anaerobic conditions. Cells were harvested in mid-log phase (OD_{600nm}=0.6) and purity was checked by Gram-stain. Bacteria cells were visualized using a phase-contrast microscope 100 × and oil immersion.

1.7 Swine dysentery (SD)

Swine dysentery (SD) is a mucohaemorrhagic colitis in growing and finishing pigs and is responsible for severe economic losses to the swine industry worldwide (ter Huurne & Gaastra, 1995). This contagious disease was first described in the USA, in Indiana in 1921 (Whiting, 1921). In 1971 the intestinal spirochaete *B. hyodysenteriae* known at that time as *Treponema hyodysenteriae* (Wu *et al.*, 2001) was found to be the causal agent of SD. Swine dysentery is considered to be the most significant cause of losses to pig production due to growth retardation, expensive medical treatment, mortality up to 30%, poor feed conversion and culling of animals (Harris *et al.*, 1972; Hidalgo *et al.*, 2009).

1.7.1 Epidemiology

Swine dysentery has been reported from pig-producing countries all over the world, including Europe, USA and Australia (Lobova *et al.*, 2004; Margawani *et al.*, 2009; Myers *et al.*, 2009). Significantly, one survey suggested that SD occurs at a frequency of 10-33% in piggeries around the world (Hampson, 2000).

Although SD can affect pigs of all ages it is mainly seen in growing and finishing pigs (6 to 20 weeks of age) and is more common in fattening than breeding herds. Normally, infection occurs via the oral route by e.g. ingestion of infected faeces. The disease is mainly introduced into a herd by so called "carrier pigs" which have already recovered from a previous *B. hyodysenteriae* infection. Investigations confirmed that asymptomatic pigs are a potential source of infection due to intermittent excretion of the organism in faeces(Griffin & Hutchings, 1980), whereby carrier animals that had recovered and remained healthy for 70 days were able to transmit SD to uninfected pigs in the herd. The transition of *B. hyodysenteriae* cells from host to host is also favoured by the ability of the pathogen to survive in the faeces at 5°C for up to 40 days (Taylor, 2004). Therefore, insufficient hygiene procedure is a significant factor causing spreading of the pathogen in the herd.

Swine dysentery is introduced onto farms by the purchase of infected pigs, by pig to pig contact as well as by contaminated boots and implements. Moreover, DNA of *B. hyodysenteriae* was isolated from faecal samples from birds and mice, which lead to the conclusion that wild rodents might be another source of infection. Mice are able to carry *B. hyodysenteriae* for about 180 days (Szancer, 2005), and it has been shown that mice infected with *B. hyodysenteriae* are pathogenic for pigs. Therefore, effective rodent control is necessary on pig farms to limit the spread of SD.

1.7.2 Clinical signs

The severity of SD can vary between infected herds, and depends on many things including the amount of stress on the pig, the group size, the weight of the pig as well as the diet (Jacobson *et al.*, 2004). In its classical form SD is characterized by inflammation, excess mucus production, and necrosis of the large intestine that results in rapid weight loss (ter Huurne & Gaastra, 1995). Clinical signs of SD usually occur within an incubation time of 10 to 14 days. Diarrhoea is the most consistent sign, which develops and persist throughout the course of the disease. The first indication of SD is soft, yellow to grey faeces as well as an increased rectal temperature of 40-40.5°C (Meyer, 1978). Initially, pigs show reduced appetite, restlessness and develop twitching of the tail. Blood, mucus and latterly necrotic material appear in the faeces. Subsequently, the pigs rapidly lose body condition. Prolonged diarrhoea usually leads to dehydration, weight loss, weakness and

eventually death. Post-mortem analysis of affected pigs will show lesions that are usually restricted to the large intestine. In extreme cases, death may result mainly due to dehydration, acidosis and hyperkalaemia (Argenzio *et al.*, 1980).

1.7.3 Pathogenesis

Infection with *B. hyodysenteriae* occurs by ingestion of faecal material. Studies have shown that an inoculum of 10^5 colony forming units (cfu) is usually sufficient to produce SD (Kinyon *et al.*, 1977). Once the bacterium is ingested, it survives the brief passage through the acidic environment of the stomach, and eventually arrives in the large intestine. The ability of *B. hyodysenteriae* to proliferate and colonize the mucosa of the large intestine requires specialized features of the pathogen and is also influenced by the resident microflora (e.g. *Fusobacterium necrophorum, Bacteroides vulgatis*). It has been shown that SD could not been experimentally produced in gnotobiotic pigs (Harris *et al.*, 1978; Whipp *et al.*, 1979) which lack commensal bacteria. So far only mild lesions were detected when gnobiotic pigs were inoculated orally with pathogenic *B. hyodysenteriae* B204 indicating that *B. hyodysenteriae* alone is able to cause SD but that the resident microflora influences the severity of the disease dramatically (Pohlenz *et al.*, 1983). Moreover, *B. hyodysenteriae* has a selective advantage in colonising the intestine by tolerating oxygen by employing the NADH oxidase which also contributes to its increased metabolic efficiency (**1.11**). The motility of *B. hyodysenteriae* provided by the PF allows the pathogen to move through viscous mucus and thus being able to gain access to the epithelial cells in the colon. Motility and the chemotaxis have been shown to be important virulence factors of *B. hyodysenteriae* enabling the pathogen to penetrate the mucus layer and invade the colonic crypts. The mentioned virulence factors will be discussed in more details in section **1.11**. The presence of *B. hyodysenteriae* at the colonic crypts stimulates goblet cell hyperplasia and an outpouring of mucus into the lumen. Clinical signs and lesion of SD start to develop when numbers of bacteria reach $10^6/cm^2$ of the mucosa (Whipp *et al.*, 1979). Moreover, *B. hyodysenteriae* cells are detectable in the faeces 1-4 days before diarrhoea appears. At this stage of the disease, a shift from predominantly Gram positive bacteria in the colon microflora of pigs to predominantly Gram negative bacteria has been detected (Pohlenz, 1984). The bacteria multiply in the crypts and invade goblet and epithelial cells, causing damage (Taylor & Blakemore, 1971). There is an associated loss of cohesion between colonic enterocytes which subsequently results in

necrosis and shedding of the epithelium. Post-mortem analyses have shown that some spirochaetes are also found in the lamina propria, particularly around blood vessels. The disruption of the epithelial cells and subsequent necrosis results in the exposure of small blood vessels, causing haemorrhage. It has been proposed that toxic components including the lipopolysaccharide (LPS) and the haemolysin are responsible for enterocyte disruption and subsequent necrosis (Lyson *et al.*, 1991).

Typical lesions have been associated with the presence of *B. hyodysenteriae* in the cytoplasm of epithelial cells and within the lamina propria in the colon, however, invasion might not be essential for lesion production (Albassam *et al.*, 1985). Furthermore, *in vitro* adherence assays of *B. hyodysenteriae* using intestinal cell lines indicated that attachment did not necessarily cause cytopathological changes or invasion of the infected cells (Bowden *et al.*, 1989). Therefore, it is not clear whether adherence of *B. hyodysenteriae* cells is essential for causing SD.

Diarrhoea and the presence of mucus and the blood in the faeces is a consequence of colonic malabsorption and erosive colitis. The malabsorption, and subsequently fluid floss, is caused by failure of the epithelial transport system to actively transport sodium and chloride from the lumen to the blood causing dehydration and resulting in death in untreated animals (Argenzio *et al.*, 1980).

1.7.4 Control of SD

To-date no SD vaccines have been registered for use worldwide; however, the disease can be controlled by improved management and a number of antimicrobials.

Medication is most effectively done by administering drugs in the drinking water. Various antimicrobials are used for the treatment of infected pigs. However, not all antimicrobials are permitted due to national regulatory constraints e.g. nitroimidazole drugs are extremely effective but are not licenced for use in pigs in the EU or the USA. In many countries tiamulin is the antimicrobial of choice for treatment of SD and usually given by injection at 10 mg/kg once day for three days or in drinking water for five days. Nevertheless, there is a concern about the reduction in susceptibility to antimicrobials of certain individual *Brachyspira* species. Resistance of *B. hyodysenteriae* has been rare to tiamulin but studies

reported decrease in susceptibility to tiamulin in isolates of *B. hyodysenteriae* in Germany, Sweden, UK (Karlsson *et al.*, 2004) and Hungary (Molnar, 1996). Studies reported that *B. hyodysenteriae* isolates were also resistant to lincomycin and thus, *B. hyodysenteriae* could not be eliminated from infected pigs using these antimicrobials that were generally considered to be most effective for treatment and eradication programmes (Szancer, 2005). Resistance is particularly evident in countries where in-feed antibiotic growth promoters (AGPs) have been withdrawn (Lobova *et al.*, 2004). The use of AGPs has been banned in the European Union (EU) since 2006. Thus, it is no longer permitted to use carbodox, olaquindox, tylosin and spiramycin for growth promotion, though tylosin and spiramycin can still be used therapeutically (Burch , 2006). Currently, a study using Thai field isolates of *B. hyodysenteriae* showed that halquinol had a low MIC for the tested strains suggesting it might be useful for the control of SD (Lugsomya *et al.*, 2012). However, the withdrawal of certain drugs and the decrease in susceptibility to the small antimicrobials arsenal are complicating the treatment of pigs suffering from SD.

Efforts have been made to prevent and control the disease using different methods. It has been shown that feeding pigs a diet containing cooked rice offers protection against infection with *B. hyodysenteriae* as well as *B. piloscoli* (Hampson *et al.*, 2000). It is suggested that the composition of diet influences the microflora of the intestine which creates unsuitable conditions for colonization by spirochaetes. *In vitro* studies have revealed that pH $\leq$ 6.0 resulted in loss of motility of *B. hyodysenteriae*. Therefore, it was thought that pigs which are fed a diet based on maize silage could be less susceptible to infection with *Brachyspira* spp.(Prohaszka & Lukacs, 1984).

Careful hygiene procedures reduce the spread of the disease, such as disinfection of pathways, and use of foot dips and pools which can reduce the transmission from pen to pen and within a herd. Moreover rodent control, renovation of stables and an environment free of stress are fundamental for an effective prophylaxis against SD.

Normally farms on which an outbreak of the disease has occurred remain infected unless depopulation of the whole herd or other disease eradication procedures is carried out. Currently, SD can be eradicated by medication or depopulation and restocking with healthy pigs (Szancer, 2005). After cleaning and disinfection, the pig house remains empty for a period of time allowing the pathogen to die out until the unit is restocked by swine

dysentery-free pigs purchased from a breeding company which are maintained in isolation. In Denmark medical eradication using tiamulin has been carried out in over 500 pig herds and is preferred due to lower costs, although restocking is much more reliable(Szancer, 2005). The standard medical eradication procedures consist of medication of breeders, piglets and weaners for 14 days, followed by depopulation of grower and finishing units with high infection pressure (Borge, 2000).

1.8 Identification of *B. hyodysenteriae*

Confirmation of SD diagnosis requires not only isolation but also identification of specific spirochaetes. This has been attempted using several methods to date, some being more effective than others.

1.8.1 Morphological characteristics

Morphological characteristics of *Brachyspira* spp. have been used to distinguish between the different species. During the last decade efforts have been made to find appropriate methods and tests to differentiate between the species.

Using electron microscopy it was possible to observe differences in the dimension and number of flagella in the bacterial cells. Compared to all other species, *B. hyodysentriae* is one of the largest spirochaetes in this genus. *B. piloscoli* is slightly shorter than *B. hyodysenteriae* and *B. innocens*. The smallest and thinnest species is *B. aalborgi*.

However, differentiation using electron microscopy is unreliable, and former reports showed that *B. hyodysenteriae* and *B. innocens* isolated from chickens with diarrhoea were not distinguishable from each other. Moreover, culture conditions seem to play an important role in determining the ultra structure of spirochaetes. The cell length varies with stage of growth and growth conditions. Wood *et al* (2006) described that straighter cells appear under suboptimal conditions. In addition, *B. hyodysenteriae* as well as other spirochaetes are able to form so-called spherical bodies. This feature is mainly seen in aging cultures and at least 1% of *B. hyodysenteriae* cells form spherical bodies in growing

culture media. The formation of the abnormal helical shape seems to be an adaptive survival response to adverse environmental conditions.

1.8.2 Biochemical differentiation

The identification of isolates of *B. hyodysenteriae* in the diagnostic laboratory is usually confirmed by biochemical tests in combination with 16S or 23S RFLP-PCR (**1.8.3**). The genus *Brachyspira* is classified into five defined biochemical groups (**Table 1.3**) that are distinguished on the basis of their ability to produce haemolysis, indole, α-glucosidase and β-glucosidase, as well as hippurate hydrolysis.

Group 1 has all the biochemical features which are usually associated with *B. hyodysenteriae, i.e.* strong β-haemolysis, positive indole production, α-glucosidase activity and lack of α-galactosidase activity. Hommez *et al.* (1998) have found some strongly β-haemolytic spirochaetes to be indole negative. Therefore, Fellström *et al.* (1999) modified the biochemical identification pattern of Group I to positive/negative for indole (Fellström *et al.*, 1999). *B. intermedia* (Group 2) is almost identical to Group 1, apart from being weakly β-haemolytic. Group 3 is divided into three subgroups a, b and c. All isolates are weakly β-haemolytic. *Brachyspira murdochii* (Group 3a) is indole, hippurate hydrolysis and α-galactosidase-negative, and can either β-glucosidase negative or positive. *B. innocens* (Group 3b and 3c) is indole and hippurate hydrolysis-negative, and positive for α-galactosidase and β-glucosidase. Group 4 *(B. piloscoli*) is can lack of indole and β-glucosidase production but positive hippurate hydrolysis as well as α- galactosidase activity. The recently recognized novel β-haemolytic *Brachyspira* spp. are not categorized in any of the known groups as they share a distinct biochemical profile.

Nevertheless, interpretation of biochemical results should be taken carefully. Identification involves difficulties due to mixtures of *Brachyspira* spp. in one herd or due to the presence of genotypically and phenotypically atypical strains in one single sample. Moreover, isolates of the generally believed weakly-β-haemolytic *B. intermedia* and a potential new species *B. sp.* SASK30446 have been reported to be strongly-β-haemolytic (Burrough *et al.*, 2012). Therefore, further and more accurate methods should be included for precise speciation.

Table 1.3: **Biochemical differentiation of *Brachyspira* spp.**

Species	Group	Haemolysis	Indole production	Hippurate hydrolysis	α-Galactosidase	α Glucosidase	β Glucosidase
B. hyodysenteriae	1	strong	-/+	-	-	-/+	+
B. piloscoli	4	weak	-/+	+	-/+	-/+	-/+
B. intermedia	2	weak	+	-	-	+	+
B. innocens	3b; 3c	weak	-	-	+	-/+	-/+
B. murdochii	3a	weak	-	-	-	-/+	-/+
B.sp SASK30446	n	strong	n	n	n	n	n
B. hampsonii	n	strong	-	-	-	-	-/+

The table is modified from (Fellström *et al.*, 1999).

n these features have not been further characterized and remain therefore unknown.

1.8.3 Nucleic acid based detection methods

It is very important to be able to distinguish *B. hyodysenteriae* from other *Brachyspira* spp. as pig faecal samples are likely to contain a mixture of several *Brachyspira* spp. Jensen *et al* (1990) investigated the use of a DNA probe for *B. hyodysenteriae*, which binds to the 16S RNA gene within the bacterial chromosome. The 16S rRNA gene offers advantages for the diagnosis of SD as well as differentiation between *Brachyspira* spp., as it contains highly conserved regions interspersed with variable regions (Haun & Goebel, 1987). Today, sequence comparison of 16S rRNA genes is commonly used for phylogenetic analyses, although a few cases have been reported in which the 16S rRNA sequences have been proven to be an insufficient tool to identify different *Brachyspira* spp. due to the highly conserved sequence of this region among the species (Burrough *et al.*, 2012).

The 23S rRNA region is another highly conserved sequence among porcine intestinal spirochaetes belonging to the same species. Barcello *et al* (2000) combined PCR amplification of the 23S rRNA gene with primer sequences shared among known *Brachyspira* spp. of swine (Barcellos *et al.*, 2000). Identification of the species was obtained after digestion of the PCR product with the restriction endonucleases *Taq*I and *Alu*I. The predicted Restriction Fragment Length Polymorphism (RFLP) patterns for *B. hyodysenteriae, B. piloscoli, B. innocens, B. intermedia* and *B. murdochii* allowed a clear difference between all tested *Brachyspira* spp. Moreover, Teran-Dianderas (1997) developed a rapid diagnostic technique designed to identify and differentiate between the three main species, *B. hyodysenteriae, B. piloscoli* and *B. innocens*, based on amplification

of the 23S rRNA gene (Haun & Goebel, 1987; Teran-Dianderas, 1997). The test involves PCR followed by RFLP using the restriction enzyme *Hph*I. *Brachyspira intermedia* and *B. murdochii* had the same restriction pattern profile as *B. innocens*; therefore, this method conveniently detects and distinguishes between the pathogenic *B. hyodysenteriae*, *B. piloscoli* and the non-pathogenic *B. innocens*. The 23S rRNA and 16S rRNA-PCR/RFLP provides a relatively simple method for detecting different species of *Brachyspira* in diseased and also in asymptomatic pigs and is thus used as a reliable diagnostic tool.

Several companies are providing diagnostic test kits in order to identify *Brachyspira* spp. infections. For instance, the company Adiagene (Saint-Brieux, France) provides a commercially available *Brachyspira* kit called "Adiavet ™ Brachy" (http://www.adiagene.com) which is able to identify the species *B. hyodysenteriae*, *B. piloscoli*, *B. intermedia* and *B. innocens* on the basis of the amplification of the 23S rRNA. Additionally, the company Genebox (Cantanhede, Portugal) (http://www1.biocant.pt/genebox/index-eng.html) provides another diagnostic kit called Brachyspira Qual PCR Box 1.0 which is used for the identification of *B. hyodysenteriae* by amplification of the *flab*1 gene which encodes flagellin.

1.8.4 Serological tests

Another tool for differentiation of *Brachyspira* spp. is serotyping. To date nine serotypes and nine serogroups have been proposed (Hampson *et al.*, 1990) Over the years, various serological assays like slide agglutination (Diarra *et al.*, 1994), immumnodiffusion (Baum & Joens, 1979) and microplate agglutination (Diarra *et al.*, 1994) with polyclonal rabbit sera have been used for diagnosis of SD. However each of the assays ultimately failed due to cross-reactivity with other intestinal spirochetes (La *et al.*, 2009). Significantly, cross-reactivity between *B. hyodysenteriae* and *B. innocens* is well recognized due to the highly conserved periplasmic flagella antigens (Miller *et al.*, 1988; Smith *et al.*, 1990). Therefore, further investigation regarding the identification of species specific antigens for *B. hyodysenteriae* is necessary.

1.9 Vaccination

It is known that a proportion of pigs which have recovered from SD are protected from disease when re-exposed to *B. hyodysenteriae* (Joens *et al.*, 1979). After pigs become infected with *B. hyodysenteriae* an immune response occurs within two or three days. Serum-specific antibodies can be detected within 7 to 10 days and may persist for up to 19 weeks. The appearance of IgG, IgA and IgM antibodies in serum and the production of IgA in gut mucosal tissue during infection might reduce the rate of further colonization of the mucosa (Rees *et al.*, 1989).

On account of the huge economic impact as well as the increasing antibiotic resistance and legal limitations of using certain antibiotics there have been many years of research towards development of a vaccine to control SD. Previous studies with *Brachyspira* vaccines have not provided full protection (Fernie *et al.*, 1983) and thus there is no effective vaccine against SD. Investigations have focused on the outer-membrane proteins (OMPs) of *B. hyodysenteriae* in order to detect potential vaccine candidates.

An outer membrane 16 kDa lipoprotein called Bhlp16 (previously known as SmpA) of *B. hyodysenteriae* was discovered by Thomas *et al.* (1992). Further investigations of this lipoprotein showed that it is apparently not present in all *B. hyodysenteriae* isolates (Turner *et al.*, 1995). Moreover using Bhlp16 as a subunit vaccine unit failed as it does not appear to be produced *in vivo* (Lee *et al.*, 2000).

Hampson and Lee produced a monoclonal antibody (mAb) clone called BJL/SHI that reacted with a preparation from all *B. hyodysenteriae* strains (Lee & Hampson, 1996). BJL/SHI mAb was shown to be of the IgM isotype and recognized a 30 kDa protein in all *B. hyodysenteriae* examined. Similar bands of 29 kDa were detected in *B. piloscoli* but they were antigenically distinct (Lee & Hampson, 1996). Subsequently, this molecule was further described as a 29.7 kDa outer membrane protein of *B. hyodysentriae*, designated Bhlp29.7 (previously known as BmpB). Recombinant Bhlp29.7 appeared to have potential as a vaccine, as pigs immunized with the protein showed reduced susceptibility to SD (Shin *et al.*, 2005). However, while vaccination with Bhlp29.7 prevented the development of severe colonic lesions, SD could still occur in the tested pigs. The use of Bhlp29.7 as a coating antigen in an ELISA for detecting pig herds with SD failed due to cross-reactivity

with other intestinal spirochaetes as described before (La *et al.*, 2009). Bhlp29.7 belongs to the methionine substrate-binding proteins (MetQ) which are found in a wide range of Gram-negative bacteria. Thus, it has been suggested that there are conserved epitopes of MetQ proteins within Bhlp29.7 which cause cross-reactivity (La *et al.*, 2005). Moreover, further investigations have shown that Bhlp29.7 has partial similarity with surface proteins of *B. innocens* B256.

1.10 Antigenic variation

It has been suggested that *B. hyodysenteriae* evades the host immune system by antigenic variation of the expression of tandemly arranged paralogous genes like *bhlp*29.7 and the genes encoding for the variable surface proteins (Vsp) formerly known as *bhmp39* and *vsp39* (McCaman *et al.*, 2003; Witchell *et al.*, 2006). Variation in the expression of key OMPs might be a reason for the failure of vaccines to provide full immunoprotection in pigs. Antigenic variation of OMPs in other pathogenic spirochaetes including *Treponema* spp. (Giacani *et al.*, 2010) and *Borrelia* spp. (Dai *et al.*, 2006*)* have been reported.

The Vsp proteins are the predominant and unique surface component of *B. hyodysenteriae* (McCaman *et al.*, 2003) and were also identified in *B. piloscoli* (Trott *et al.*, 2001). Interestingly, the *B. hyodysenteriae* B204 genome encodes eight *vsp* genes, which have been shown to share high sequence similarity among each other. These eight genes are divided into two paralogous loci termed *vspA-D* and *vspE-H* (Cullen *et al.*, 2004). Further *in vitro* studies have shown that VspF and VspH were the transcripts most abundantly expressed by *B. hyodysenteriae* B204. Moreover, the data showed that the *vspE-H* genes produced a monocistronic transcript. Each gene seems to be regulated individually, supporting the theory that they are involved in antigenic variation (Witchell *et al.*, 2006). Additionally the *vsp* genes have been detected in other *B. hyodysenteriae* strains and are annotated in the genome WA1 (Bellgard *et al.*, 2009). However, further investigation revealed that the protein sequences of the different Vsp were highly conserved among strains but the number of genes coding for these proteins differed (Witchell *et al.*, 2011). Witchell *et al* (2011) identified two additional genes termed *vspI* and *vspJ* in the *B. hyodysenteriae* strains WA1, B204 and X576 which were separated from the other known *vsp* gene clusters. Although annotation of the genome of WA1 included the gene *vspH* (BHWA1_02382), sequence alignments showed that it shares only 60% sequence

similarity with *vspH* of strain B204 but 96% sequence identity with the gene *vspI*. Thus, the genes *vspG* and *vspH* were missing in the genomes of *B. hyodysenteriae* WA1 and X576 whereby *vspD* was also absent in X576. However, the biological role of these secreted proteins remains still hypothetical.

1.11 Potential virulence factors

Brachyspira hyodysenteriae is recognized as the most pathogenic *Brachyspira* species. Studies have shown that *B. hyodysenteriae* contains more genes involved with lipopolysaccharide biosyntheses, motility, chemotaxis and adhesion than *B. piloscoli* and *B. murdochii* (Wanchanthuek *et al.*, 2010) which might correspond to greater virulence potential.

1.11.1 Haemolysis

An early method of differentiating between pathogenic and non-pathogenic *Brachyspira* spp. species was on the basis of haemolytic activity. *Brachyspira hyodysenteriae* appears strongly β-haemolytic on blood agar whereas all of the other *Brachyspira* species are only weakly-β-haemolytic intestinal spirochaetes (WBHIS). Studies have shown that strongly–β-haemolytic strains of *Brachyspira* spp. generally induce significantly greater caecal inflammation than weakly-β-haemolytic isolates (Burrough *et al.*, 2012). The presence of strong haemolytic activity is therefore thought to be associated with pathogenicity (Hughes, 1975). However, weakly-haemolytic spirochaetes have been found to cause disease, too. For instance, *B. piloscoli* causes IS in pigs, which is a milder form of colitis and diarrhoea. Thus this classification is useful to distinguish between *B. hyodysenteriae* and other *Brachyspira* species but is not adequate for further differentiation. The haemolysin is cytotoxic for a number of cell lines (Kent & Lemcke, 1984). Hybridization experiments identified three separate haemolysin genes, referred to as haemolysin A (*tlyA*: BHWA1_00238), haemolysin B (*tlyB*: BHWA1_01228) and haemolysin C (*tlyC*: BHWA1_01427) which encode gene products with molecular masses of 26.9, 93.3 and 30.8 kDa, respectively. However, none of the three genes have been linked directly by protein sequencing to a *B. hyodysenteriae*-specific gene product displaying haemolytic activity, or to the native β-haemolysin (Hsu *et al.*, 2001). To-date seven potential

haemolysins genes are known in *B. hyodysenteriae*: The three previously mentioned, an acyl carrier protein containing beta- haemolysin (*hylA*: BHWA1_02643), genes encoding putative haemolysin III (BHWA1_00448 and BHWA1_01870) and a putative haemolysin CBS domain containing protein (BHWA1_00587). The *tlyA* gene is required for strong haemolysis and is present in all tested *B. hyodysenteriae* strains and *B. intermedia* but is absent in *B. innocens, B. piloscoli* and *B. murdochii* (Hsu *et al.*, 2001). Furthermore, it has been suggested that *B. innocens* contains genes related to *tlyB* and *tlyC* (ter Huurne & Gaastra, 1995). Immune serum from infected pigs did not recognize *tlyA*, suggesting that it might not be a strong immunogen. Hyatt *et al.* (1994) produced a *tlyA* mutant from *B. hyodysenteriae* strain B204 and tested its virulence in pigs. The mutant strain was able to colonize but did not cause SD. Moreover, pigs infected with a *tlyA* mutant strain were partially-protected against challenge with virulent *B. hyodysenteriae* strains (Hyatt *et al.*, 1994). Interestingly, haemolysin extracted from an avirulent strain of *B. hyodysenteriae* VS1 was less toxic for cell cultures (Kent & Lemcke, 1984), supporting the hypothesis that haemolysin is an important virulence factor of *B. hyodysenteriae*.

1.11.2 Chemotaxis and Motility

Chemotaxis and motility have been shown to be essential for the colonization of several pathogens and thus play an important role in virulence. The *B. hyodysentriae* WA1 genome contains a total of 84 genes (3.5% of the genome) which are related to chemotaxis and motility functions (Bellgard *et al.*, 2009).

Motility resulting from the periplasmic flagella (PF) of *B. hyodysenteriae* is thought to play an important role in colonization of the intestine and therefore in the enteropathogenicity of this spirochaete. Due to the unique structure of PF, *B. hyodysenteriae* is highly motile in viscous material and therefore able to colonize the crypt of Lieberkuehn (Kennedy *et al.*, 1988). Genome sequencing of WA1 has shown that 22 out of 24 defined core genes (Liu & Ochman, 2007) involved in the flagella biology of bacteria are present in *B. hyodysenteriae* and are listed and discussed in **Chapter 4**. The flagella rod usually is composed of four substructural subunits FlgG, FlgC, FlgH and FlgI. The assembly genes *flgH* and *flgI* responsible for the L and P ring proteins in the outer-membrane were absent and known to be deficient in other spirochaetes (Bellgard *et al.*, 2009). The reason for this might be that spirochaetes flagella compared to eubacterial flagella does not penetrate through the outer-

membrane and elongate outside the cell (Chevance *et al.*, 2007). Kennedy *et al.* (1988) proposed three different mechanisms by which spirochaetes could colonise the intestinal mucosa, these being 1) specific or non-specific adhesion to epithelium or mucus, 2) co-adhesion to adherent microorganisms or 3) chemotactic and/or motility-regulated mucus association. Although *B. hyodysenteriae* cells have been seen in and around necrotic epithelial cells as well as lamina propria, no evidence of deep invasion of tissue has been detected. Milner and Sellwood (1994) hypothesised that mucus colonization alone may be the only mechanism by which *B. hyodysenteriae* associates with the intestinal epithelium and adhesion may not be relevant. Adhesion of *B. hyodysenteriae* to epithelia cells was demonstrated *in vitro* showing optimal adhesion under neutral pH conditions. Moreover the studies indicated that the hyperimmune and convalescent sera reduced the adhesion by 80% (Bowden *et al.*, 1989). However, none of the studies gave evidence of invasive attachment of epithelia cells. Therefore, chemotaxis alone could enable *B. hyodysenteriae* to colonise the mucosal surface (Milner & Sellwood, 1994).

Brachyspira hyodysenteriae PFs are composed of four filament proteins; FlaA and three FlaB proteins. Former investigations provided evidence that FlaA comprises the sheath and that FlaB proteins form the core (Li *et al.*, 2000). In previous studies, the effect of a single deletion mutation in each of the filament genes of *B. hyodysenteriae* was analyzed in detail. Mutagenesis of all three filament genes resulted in decreased motility compared to the wild-type (Li *et al.*, 2000; Rosey *et al.*, 1996). Rosey *et al.* (1996) created a double *flaA/flaB1* mutant, and used it to show that this mutant was less virulent in mice (Rosey *et al.*, 1996). Li *et al.* (2000) found out that only the *flaB1/flaB2* double mutant was completely non-motile whereas the single mutants of *flaB1* and *flaB2* remained motile (Li *et al.*, 2000). Thus, the data suggests that the protein functions of FlaB1 and FlaB2 overlap such that neither of the proteins is essential for cell motility and therefore for virulence.

A total of 46 genes encoding methyl-accepting chemotaxis proteins (MCPs) were identified in the *B. hyodysenteriae* WA1 genome which were twice as many as identified in *L. interrogans*, *T. pallidum* and *B. burgdorferi* indicating the need for the bacteria to adapt and survive in the complex environment of the large intestine. Chemotaxis assays have shown that virulent *B. hyodysenteriae* strains were significantly more chemotatic than avirulent strains of *B. hyodysenteriae* and *B. intermedia* (Milner & Sellwood, 1994). Interestingly, the non-pathogenic strain *B. innocens* B256 did not show any chemotactic response in hog gastric mucin (HGM).

1.11.3 Proteases

Proteases have been shown to be an important virulence factor in other spirochaetes by increasing their tolerance of oxidative stress (Guyard *et al.*, 2006) and contributing to the degradation of host cells (Lee *et al.*, 2002). In the WA1 genome 15 proteases were identified that may be involved in causing disease due to destruction of host tissue. The serine protease BHWA1_00767 was found to have ≥ 42% homology with *htrA* which is known to be an important virulence determinant in *Salmonella enterica* (Bellgard *et al.*, 2009).

1.11.4 NADH oxidase

The production of NADH oxidase of *B. hyodysenteriae* is considered to be an important factor in the pathogenesis of SD. The ability to withstand oxidative stress enables *B. hyodysenteriae* to colonize the large intestine of the pig. NADH oxidase has been described as a mechanism by which *B. hyodysentriae* cells are able to tolerate oxygen, by using the enzyme as an antioxidant defence system or take advantage of it as an alternative NADH–regenerating pathway (Stanton *et al.*, 1999). Additional oxidative stress mechanisms like NADH peroxidase, superoxide dismutase (SOD) and catalase appear to be important adaptations enabling *B. hyodysenteriae* to establish and persist among the oxygen-respiring tissues of the swine (Stanton, 1989). Stanton *et al.* (1999) isolated and characterized mutant strains lacking oxidase activity (encoded by the *nox* gene) which exhibited reduced virulence for swine in comparison with the wild-type strain (Stanton *et al.*, 1999). Moreover fewer animals were colonized, and infected animals exhibited milder signs of SD. Thus, these results provided evidence that NADH oxidase serves to protect *B. hyodysenteriae* cells against oxygen toxicity and that the enzyme, in that role, contributes to the pathogenic ability of the spirochaete.

1.11.5 Iron Transport systems

Iron is an essential element which is required by the majority of organisms. Acquisition of iron from host tissues by invading bacteria is an essential step in infection as most of the host-iron is stored intracellular as ferritin, haem compounds and the glycoprotein's

transferrin and lactoferrin, and is therefore unavailable to the microorganism (Weinberg, 1984). In Gram-negative bacteria, active transport of molecules from the periplasm to the cytosol occurs via well-conserved systems (Higgins, 1992). Transport of free-iron from the periplasmic space into the cytoplasm is proposed to occur by a classic active transport process involving a periplasmic binding protein, a specific cytoplasmic permease, and an energy-supplying nucleotide binding protein. This active ABC transporter system is widely used among pathogenic Gram-negative bacteria (Dugourd *et al.*, 1999). Dugourd *et al.* (1999) described an ABC importer system of *B. hyodysenteriae* implicated in iron import (BIT-system). The BIT-system includes an ATP-binding protein (BitD), two hydrophobic cytoplasmic membrane permeases (BitE and BitF) and at least three lipoproteins (BitA, BitB and BitC) with homology to iron periplasmic binding proteins of *Actinobacillus pleuropneumonaie* (Dugourd *et al.*, 1999). Thus, it is thought that the transport of iron in *B. hyodysenteriae* occurs by a similar mechanism to that observed in other Gram-negative bacteria. Spirochaetes including *Treponema* and *Leptospira* have been shown to obtain iron via lactoferrin-binding proteins, hemophores or xenosiderophores. To-date the BIT system is the only described mechanism by which *Brachyspira* is known to acquire iron. Due to the requirement of iron for many microorganisms and limited knowledge of iron-uptake in *Brachyspira* spp. further investigations and identification of iron-acquisition systems would enhance the understanding *B. hyodysenteriae* pathogenicity which will be discussed in **Chapter 5**.

1.11.6 The gene transfer agent VSH-1

It has been shown that a 16.3 Kb region of a prophage-like agent named VSH-1 (virus of *Serpulina hyodysenteriae*) is integrated into the host genomes of *B. hyodysenteriae* 204 and WA1 and is likely to be involved in natural gene transfer and recombination within the species (Humphrey *et al.*, 1997; Matson *et al.*, 2007). Gene transfer agents (GTA) have been described for diverse anaerobe and facultative anaerobe bacteria species like *Rhodobacter capsulatus* (Yen *et al.*, 1979), *Methoanococcus voltae* (Eiserling *et al.*, 1999) and *Desulfovibrio desulfuricans* (Rapp & Wall, 1987), and have shown to enable packing of short and random DNA fragments. Hence they are important vehicles for horizontal gene transfer and provide advantages for their bacterial host *e.g.* influencing the bacterial colonization and invasion, enhancing bacterial resistance to phagocytes (Kaneko *et al.*, 1997) and antibiotic resistance (Ubukata *et al.*, 1975). Moreover, phages are known to

contribute to evolution and virulence of many pathogens. The prophage-like agent VSH-1 has been purified and characterized by treating *B. hyodysentriae* cells with mitocymicin C (Humphrey *et al.*, 1997). Mitomycin C is known to be a DNA damaging agent which triggers an SOS response to maintain intact DNA described and studied mainly in *E. coli* (Vericat *et al.*, 1984). In addition VSH-1 virions could be detected by using agarose gel electrophoresis (Turner & Sellwood, 1997) and co-culturing of *B. hyodysenteriae* cells (Stanton *et al.*, 2001). Previously, it was also shown that carbadox- and metronidazol induce the production of the VSH-1 phage (Stanton *et al.*, 2008). Induction of the phage results in random packing of 7.5 kb of genomic *B. hyodysenteriae* DNA (Humphrey *et al.*, 1995) which enables transfer of chromosomal genes, including antibiotic resistance genes, between *B. hyodysenteriae* isolates (Humphrey *et al.*, 1997; Matson *et al.*, 2007; Stanton *et al.*, 2008). Studies of the VSH-1 phage indicate that it is a defective prophage which is incapable of self-replication and thus replication is likely to be dependent upon chromosomal replication. Purified VSH-1 virons have been shown to be avirulent when added to cultures of *B. hyodysenteriae* cells (Humphrey *et al.*, 1997) which is typical feature among GTAs (Bertani, 1999). Analysis of VSH-1 has revealed that the agent consists of three clusters with genes encoding for the head (seven genes), tail (seven genes) and lysis (four genes). However, seven ORFs remain still undefined (Matson *et al.*, 2007). None of the head and tail proteins shared significant amino acid similarity with any of the proteins in GenBank. In addition two genes encoding for endolysin (*lys*) and a class II holin protein (*hol*) could be identified to play an important role in the release of VSH-1 virions from *B. hyodysenteriae* cells (Stanton *et al.*, 2009). The VSH-1 element *vsp38* was found in *B. hyodysentriae*, *B. piloscoli*, *B.intermedia*, *B. innocens B. murdochii* and *B. alvinpulli* suggesting that the VSH-1 agent is widespread among *Brachyspira* spp. (Calderaro *et al.*, 1998a; Calderaro *et al.*, 1998b; Stanton *et al.*, 2003)). Interestingly, none of the known elements could be found in *Treponema*, *Borrelia* and *Leptospira* (Stanton *et al.*, 2003). Moreover, all genes detected in the VSH-1 element in *B. hyodysenteriae* B204 could be identified in *B. hyodysenteriae* WA1, *B. intermedia* HB60 and *B. piloscoli* 95/100 (Motro *et al.*, 2009). Nevertheless, rearrangements, differences in the amino acid sequences of individual gene products as well as presence and absence of certain genes on the VSH-1 agents in all tested strains were detected mirroring the phylogeny of the three species (Motro *et al.*, 2009). The novel VSH-1 is the only known gene acquisition mechanism of *B. hyodysenteriae* and is likely to contribute to the recombinant and diverse population of this pathogen (Trott *et al.*, 1997).

1.12 Non-typical *B. hyodysenteriae* strains

In a number of rare cases, so called atypical *B. hyodysenteriae* strains have been identified in the UK (Thomson *et al.*, 2001). *Brachyspira hyodysenteriae* strains were isolated from pigs with diarrhoea. Standard diagnostic tests resulted in two different propositions about the species of *Brachyspira* present. Isolates had phenotypic characteristics consistent with *B. hyodysenteriae* whereas the 23S rRNA-based PCR test indicated genetic sequences consistent with *B. innocens*. Further sequencing studies of the 23S rRNA gene showed that all isolates had the same single base change (at bp 1114 from G to A) but seven base changes from *B. hyodysenteriae* $B78^T$. PCR results as well as phylogenetic analysis based on 23S rRNA sequences suggested that these isolates were a subset of *B. innocens*. In contrast, 16S rRNA sequences showed that all isolates clustered in the main *B. hyodysenteriae* cluster. The literature indicates that these atypical *B. hyodysenteriae* strains constitute a very small percentage of the *Brachyspira* population (≤1%), but knowledge of their existence is important especially for using the 23S rRNA method.

1.12.1 Avirulent *B. hyodysenteriae* strains

Brachyspira hyodysenteriae is regarded as a primary pathogen. Pigs infected with *B. hyodysenteriae* usually develop SD, however, in the early 80's an avirulent strain of *B. hyodysenteriae* was isolated from herds free of SD in Mexico (Lyson, 1982). Varying degrees of virulence have been detected within *B. hyodysenteriae* and atypical *B. hyodysenteriae* have been recognized (Hampson, 1997; Thomson *et al.*, 2001). Variation in virulence among *Brachyspira* spp. has been studied in piglets and mouse models (Achacha *et al.*, 1996). Achacha *et al.* (1996) found that piglets inoculated with the reference strains B234 and A-1 did not show any clinical signs of SD, and remained culture negative. Moreover, attenuation of pathogenic *B. hyodysenteriae* isolates B78 and A-1 has been recognized after ≥35 times laboratory passages (Hyatt *et al.*, 1994; Kim *et al.*, 2010). Thus, it has been suggested that some *B. hyodysenteriae* strains are more likely to undergo spontaneous mutation than others, and become less virulent as a result. Researchers have concluded that non-pathogenic *B. hyodysenteriae* might form part of the normal flora of pigs, due to confirmation of the presence of *Brachyspira* spp. using the fluorescent antibody test (FAT) in swine without any clinical signs of SD (Mechow, 1975). Hampson *et al.* (2007) proved in a pig experiment that two avirulent strains of *B. hyodysenteriae*

(VS1 and SA3) remained non-pathogenic, whereas pigs infected with virulent strains developed bloody diarrhoea and mucus production (Hampson, 2007). At post-mortem only one of 20 pigs inoculated with avirulent *B. hyodysenteriae* strain had mild localized lesions. In comparison seven of ten pigs infected with the virulent *B. hyodysenteriae* isolate showed typical lesions throughout the colon and caecum as well as mucohaemorrhagic colitis. The strain *B. hyodysenteriae* SA3 was demonstrated to cause less severe lesion in chickens and did not significantly depress growth rates compared to the virulent strain WA15 (Trott & Hampson, 1998). This data confirms that mild virulent *B. hyodysenteriae* isolates are less able to induce SD which might be associated with a lack of colonization due to reduced motility and chemotaxis. Chemotaxis and motility has been studied in some porcine spirochaetes to investigate the importance of colonization (Millner *et al.*, 1994). It was shown that virulent strains of *B. hyodysenteriae* were significantly more chemotactic than the avirulent strains SA3 and VS1. Interestingly, *B. innocens* B256 is reported to be non-pathogenic and was not chemotactic at all (Milner & Sellwood, 1994). All strains possessed similar numbers of endoflagella. Therefore, motility probably does not play an important role in chemotactic behaviour. Nevertheless, pathogenic *B. hyodysenteriae* has been observed to be consistently more frequently present within the crypt than presumptive avirulent isolates (J. R. Thomson, personal communication).

The presence of mildly virulent or avirulent *B. hyodysenteriae* strains poses a major problem for the current diagnosis of SD. Previous studies have shown that the majority of detected avirulent strains did not harm the pig as no typical sign of SD could be observed. To-date nothing is known as to whether these asymptomatic strains belong to the normal pig flora and therefore pose no risk for the pig health and industry. However, pig herds carrying these asymptomatic strains present a threat and make selling these animals impossible. Even though the pig appears to be healthy, the diagnosis leads to expensive medical treatment or even pig loss to eradicate the pathogen. The phenotypic characteristics of the virulent strains are indistinguishable from those of typical virulent strains.

1.13 Project objectives and aims

The existence of avirulent *B. hyodysenteriae* strains brings the need for a diagnostic which would be able to distinguish between virulent (isolated from a pig showing typical sign of SD) and avirulent strains (isolated from a pigs which did not show or develop any characteristical symptoms of SD).

Therefore, the primary objective of the project was to identify substantial differences between virulent and presumably avirulent *B. hyodysenteriae* isolates using genomics and proteomics approaches that can subsequently be used towards disease control through a potential differential test or vaccination by:

- Sequencing and annotation of a genome of one representative of virulent isolate (P8544) and one genome of a representative of putative avirulent isolate (P7455) of *B. hyodysenteriae*.

- Comparing the genomes and gene content including the published pathogenic *B. hyodysenteriae* strain WA1 to detect genes which are shared and distinct in each isolate.

- Survey of the identified genes being present/absent by comparative genomics in a wider panel of virulent and putative avirulent *B. hyodysenteriae* isolates in order to verify whether putative differential markers are truly typical for each group of isolates.

- Investigation and comparison of the proteome profile of the outer-membrane and whole cell of the representative virulent isolate P8544 and the representative putative avirulent isolate P7455 under iron-restricted and iron-replete growth conditions.

Chapter 2: Materials and Methods

2.1 Source of general reagents and chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Restriction endonucleases, 25 mM $MgCl_2$, 1 kb DNA ladder, 100 bp DNA ladder and 6 × loading dye were purchased from Promega UK Ltd (Hampshire, UK). Kits for extraction of RNA were obtained from Ambion (West Sussex, UK) unless otherwise stated.

Sterilisation of media and reagents was achieved by autoclaving or by filtering. In the latter case, volumes of less than 100 ml were sterilised by filtration using 0.2 µm pore diameter syringe filters (Milipore; Watford, UK) and stored at 4°C until required. Larger volumes were drawn through 0.2 µm Stericup® filter units (Millipore) under vacuum. Unless otherwise stated, all large volumes of media, buffers and other solutions were sterilised by autoclaving at 121°C for 15 minutes at a pressure of 15 psi.

Where antibiotics were required to be added to growth media, concentrated stock solutions of 10 or 100 mg/ml were used. Spectinomycin and ampicillin stocks were prepared by dissolving powder in distilled water. Rifampicin stock solutions were prepared in methanol. Aliquots of 1 ml were prepared and stored at -20°C until required.

Oligonucleotide primers were synthesised to-order by Sigma-Genosys Ltd. (Haverhill, Suffolk, UK). Stock solutions were prepared by reconstituting lyophilised primers to 100 µM in ddH_20. Oligonucleotides were subsequently stored at -20°C until required.

2.2 *Brachyspira hyodysenteriae* field isolates

Bacterial strains were obtained from the Scottish Agriculture College (SAC) Veterinary Science Division, Edinburgh. The strains were isolated from pigs from different farms across different geographical regions, including Scotland, England, the EU and other non-EU countries, between the years of 1997 and 2009. The definition of avirulent strains is explained in **Table 2.1**. All field isolates are summarized in **Table 2.2** and **Table 2.3** .

2.2.1 Characterisation and definition of avirulent *B. hyodysenteriae* isolates

Avirulent or low-virulence *B. hyodysenteriae* isolates were defined according to the characteristics described in **Table 2.1**.

Table 2.1: Definition of avirulent *B. hyodysenteriae* field isolates*.

Factor	Characteristic
Herd History	• No history of SD in the herd, or in grow-out units, or herds supplied with pigs from the herd • No clinical evidence of diarrhoea in wean-to-finish pigs at veterinary inspection of the herd when samples taken • No antibiotic medication used • Herds used standard commercial compound pig rations with no additives that might have suppressed infection • Isolates derived from fresh faecal samples from normal pigs that were tested as part of routine health screening measures • After *B.hyodysenteriae* isolation, a minimum of 6 months of continued clinical disease freedom in the absence of any intervention measures had passed before considering the isolate 'low virulence/avirulent'
Laboratory Tests	• The presence of *B. hyodysenteriae* was initially detected by PCR testing (23S-RNA) • The isolates that were subsequently recovered had phenotypic characteristics that were typical of *B.hyodysenteriae* • A notable feature was generally reduced viability on subculture, with some isolates becoming weaker and sometimes dying off • Recovery of isolates stored at -80°C under standard conditions was often poor • The morphological appearance of organisms by light microscopy was typical of *B. hyodysenteriae.*
Infected Pigs	• Pigs carrying and shedding these isolates showed no adverse impact on health, growth or performance, as reported by highly-experienced pig veterinarians who attended the units • On post mortem examination, the macroscopic appearance of the caecum and colon of such pigs were normal, the content was of normal consistency with no evidence of increased mucus or blood • On histopathological examination of the colon, there was no appreciable colonic crypt hyperplasia or goblet cell hyperplasia • The number and types of mucosal cellular infiltrates were either within normal limits or there were sparse/low-grade or mild lymphocytic infiltrates only • Silver staining showed large spirochaetes typical of *B.hyodysenteriae* on the surface epithelium and at the openings of colonic crypts. The spirochaetes did not appear to have entered into the body or base of the crypts (which usually happens in *B.hyodysenteriae* infections)

*J. R. Thomson, personal communication.

Table 2.2: Overview of *B. hyodysenteriae* field strains isolated from symptomatic and asymptomatic pigs.

Strain*	Origin	Unit	Year	clinical history
P7455	England	32	2008	Non-clinical
P8544	England	33	2009	Virulent
P7377/3	England	32	2008	Non-clinical
P8226/7	EU	34	2009	Virulent
QCR1	England	35	2008	Virulent
WA1	Australia	?	2007	Virulent

* The isolates were available as glycerol stocks and routinely cultured during this study.

Table 2.3: **Panel of *B. hyodysenteriae* field strain genomic DNA isolated from symptomatic and asymptomatic pigs.**

Strain*	Origin	Unit	Year	clinical history
P1093/6/01	Scotland	1	2001	Non-clinical
P949/4/00	Scotland	2	2000	Non-clinical
P949/5/00	Scotland	2	2000	Non-clinical
P949/9/00	Scotland	2	2000	Non-clinical
P944/14/00 †	England	3	2000	Non-clinical
P5943/07#	EU	4	2007	Virulent
P6129/2/08	Scotland	5	2008	Virulent
P6812/1/08#	England	6	2008	Virulent
P6858/08#	England	7	2008	Virulent
P7124/08	England	8	2008	Virulent
P7210/1/08#	Scotland	9	2008	Virulent
P7286/1/08	Scotland	10	2008	Virulent
P7309/1/08#	England	11	2008	Virulent
P7271/1/08	England	12	2008	Virulent
P7346/08	England	13	2008	Virulent
P7343/4/08	England	14	2008	Virulent
P7381/2/08	England	15	2008	Virulent
P7458/1/08#	England	16	2008	Virulent
P7458/2/08	England	16	2008	Virulent
P5683/07#	EU	17	2007	Virulent
P278/97	Scotland	18	1997	Non clinical
P271/97	Scotland	19	1997	Non clinical
P265/97	Scotland	20	1997	Non clinical
P271/98	Scotland	21	1998	Non clinical
P246/2/97	Scotland	22	1997	Non clinical
P935/LI/00	England	23	2000	Non clinical
P949/3/LI/00†	England	2	2000	Non clinical
P354/2/97	England	24	1997	Non clinical
P264/97	Scotland	25	1997	Non clinical
P257/97†	Scotland	19	1997	Non clinical
P252/A/97	Scotland	22	1997	Non clinical
P252/B/97†	Scotland	22	1997	Non clinical
P935/LI/00†	Scotland	23	2000	Non clinical
P935/2/00	Scotland	23	2000	Non clinical
P944/15/00	England	3	2000	Non clinical
P7649/2/09	England	25	2009	Non clinical
P7646/5/09#	England	26	2009	Virulent
P7645/09#	England	27	2009	Virulent
P7624/1/09	Scotland	28	2009	Virulent
P7620/1/09	Scotland	29	2009	Virulent
P7563/3/08#	Non-EU	30	2008	Virulent
P7486/2/08	England	31	2008	Virulent
QCR2	England	36	2008	Virulent
P8408	England	33	2009	Virulent

*Only genomic DNA was available for these strains, not viable organisms.

†These isolates were confirmed to be non-pathogenic by pig challenge experiments.

#Isolates analysed by Multilocus Sequence Typing as part of a distinct study (Figure 3.4).

2.3 Microbiological techniques

2.3.1 Bacterial storage

Bacterial strains and isolates were stored as 20 % (v/v) glycerol stocks. Stocks were prepared by aliquoting 750 µl of overnight bacterial cultures into cryovials containing 250 µl of sterile 80 % (v/v) glycerol. After mixing, each glycerol stock was placed at -80 ^{o}C until required. Glycerol stocks were generally utilised for inoculation of starter cultures for experimental work.

2.3.2 General culture and maintenance of *B. hyodysenteriae*

Generally, *B. hyodysenteriae* was routinely propagated on Blood Agar (BA) plates (Sheep Blood agar No 2, E&O laboratories, UK) supplemented with 5 % (v/v) sheep blood. Frozen glycerol stocks of *B. hyodysenteriae* were allowed to thaw at RT and 100 µl of spirochaete suspensions were then streaked onto BA plates. The inoculated plates were then incubated under anaerobic conditions in 3.5 l AnaeroJars, using AnaeroGen™ sachets (Oxoid, Hampshire, UK) at 37°C for up to 10 days. Anaerobic conditions were confirmed using an anaerobic indicator strip (Oxoid) which turned from pink to white in the absence of oxygen. For growth curve experiments and generating cultures of larger volumes, an anaerobic workstation was used as described under section **2.3.4**. Subsequently, the purity of each isolate was checked and sub-cultured. Liquid cultures of *B. hyodysenteriae* were generally propagated in Brain Heart Infusion broth (BHI) supplemented with 5% (v/v) foetal calf serum (FCS; Promega UK Ltd). The BHI medium was prepared in-house from powder (Oxoid) according to the manufacturer's guidelines. The medium was sterilised by filtration, as described in section **2.1**. Where required, BHI was supplemented with antibiotics, to the following final concentrations: spectinomycin (400 µg/ml) and rifampin (15 µg/ml). The growth derived from two BA plates was routinely used to prepare *B. hyodysenteriae* 2 to 5 ml suspensions for inoculation of liquid media. Previous experience in our lab has shown that it is difficult to grow *B. hyodysenteriae* in large volumes; therefore, starter cultures of between 5 to 10 ml were used to inoculate larger volumes of BHI in 25 cm^2 tissue culture flasks (Corning, UK). Cultures were gently agitated at *ca.* 200 rpm during growth, and were incubated until mid-log phase under the same conditions as

described above. Subsequently, the purity of cultures was checked by Gram-stain before proceeding with further experiments.

2.3.3 Confirmation of *B. hyodysenteriae*

Faecal samples may contain a mixture of *Brachyspira* species. Although the species differ in length and size it is not possible to distinguish between the different species under the light microscope. Therefore, to confirm the presence and purity of each isolate of *B. hyodysenteriae*, the following tests were performed:

2.3.3.1 *Gram-stain*

To check the purity of liquid cultures, 500 µl were centrifuged at 14,000 rpm for 3 min. Supernatants were removed and the pellets used for further tests. Cultures were tested for purity using a Gram-stain Kit (Sigma). A sterile loop was used to transfer a drop of saline onto a clean microscope slide. Then, another sterile loop was used to retrieve a small amount of culture from a plate, which was dispersed in the saline by gentle agitation of the loop. Cells were heat-fixed over a Bunsen flame, and then the dry smear was flooded with Crystal Violet for 10 sec and washed briefly to remove excess stain. Then, the slide was flooded with iodine solution and incubated and washed as described before. Afterwards the slide was decolourised with acetone until the moving dye front had passed the lower edge of the section. Subsequently, the decolourizer was rinsed off with tap water and the slide was flooded with Safranin for 15 sec. After rinsing off the stain with tap water, the slide was blotted dry using blotting paper. Following this, the slide was examined by light microscopy (2.3.4).

2.3.3.2 *Microscopy of B. hyodysenteriae*

To confirm that pure cultures of *B. hyodysenteriae* were obtained, Gram-stained cultures (**2.3.3.1**) were examined using an Olympus BH-2 light microscope (Olympus, UK) with oil immersion using a 100 × objective lens. Thus, it was possible to distinguish *B. hyodysenteriae* from other bacteria (including Gram-positive organisms) on the basis of

stain colour and their unique helical cell morphology. Further characterization and validation of the isolates as being *B. hyodysenteriae* was carried out as described below.

2.3.3.3 *Biochemical tests*

Brachyspira isolates were identified according to phenotype and molecular-based techniques such as those described in the **Chapter 1.**

2.3.3.4 *β-haemolysis*

The capability of *B. hyodysenteriae* to be strongly β-haemolytic was checked by streaking isolates on BA plates and comparing them to the mildly β-haemolytic type strain of *B. innocens* 29796 ATCC **(Table 2.3).**

Table 2.4: Bacterial strains used as positive and negative controls for the biochemical profiling.

Bacterial strain	Source
B, innocens 29796	ATCC
E.coli 10419	NCTC
Salmonella poona 4840	NCTC

2.3.3.5 *Indole spot test*

The indolee spot test is routinely-used in the grouping and identification of anaerobic bacteria; the test works by virtue of the colourimetric detection of indolee, which is split from tryptophan by certain organisms producing a tryptophannase. A 13 mm diameter filter disc (BD Diagnostics; Oxford, UK) was placed onto an agar plate, within an area of good spirochaete growth. After 5 min the disc was removed and one drop of 1 M HCl was added to the surface of the disc. A positive reaction was indicated by the formation of a blue colour within one minute, whereas a negative reaction was shown by the formation of a pink colour within 1 min. Control reactions were conducted using *Escherichia coli* NCTC 10419 (positive control) and *Salmonella poona* NCTC 4840 (negative control) (**Table 2.3**).

2.3.3.6 *Hippurate hydrolysis*

The ability to hydrolyse hippurate was used to distinguish *B. hyodysenteriae* from some other anaerobic bacteria frequently associated with faeces (e.g. *Campylobacter*). For this test, 1 ml of saline was inoculated with a small amount of material from a culture plate using a sterile swap. A sodium hippurate tablet (BD Diagnostics) was added to the solution which was then incubated anaerobically for 2 hours at 37°C. After incubation, 5 drops of Ninhydrin reagent (Ninhydrin 0.88 g, butanol 12.5 ml, acetone 12.5 ml) were added. The suspension was mixed by gentle shaking and incubated for 1 min at 37°C to detect hippurate hydrolysis. A positive reaction was indicated by the formation of a deep blue or purple colour, whereas a negative reaction was indicated by the solution turning light blue or remaining colourless.

2.3.3.7 *Digestion of 23S rRNA PCR amplicon*

All 23S rRNA fragments were amplified from target strains by Polymerase Chain Reaction (PCR). PCR amplicons were subjected to restriction endonuclease digestion to allow differentiation between *B. hyodysenteriae*, *B. innocens* and *B. piloscoli* (Barcellos *et al.*, 2000) based upon digest profiles.

Polymerase Chain Reaction was performed using the primers SF1 and SR1 which allow the amplification of a highly conserved region between nucleotides 874 and 1,429 of the 23S rRNA gene of *Brachyspira* spp., giving rise to a characteristic product of 555 bp (Teran-Dianderas, 1997). For PCR, MegaMix-Blue (Microzone Ltd, UK) was used, containing 200 µM of each dNTP, 2.5 mM $MgCl_2$, *Taq* polymerase (1 U/50 µL), and supplemented with 0.2 mM of each primer and DNA template. Thermal cycling was carried out for an initial cycle of 4 min at 94°C (denaturation), 1 min at 60°C (annealing), and 2 min at 72°C (extension), followed by a further 39 cycles of 1 min denaturation, 1 min annealing and 2 min extension at the same temperatures. Subsequently PCR amplicons were stored at 4°C until needed.

Each 23S rRNA amplicon was analysed by Restriction Fragment Length Polymorphism (RFLP). Restriction endonuclease digestions were conducted according to the

manufacturer's instructions. Reactions (20 µl) were carried out in 0.5 ml microcentrifuge tubes containing 40 × reaction buffer, 2.5 units of *Hph*I (New England Biolabs, UK), 3 µl PCR product and ddH_20 to 20 µl. Restriction endonuclease digests were incubated at 37°C for 90 min, then electrophoresed through 10 % (w/v) polyacrylamide gels **(Appendix 1)** and visualized by silver staining **(2.9.2)**. The *HphI* recognition site (5'-GCTGA-3') is present at different positions in the 555 bp product of different *Brachyspira* spp.; thus, different RFLP patterns were obtained which were unique for each species (Barcellos *et al.*, 2000). The RFLP analysis of *B. hyodysenteriae* revealed 3 fragments of 298 bp, 201 bp and 51 bp, while *B. innocens* gave rise to 2 fragments of 290 bp and 267 bp, and *B. piloscoli* gave rise to 3 fragments of 472 bp, 57 bp and 29 bp.

2.3.4 Growth curve determination

The Whitley A35 anaerobic workstation (Don Whitley Scientific; Shipley, UK) was used to analyse growth characteristics of *B. hyodysenteriae*, as well as generating larger volumes of cultures for proteomic analyses and gene expression experiments.

Cultures were routinely propagated under an anaerobic atmosphere containing a standardized mixture of 10% hydrogen, 10% carbon dioxide and 80% nitrogen (BOC, UK). Five ml starter cultures of *B. hyodysenteriae* were prepared as described in section **2.3.2** and incubated in the anaerobic chamber. When cells reached mid-log-phase, 20-50 ml (depending on the experiment) of BHI supplemented with 5% (v/v) FCS (and other reagents as required) were inoculated with 0.5-1 ml of the starter cultures. The $OD_{600\ nm}$ was measured at regular time intervals (usually every 2 hours) to monitor the growth rate. Growth experiments were carried out in triplicate, where three independent cultures from three independent starter cultures were grown for each condition. The average absorbance of the three biological replicates was used to generate growth curves for each growth condition. During the experiment, the purity of the cultures was checked by Gram-stain (**2.3.3.1**) every twelve hours to ensure that they did not contain any contaminants.

2.3.5 Determination of Colony forming units (CFU) of *B. hyodysenteriae*

In order to count viable cells of *B. hyodysenteriae*, 1 ml of culture was taken at time points 0, 16, 24 and 40 hrs and centrifuged at 17,970 × *g* after first determining the OD_{600nm} value. The pellet was washed with 1 × PBS and resuspended in 1 ml of 1× PBS. Bacteria were then 10-fold serially diluted (from 10^1 to10^{10}) by transferring 100 µl of bacterial suspension to 900 µl of 1× PBS, vortexing to mix, and so on. Following this, 10 µl of each dilution was spotted in triplicate onto the surface of a BA plates, allowed to air dry and incubated anaerobically as described in **section 2.3.4** for 8 days. In total, three biological replicates were carried out for each strain at each time point. The number of colonies in the highest dilution yielding ≥10 colonies per drop was counted, and the CFU/ml was calculated from the mean of the number of colonies in three replicates. Plate counts were determined for three distinct experiments, and the mean of these experiments calculated.

2.4 Nucleic Acid extraction and Purification

2.4.1 Extraction and purification of genomic DNA

To provide material for DNA sequencing and PCR experiments, genomic DNA was extracted from *B. hyodysenteriae* isolates using a phenol-chloroform method as follows: Five ml cultures of *B. hyodysenteriae* were propagated in BHI with 5% (v/v) FCS for 18-20 hrs at 37°C. Cells were harvested by centrifugation at 3,893 × *g* for 10 min at 4°C. A fresh lysozyme solution (2.5 mg/ml) was made up in TE buffer (**Appendix 1**), and each bacterial pellet was suspended in 1 ml of the lysozyme buffer and was incubated for 30 min at 37°C. Then, 50 µl of Proteinase K (500 ng/µl) and 50 µl of 10% (w/v) N-lauryl-sacrosine solution were added, and incubation was conducted overnight at 55°C. Afterwards, 4 µl of RNAse A (100 mg/ml) was added to each sample which was then incubated at 37°C for a 10 min. Following this, DNA was extracted twice with 500 µl phenol:chloroform:isoamyl alcohol (25:24:1). The solution was mixed gently and incubated on ice for 10 min. The samples were centrifuged at 17,970 × *g* in a microcentrifuge for 5 min at RT, then the top layer of each sample was transferred into a clean tube and the extraction step repeated. Subsequently, the top layer of each sample was transferred into a clean tube and a further round of extraction was carried out with chloroform:isoamyl alcohol (24:1). Finally, DNA was precipitated by addition of 1 ml of

absolute ethanol and 50 µl of 3 M sodium acetate (pH 5.2). Precipitated DNA was harvested by centrifugation at 17,970 × *g* in a microcentrifuge for 20 min at 4°C. The resulting pellets were washed with 500 µl 70% (v/v) ethanol and centrifuged as before for 10 min. Afterwards, DNA pellets were air-dried at RT for 15 min prior to re-suspension in 100 µl TE-buffer (pH 8.0). The DNA was quantified and the purity determined using a NanoDrop ND-100-Vis spectrophotometer (NanoDrop technologies, Delware, USA). DNA samples were stored at -20°C until required.

2.4.2 Extraction and purification of RNA

RNA was extracted from *B. hyodysenteriae* cultures using the RiboPure™-Bacteria Kit (Ambion; Paisley, UK). Briefly, a 20 ml culture of the desired strain was prepared by inoculation of the desired medium. After the required incubation period, *B. hyodysenteriae* cells were harvested at 3,893 × *g* for 10 min. The pellet was re-suspended in 350 µl RNAwiz and vortexed vigorously for 10-15 sec. Following this, the cells were transferred to a new tube containing 250 µl ice-cold Zirconia Beads. The bacterial cells were homogenized using a Fastprep® instrument (Q.Biogene; Middlesex, UK) for 3 × 20 sec at high speed rating. Subsequently, the Zirconia Beads were pelleted by centrifugation in a microcentrifuge for 5 min at 4°C, and the lysate was transferred to a new tube. Afterwards 0.2 volumes of chloroform were added to the bacterial lysate and mixed vigorously. After 10 min incubation at RT the aqueous and organic phases were separated by centrifugation at 17,970 × *g* in a microcentrifuge for 5 min at 4°C. The aqueous phase, containing the partially purified RNA, was transferred to a clean tube and a ½ volume of absolute ethanol was mixed thoroughly with the sample. Following this, the sample was transferred to a RiboPure Cartridge filter and centrifuged for 1 min. The flow-through was discarded and the column was washed with 700 µl wash solution 1. The sample was then centrifuged under the same conditions as described previously until all liquid had passed through the filter. Additionally, the filter was washed twice with 500 µl of wash solution 2/3. The Cartridge filter was centrifuged after the second wash to remove excess wash and transferred to a fresh 1.5 ml tube. Subsequently, the RNA was eluted by adding 50 µl of pre-heated (95°C) ddH_2O to the centre of the filter and centrifuging for 1 min at 17,900 × *g*. Prior to further RNA analysis, a DNase I treatment **(2.4.3)** was performed immediately

to remove trace amounts of genomic DNA from the eluted RNA. Afterwards the RNA was stored at -20 ^{o}C until required.

2.4.3 Dnase I treatment

DNase I is an endonuclease that non-specifically cleaves single and double stranded DNA. The reagents for the treatment were provided in the RiboPure™-Bacteria Kit. The DNase treatment was carried out by adding 1/9 volume of the DNase buffer and 2 U/µl of DNase I to the eluted RNA. The mix was gently shaken and incubated for 30 min at 37°C to digest the genomic DNA. Subsequently, a volume of DNase Inactivation Reagent was added, equal to 20% of the buffer volume containing the RNA. The mixture was vortexed briefly and stored at RT for 2 min. Afterwards, the sample was centrifuged for 1 min to pellet the DNase Inactivation Reagent and the RNA solution was transferred to a new RNase-free tube. Prior to quantitative reverse transcription real-time PCR (qRT-PCR), standard PCR was carried out using the isolated and treated RNAs as a template to ensure that the RNA samples were pure and did not contain any trace of DNA.

2.4.4 Polymerase Chain Reaction

Polymerase Chain Reactions were conducted using different DNA polymerase enzymes according to the downstream applications for which amplified DNA was to be used. The procedure was routinely performed in 50 µl reaction volumes using 0.25 ml flat-topped PCR tubes (Elkay Lab Products (UK) Ltd; Hampshire, UK) or 96-well ABgene® PCR plates (Thermo Scientific, UK). In addition, template DNA varied in concentration (and hence volume) according to source. Thermal cycling was conducted using a GeneAmp® PCR system 9700 thermocycler (Applied Biosystems; Foster City, California, USA).

2.4.4.1 *Primer design*

Oligonucleotide primers were manufactured to order by Sigma-Genosys Ltd. (Haverhill, Suffolk, UK) and are described in **Table 2.5**, **2.6**, **2.7**, **2.8** and- **2.9**. Lyophilised primers were reconstituted to 100 pmol/µl in ddH_2O. Working solutions of primers were produced

by further diluting in ddH_2O to a concentration of 10 pmol/µl. Primer stocks and working solutions were stored at -20 ^oC until required.

2.4.4.2 *PCR using KOD Hot Start Master Mix*

Polymerase Chain Reaction was performed using KOD Hot Start DNA Polymerase (Novagen; Nottingham, UK), which is a premixed complex of KOD DNA Polymerase and two monoclonal antibodies that inhibit the 3'→5' exonuclease activity of DNA polymerase at ambient temperatures (Mizuguchi *et al.*, 1999). Standard reactions were conducted in 50 µl volumes, containing 1× KOD Hot Start Polymerase buffer, 1.5 mM $MgSO_4$, 0.2 mM each of dATP, dTTP, dCTP and dGTP, 0.2 µM of each sense and anit-sense oligonucleotide primer, 100 ng purified genomic DNA, 35 µl purified water and 0.02 U of KOD DNA Polymerase. A standard thermal cycling protocol included an initial denaturation of 94°C for 2 min, followed by 30 cycles of a denaturation step of 94°C for 20 sec, an annealing step of 54-60°C for 10 sec, and an extension step at 72°C for 1 min. On completion of thermal cycling, a final extension step of 72°C for 7 min was also included.

Table 2.5: **Primer sequences utilised for the amplification of the 23S rRNA region.**

Primer	Sequence 5' – 3'	Target	Size (bp)	Reference
SF1	CAGCTAAGGTCCCAAAATCTATGT	BHWA1_02698	555	This study
SF2	GAACCCGAAAGCCCAGTCAC	BHWA1_02698		This study

Table 2.6: **Primers for screening of target genes associated with virulence.**

Primer	Sequence 5' – 3'	Target	Size (bp)	Reference
flgG-F	GCGGTATGAATGGTATGCAG	BHWA1_00916	608	(Bellgard *et al.*, 2009)
flgG-R	TCCATTAGCACCTGGTTCG	BHWA1_00916		(Bellgard *et al.*, 2009)
BmpB-F	GCGGAAATACTTCTTCTGGTG	BHWA1_01754	568	(Bellgard *et al.*, 2009)
BmpB-R	CCAGGATTCAAACCGAAGTC	BHWA1_01754		(Bellgard *et al.*, 2009)
mViN-F	GCATTTGCTGGAGCTATTGG	BHWA1_02582	676	(Bellgard *et al.*, 2009)
mViN-R	TCCTAGAATCGGCATTGCTG	BHWA1_02582		(Bellgard *et al.*, 2009)
vspI-F	AGCAGAAGCGGAGATTTGAC	BHWA1_02392	586	(Bellgard *et al.*, 2009)
vspI-R	AAGGCGGTATTCTTACAGGC	BHWA1_02392		(Bellgard *et al.*, 2009)
arp-F	TTATGGCAGAGCAGATGTAG	BHWA1_00980	1122	(Bellgard *et al.*, 2009)
arp-R	TCAGCATTATAAGAAGCAGC	BHWA1_00980		(Bellgard *et al.*, 2009)
spp.A-F	CAGATCAATGGCGGCAAG	BHWA1_00767	500	(Bellgard *et al.*, 2009)
spp.A-R	CATCATAAGGGAAAGGCAGG	BHWA1_00767		(Bellgard *et al.*, 2009)
clpX-F	TCATAAGCAGCGGAGAAGC	BHWA1_01231	1108	(Bellgard *et al.*, 2009)
clpX-R	AACTCCTTCTCGCTGAATGC	BHWA1_01231		(Bellgard *et al.*, 2009)
pBW-F	GCGGACGAGATGAAGCTGAG	pBWA1_02682	700	(Bellgard *et al.*, 2009)
pBW-R	CGTACCCAACCATCAGAGGC	pBWA1_02682		(Bellgard *et al.*, 2009)
BitC-F	GGGCGGAACTATAAGTATGG	BHWA1_00869	379	(Bellgard *et al.*, 2009)
BitC-R	ATAAACAGCAGAAGAACCG	BHWA1_00869		(Bellgard *et al.*, 2009)
mglB-F	GACCCTCAAGCTGCTCAAAC	BHWA1_02552	698	(Bellgard *et al.*, 2009)
mglB-R	GGAACGCGAACTGCTTTAAC	BHWA1_02552		(Bellgard *et al.*, 2009)
F1	ATATGCAAGGAAAAATCGTAGTAG	BHWA1_02552	256	(Walker, 2001)
F2	ATTTTATCGCCGTATTTTTGTATC	BHWA1_02552		(Walker, 2001)

Table 2.7: Primers for the speculative amplification of target genes in the chromosome of *B. hyodysenteriae* P7455.

Primer	Sequence 5' – 3'	Target	Size (bp)	Reference
BHWA1_00373-F	CCATTTAGATCACTGAGAG	Bhyo8544_0386	365	This study
BHWA1_00373-R	GCTAGATAATGGAAAATCTTC	Bhyo8544_0386		This study
BHWA1_00494-F	GTATCATTCAAGACAGATGG	Bhyo8544_1628	245	This study
BHWA1_00494-R	TCTTTAGCATTAAGATCAGC	Bhyo8544_1628		This study
BHWA1_00637-F	CGTAGTGAATTATCATTGAG	Bhyo8544_1442	667	This study
BHWA1_00637-R	GAGCAAGGAATTATTGATAC	Bhyo8544_1442		This study
BHWA1_00637-F1	GGCATATTTGAAATTTCCGAAAG	Bhyo8544_1442	713	This study
BHWA1_00637-R2	CATGGTGGAAGATTTATGGCAG	Bhyo8544_1442		This study
BHWA1_00638-F	CCAAATATATCTTGTTATTCAG	Bhyo8544_1443	271	This study
BHWA1_00638-R	ATAAATCAAAAATATCCTGTG	Bhyo8544_1443		This study
BHWA1_00723-F	ACCTGGATTTATTATTATAACTG	Bhyo8544_0808	282	This study
BHWA1_00723-R	CTATGAAGCAAATATCCTATTAC	Bhyo8544_0808		This study
BHWA1_01319-F	CATATTAGAAACGGATTCAC	Bhyo8544_1746	417	This study
BHWA1_01319--R	GCAGGTTCATAAACAATTAC	Bhyo8544_1746		This study
BHWA1_01320-F	AGTGCTAAAACAGGAAAAC	Bhyo8544_1747	447	This study
BHWA1_013207-R	ATACAGACATTCAGCCATAC	Bhyo8544_1747		This study
BHWA1_01359-F	GTTCATAAGGGAGTATCAGG	Bhyo8544_1786	343	This study
BHWA1_01359-R	GATAGGCTCCGTACATAGAG	Bhyo8544_1786		This study
BHWA1_02035-F	GGAGCCGATGTCAATTTACC	Bhyo8544_2701	112	This study
BHWA1_020357-R	TAGCTTCAATGTCCGCACC	Bhyo8544_2701		This study
BHWA1_02574-F	TAGGTTTCAAAGAATTAAGGC	Bhyo8544_1369	97	This study
BHWA1_02547-R	GTTAAATCTTCATTATGAACAGG	Bhyo8544_1369		This study
02689-F2	GACGAGGAGATTATATGTATGCG	pWA1_02689	1332	This study
02689-R2	CATATTAGGTCTGGTCTGGTCTG	pWA1_02689		This study
02686-F	GACAAACCAATTAAAGATTCATAC	pWA1_02689	2373	This study
02686-R	CTCATAAGTTAGAGAGTCATTGC	pWA1_02689		This study
20-F2	GCTTGGTGGATACCTATAAGAAG	pBhyoa7455_20	688	This study
20-R2	GAACACCTCCATATAAACCAGG	pBhyoa7455_20		This study

Table 2.8: **Primer sequences utilised for housekeeping genes for *q*RT-PCR.**

Primer	Sequence 5' – 3'	Target	Size (bp)	Reference
GyB-F	TGTTGATATGCATCCGAAACTTAAA	BHWA1_00601	105	(Witchell *et al.*, 2006)
GyB-R	ACGCCTACACCATGCAAACC	BHWA1_00601		(Witchell *et al.*, 2006)
16S-F	GCCGCGGTAATACGTAGG	BHWA1_02699	186	This study
16S R	CGATATCTGCGCATTCCAC	BHWA1_02699		This study
recA-F	TTACGGACATGAGGCTTCTG	BHWA1_01107	188	This study
recA-R	CAAGAGCTTCTTCACCGCTG	BHWA1_01107		This study

Table 2.9: **Primer sequences for target genes of interest for qRT-PCR**.

Primer	Sequence 5' – 3'	Target	Size (bp)	Reference
BitC-F	TAGCAGCTGGAACAGGAGAAC	BHWA1_00869	96	This study
BitC-R	GCCATACTTATAGTTCCGCCC	BHWA1_00869		This study
FeoB-F	GAATGTAATAATGGCGGCGAG	BHWA1_00859	292	This study
FeoB-R	CAAGCCAGCCCTGAGGATAAG	BHWA1_00859		This study
BHWA1_00040-F8	TATAGGCGGTGAAAGCGAGG	Bhyo8544_1172	269	This study
BHWA1_00040-R8	TTGCTGCAACCATTAGCTGG	Bhyo8544_1172		This study
BHWA1_00040-F7	ATAGGCGGTGAAAGTGAGGC	Bhyo7455_1934	198	This study
BHWA1_00040-R7	ATCCACCTGACTGCTGCATC	Bhyo7455_1934		This study
BHWA1_00042-F8	TTCCGGCACTTACCTCTATG	Bhyo8544_1170	118	This study
BHWA1_00042-R8	CAATGCATGCTCCTGTAGG	Bhyo8544_1170		This study
BHWA1_00042-F7	TCCGGCACTCACTTCTATG	Bhyo7455_1932	148	This study
BHWA1_00042-R7	ATTCACCATAACAATGCATGC	Bhyo7455_1932		This study
BHWA1_00535-F	ATAGGTCCTAACGGATGCGG	Bhyo8544_1603	331	This study
		Bhyo7455_0041	331	
BHWA1_00535-R	GCAATTCTCCGCCTGAAAG	Bhyo8544_1603		This study
		Bhyo7455_0041		
BHWA1_00536-F	GCGGTTTTAGTGGCATTTAC	Bhyo8544_1602	211	This study
		Bhyo7455_0040	218	
BHWA1_00536-R8	CCCCTATCACTGCCGTAAC	Bhyo8544_1602		This study
BHWA1_00536-R7	AAAGGAGCCCCTATCACTG	Bhyo7455_0040		This study
BHWA1_00888-F	ATAGGAGTGGCAGGGCTTC	Bhyo8544_2174	286	This study
		Bhyo7455_1782	286	
BHWA1_00888-R	ATGCCCCAACTGCTCTTG	Bhyo8544_2174		This study
		Bhyo7455_1782		
BHWA1_00891-F8	TGCGGAGATAAAGCTGATG	Bhyo8544_2171	143	This study
BHWA1_00891-R8	TCAACCCAATGCTCCATAC	Bhyo8544_2171		This study
BHWA1_00891-F7	GGAGCATTGGGTTGATACAG	Bhyo7455_1785	296	This study
BHWA1_00891-R7	CGAAAGCAGGATGAGTTACC	Bhyo7455_1785		This study

2.4.4.3 *Long-Range PCR*

Long-range PCR was conducted using the Expand Long-Template PCR System (Roche Diagnostics Ltd.; Burgess Hill, UK), which comprises thermostable *Taq* DNA polymerase and *Tgo* DNA polymerase (a thermostable DNA polymerase with proofreading activity). Typical reactions contained 1× reaction buffer with $MgCl_2$, 500 µM each of dATP, dTTP, dCTP and dGTP, 0.3 µM of each sense and anti- sense oligonucleotide primer, 200-400 ng genomic DNA and 0.075 U/µl Expand Long Template enzyme mix.

The thermal cycling protocol included an initial denaturation of 94°C for 2 min, followed by 10 cycles of a denaturation step of 94°C for 10 s, an annealing step of 60°C for 30 sec, and an extension step at 68°C for 45 sec. This was then followed by another 25 cycles of a denaturation step of 94°C for 15 sec, an annealing step 60°C for 30 sec, and an extension step at 68°C for 45 s plus 20 sec for each elongation cycle. On completion of thermal cycling, a final extension of 72 °C for 7 min was also included.

2.4.5 Analysis of gene expression

Expression of target genes was assessed by quantitative reverse-transcription real-time PCR, which was carried out in a one-step cycling protocol using the Quanti Fast® SYBER® green RT-PCR kit (Qiagen; West Sussex, UK). The mix comprised HotStar Taq *Plus* DNA polymerase which has no enzymatic activity at ambient temperatures to prevent formation of primer-dimers during the reverse-transcription cycle. The enzyme is activated by a hot start incubation cycle (5-minute, 95°). At the same time the reverse-transcriptase is inactivated, ensuring separation of both enzymes, and thus allowing both steps to be performed sequentially in one single tube. Oligonucleotide primers were specific for the target genes of interest **(Table 2.9)**. The specifity of each amplified PCR product was assessed by performing a melting curve analysis to ensure that a homogenous product was being generated.

2.4.5.1 *Quantitative real time PCR (qRT-PCR)*

qRT-PCR was performed according to the manufacturer's supplied instructions. Briefly, standard reactions were carried out in 25 µl containing 40 ng of pure RNA, 1× QuantiFast SYBR Green RT-PCR mix, 0.25 µl QuantiFast Mix and 1 µM of each sense and anti-sense oligonucleotide primer. Thermal cycling was conducted in a Chromo4 Continuous Fluorescence detector system CFD-3240 (MJ Research; Massachusetts, USA). The cycling protocol included a reverse transcription step of 10 min at 50°C, which was then followed by an initial denaturation step of 95°C for 5 min to activate the polymerase. Afterwards 40 cycles were performed including a denaturation step of 95°C for 10 sec and a combined annealing and extension step 60°C for 30 sec.

2.4.5.2 *Housekeeping gene (HKG) selection*

In order to find a housekeeping gene (HKG) which was most stably expressed in the tested isolates, as well as under different growth conditions, three different HKG were examined by qRT-PCR. Chosen candidate reference genes were amplified from RNA extracted from three biological replicates of each of *B. hyodysenteriae* P8544 and P7455. The raw, non-normalized data, in the form of Ct-values, were then used to predict the most stable HKG using the commercially available software geNorm^{Plus} (http://www.biogazelle.com/genormplus). This software predicts the gene expression stability (M) of the candidate reference genes by calculating the pair-wise variation of each gene with that of all other tested genes. The HKG with the lowest M value are the most stable ones (Vandesompele *et al.*, 2002).

2.4.5.3 *qRT-PCR efficiency analysis*

The efficiency of qRT-PCR depends on different criteria like the quality of RNA and the amount of cDNA used, which in turn has a major impact on the accuracy of the calculated expression results.

Prior to gene expression analysis, the amplification efficiency of each HKG and target gene of interest was determined as follows: A dilution series (1; 1:10; 1;100;1:1000) of extracted RNA samples served as template and reactions were carried out in triplicate. The Ct values were exported to the software GraphPad Prism 5 to determine the amplification efficiency (E) for each primer pair, where $E = 10^{(-1/slope)}$ as determined by linear regression analysis of a dilution series of reactions. Generally, efficiency of between 90 and 110% were considered to be acceptable.

2.4.5.4 *Relative quantification of target gene expression*

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and gives a result relative to the levels of an internal control RNA. In the current study the ΔΔ Ct method with kinetic PCR efficiency correction was used to allow more precise gene expression analysis by taking the efficiency of the target gene (E_t) and the efficiency of the reference gene (E_r) into account (Pfaffl, 2001). The following equation shows the most convenient mathematical model, which includes the efficiency correction of target and reference gene (Pfaffl, 2001).

$$\text{Ratio} = \frac{E_t^{\Delta Ct_{\text{target (control - treated)}}}}{E_r^{\Delta Ct_{\text{reference (control - treated)}}}}$$

Differences in the target gene expression (Δ Ct) were calculated by transforming the difference in Ct values of iron-replete (control) versus iron-restricted (treated) based on its real-time PCR efficiencies. Fold changes in target gene expression were then normalized against the HKG GyraseB (reference gene).

2.5 Agarose gel electrophoresis

2.5.1 Analysis of DNA samples

Routinely, DNA samples were analysed by electrophoresis through agarose gels, using the Mini-Sub Cell GT electrophoresis apparatus (BioRad, UK). Briefly, agarose gels were

prepared, using a molecular biology-grade agarose concentration of 1 % (w/v) in 0.5× Tris/acetate/EDTA (TAE) buffer. To facilitate visualisation of DNA, a 1:10,000 dilution of GelRed (Cambridge Bioservices,UK) was added to gels prior to pouring. Electrophoresis was conducted at 100 V, following which DNA was visualized over a UV transilluminator (GRI AlphaInnotech; UK).

2.6 DNA clean up

2.6.1 PCR purification

For sequencing, PCR products were purified using the QIAquick PCR purification kit (Qiagen; West Sussex, UK). Briefly, 5 volumes PBI buffer were added to 1 volume of PCR reaction mix. The sample was mixed and then applied to a QIAquick spin column and centrifuged at 13,000 × *g* for 1 min. Subsequently, the DNA was washed by adding 0.75 ml Buffer PE to the column-bound DNA. After centrifugation for 1 min, as above, the flow through was discarded and the centrifugation was repeated. The column was placed into a clean 1.5 ml microcentrifuge tube. Finally, DNA was eluted by adding 50 µl of ultrapure water to the centre of the column membrane and centrifuging for 1 min as described above.

2.7 DNA Sequencing

2.7.1 Sequencing of PCR products

Contract sequencing of PCR products was conducted by the University of Cambridge (Department of Biochemistry). DNA was always sequenced from both ends to increase confidence in obtained data. Sequence data were analyzed using the Clone Manager Professional Suite, Version 9 (Scientific & Education Software Ltd., USA).

2.7.2 Whole genome sequencing

Genomic DNA for whole genome sequencing was prepared as described in section **2.4.1**. Ten µg of genomic DNA from each isolate was supplied to 'the GenePool' Next generation Sequencing and Bioinformatics facility at the University of Edinburgh where

contract sequencing was conducted using Illumina SOLEXA apparatus and indexed, paired-end 50+50 parameters, which generated read pair lengths of approximately 50 bp with at least 40-fold coverage of each genome. Sequencing reads were assembled by GenePool using VELVET 0.6 (Zerbino &Birney, 2008).

2.8 Proteomic techniques

2.8.1 Extraction of outer-membrane protein from *B. hyodysenteriae*

In order to identify differences in the outer-membrane protein (OMP) profiles of virulent *B. hyodysenteriae* P8544 and avirulent *B. hyodysenteriae* P7455 when propagated under a variety of environmental conditions, each strain was grown under iron-replete and iron-restricted conditions, and the resulting OMP profiles compared.

In this study, a rapid and non-quantitative shotgun proteomics-based approach was used to catalogue the protein complement of the *B.hyodysenteriae* OM fraction. This approach, comprised SDS-PAGE, one-dimensional monolithic column liquid chromatography, electrospray ionisation (ESI) and fast MS/MS scanning, is colloquially termed "sawn-off shotgun proteomics analysis" (SOSPA). This approach enables the analysis of membrane-associated and other hydrophobic proteins while simultaneously combining rapidity with breadth of coverage. Software developed in-house was deployed to survey the resulting SOSPA-generated data with a view to identifying homologous proteins within selected *Brachyspira* genomic sequences.

The OMPs of *B. hyodysenteriae* P8544 and P7455 were isolated using 1% (v/v) Triton-X-114 (Haake *et al.*, 2000). This non-ionic detergent solubilises membrane proteins and separates them by phase separation. Triton-X-114 has the unique feature of being homogenous at 0ºC but separates into a hydrophilic aqueous phase and detergent-rich hydrophobic phase above 20ºC. Membrane proteins typically partition into the detergent-fraction which allows distinction between cytoplasmic and outer-membrane proteins.

Briefly, three 25 ml cultures of each of *B. hyodysenteriae* P8544 and P7455 were grown under iron-restricted and iron-replete conditions. The cultures were harvested at the same point within the logarithmic phase of growth (OD600nm=0.6) and pelleted by

centrifugation at 3,893 × *g* for 15 min. The pellets were washed 3 times in ice-cold 1× PBS containing 5 mM $MgCl_2$. Subsequently, proteins were extracted by dissolving the pellets in 1.5 ml of extraction solution **(Appendix 1)** containing 1 % (v/v) Triton-X-114 and incubated for 30 min at 4°C. Insoluble material was removed by centrifugation at 17,000 × *g* for 10 min. Following this, the supernatant was transferred to a new tube and mixed with half of the volume of 20 mM $CaCl_2$. The samples were incubated at 37°C for 30 min and then centrifuged at 17,970 × *g* in a microcentrifuge for 30 min at room temperature, forming an upper aqueous phase and a lower detergent phase. Each of the fractions was precipitated with 10 volumes of acetone on ice for 60 min. These fractions were centrifuged at 17,970 × *g* at 4°C for 30 min, and the pellets were air-dried and dissolved in 50 µl of 2% (w/v) SDS prior to analysis by polyacrylamide gel electrophoresis and LC-ESI-MS/MS (**2.10.2**).

2.8.2 Sodium dodecyl sulphate-polyacrlyamide gel electrophoresis (SDS PAGE)

Electrophoretic separation of *B. hyodysenteriae* proteins under denaturating conditions was performed by SDS-PAGE using the Xcell SureLock mini-cell apparatus (Invitrogen). Pre-cast polyacrylamide NuPAGE®Novex® 4-12% Bis-Tris gradient mini gels (Invitrogen) were used to separate protein samples. Gels were placed into the electrophoresis tank according to manufacturer's instructions. The inner and outer chambers were filled with 1x NuPAGE®MES running buffer (Invitrogen) **(Appendix 1)**. For reduced samples, 500 µl of NuPAGE® Antioxidant was added to the upper chamber. Samples were prepared by adding 5 µl of NuPAGE LDS sample buffer and 2 µl of reducing agent to 13 µl of protein sample. Following this the samples were heated in a hot block for 10 min at 70°C, briefly centrifuged in a microcentrifuge at 17,970 × *g*, and loaded into the wells of the gel. Protein M_r markers were chosen according to the type of post-staining technique to be used; SeeBlue® Lus2 pre-stained standards (Invitrogen) were used for Coomassie staining and Mark 12™ standards (Invitrogen) were used for silver staining.

2.8.3 2D Gel Electrophoresis (2-DGE)

The method used for 2D-gel electrophoresis (2-DGE) was based on that recommended by Amersham Bioscience. For whole-cell separation, 3 biological replicates of 5 ml of *B. hyodysenteriae* P8544 and P7455 cultures were grown under standard conditions and iron-restricted conditions until cells reached an OD_{600nm} value of 0.6. Following this, cultures were harvested by centrifugation 3,893 × *g* and washed three times with ice-cold 1× TBS buffer. For protein extraction, the pellet was re-suspended in 250 µl of extraction buffer **(Appendix 1)** and incubated for 30 min at room temperature and vortexed every 10 min until dissolved. Afterwards the dissolved sample was centrifuged for 10 min and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube prior to clean up.

Protein samples were purified using the 2-D Clean-up Kit (Amersham) to remove remaining salts, lipids and nucleic acids which would interfere with further protein identification and characterization. Following this, the protein sample was purified and the samples were treated according to the manufacturer's instructions. Briefly, 100 µl aliquots containing 400 µg of proteins were mixed with 300 µl precipitant solution, vortexed thoroughly and incubated on ice for 15 min. Subsequently, 300 µl of co-precipitant was added and the mixtures were vortexed briefly. The tubes were centrifuged at 17,970 × *g* for 5 min in a microcentrifuge. Afterwards, the supernatants were completely removed, and the samples pulsed in a microcentrifuge to recover residual liquid. The pellet was then carefully layered with 40 µl of co-precipitant and the tubes were left on ice for 5 min. Following this, the tubes were centrifuged for 5 min as described above and the supernatant was discarded. Additionally, 25 µl of ddH20 was added and tubes were vortexed briefly to disperse the pellet. Following this, 1 ml of chilled wash buffer (-20°C) and 5 µl of wash additive was added to the tubes and vortexed until the pellet was fully dispersed. The samples were then incubated at -20°C overnight. Then, tubes were centrifuged as previously described and the wash buffer was discarded. The pellet was air-dried for no more than 5 min and re-suspended in 450 µl of rehydration buffer **(Appendix 1)**.

Normally, 5 µl of protein samples were used for protein quantification using the Ettan TM 2-D Quant Kit (Amersham), according to the manufacture's guidelines. Briefly, a set of bovine serum albumin (BSA) standards was prepared, ranging from 10 µg to 50 µg in 1.5

ml microcentrifuge tubes. Following this, 500 µl of precipitant was added to 5 µl of protein sample and standards (which were usually set up in duplicate). All samples were vortexed and incubated for 3 min at room temperature. Additionally 500 µl of co-precipitant was added to each tube, vortexed briefly and centrifuged at 17,970 × *g* for 5 min to sediment the proteins. The supernatant was decanted and the samples were centrifuged as previously described before again removing any remaining liquid. Subsequently, 100 µl of copper solution and 400 µl of ddH20 were added to each tube to dissolve the pellets (aided by vortexing). Afterwards, 1 ml of working colour reagent was added to each tube and mixed immediately by inversion. The samples were incubated at room temperature for 15-20 min and the absorbance of each sample and standard was read at 480 nm using water as a reference. The concentrations of the samples were calculated by generating a standard curve by plotting the absorbance of the standards against the quantity of the protein to predict the protein concentration of each sample. The volume of each clean protein sample was then adjusted to a final concentration of 400 µg in 450 µl in rehydration buffer. The resultant mixture was vortexed for 30 sec and subsequently centrifuged to reduce foam and separate insoluble material. Subsequently, commercially-available pre-cast 24 cm pH 4-7 gradient Immobiline ™ Drystrips (Amersham) were rehydrated for a minimum of 12 hrs in a DryStrip reswelling tray channel with the gel facing down (Amersham).

2.8.3.1 *First dimension separation*

Since 2-DGE analysis of P8544 and P7455 was conducted in two different laboratories, two different methods for both first dimension separation and second dimension separation were performed.

2.8.3.1.1 First dimension separation of B. hyodysenteriae P8544 proteins

First dimensional separation of *B. hyodysenteriae* P8544 proteins was carried out using the IGPhor isoelectric focusing unit in the proteomic laboratory of the Moredun Research Institute. The slots of the IPG DryStrip holder were filled up with 108 ml of Immoboline Drystrip cover fluid and placed onto the IGPhor isoelectric focusing unit. After rehydration, the IPG DryStrips were transferred to the manifold with the gel facing up in the tray with the anodic end of the strip resting on the appropriate mark etched on the

bottom of the manifold track. Isoelectric focusing was carried out using a gradually-increasing voltage: 500 V for the first 7 hrs, 1000 V for 1 hr; then gradient 8000 V for 3 hrs and 8000 V for 5 ½ hrs.

2.8.3.1.2 First dimension separation of B. hyodysenteriae P7455 proteins

Brachyspira hyodysenteriae P7455 protein samples were rehydrated and separated in the IGPhor isoelectric focusing unit in the proteomic laboratory of Dr. Richard Burchmore at the University of Glasgow (Institute of Infection, Inflammation and Immunity). Firstly, 450 µl of rehydration solution was pipetted into an individual Strip Holder. The Immobiline DryStrip gel was then positioned carefully into the holder with the strip facing down and the anodic end of the strip directed towards the pointed end of the Strip Holder. Isoelectric focusing was performed as follows: Rehydration was carried out for 12 hrs at 30 V followed by a gradually increasing voltage, 300 V for 2 hrs, 600 V for 2 hrs, 1000 V for 2 hrs, gradient 8000 V for 3 hrs and 8000 V for 8 hrs. The temperature was maintained at 20 °C. On completion of the run, the strips were stored at -80°C until electrophoresis in the second dimension could be carried out.

2.8.3.2 *Second dimension separation*

Prior to the second dimension separation a two-step equilibration process was performed to saturate the IGP Strips with the SDS buffer system. Strips were allowed to warm up to room temperature and incubated on a shaking platform for 15 min in 10 ml SDS buffer equilibration solution 1 (**Appendix 1**) containing 0.5% Dithiothreitol. The reducing agent ensured that disulfide bridges were broken (as compared to the effect of SDS which denatures proteins by the induction of a net negative charge). The equilibration solution 1 was discarded and replaced with 10 ml of equilibration solution 2 **(Appendix 1)** containing 4.5% Iodacetamide which alkylates the thiol groups on the proteins, preventing their reoxidation during electrophoresis. The strips were incubated under the same conditions as described above.

The second dimension was carried out in two different electrophoresis systems. Samples corresponding to *B. hyodysenteriae* P7455 were separated using the Ettan DALTtwelve

system whereas protein samples of *B. hyodysentriae* P8544 where separated using the Multiphor II electrophoresis flat-bed unit (GE Healhtcare).

Proteins of P7455 were electrophoresed through self-cast 12.5% (w/v) polyacrylamide gels. Prior to gel casting, glass plates were treated with Bind Silane to ensure that gels stuck to the plates throughout the whole procedure. Prior to positioning of the pre-cast gels, 1 ml of gel buffer was applied to the glass plates as a streak along the centre and the right hand spacer. The gel was then positioned onto the glass plates ensuring that it was orientated with the cathodic edge of the gel towards the cathodic edge of the cassette. Following this, a roller was used to remove bubbles and excess buffer by pressing firmly against the entire gel. After the gels were adhered to the glass, the cassettes were closed and the procedure was repeated until all six gels were set up. Then, the IPG strip was removed from the equilibration buffer and quickly dipped in 1× cathodic buffer for lubrication. Afterwards, the IPG strip was applied on top of the (cathodic) surface of the gel and pushed gently down so that the entire lower edge of the strip was in contact with the top surface. The IPG strip was sealed by pipetting 1 ml of agarose Sealing Solution across the entire IPG strip which was heated up to 90°C prior to use. The procedure was repeated for the remaining IPG strips. Subsequently, the loaded gel cassettes were inserted into the electrophoresis unit. When all six gels were inserted, the buffer level was adjusted using ddH_20 up to the fill line. The upper chamber was put on and then filled with 1× cathodic buffer.

The Ettan DALTtwelve system was run overnight at 80 V, 10 mA/gel and 1 W/gel for the first hours at 30°C. Afterwards the settings were adjusted to 150 V, 12 mA/gel and 2 W/gel for a further 17 hrs at 30°C. The run was finished when the bromphenol blue line reached the bottom of the gels.

Protein samples of P8544 were horizontally separated by mass using 245 x 180 x 0.5 mm ExcelGel® XL SDS 12-14 precast polyacrylamide gradient gels. The cooled flat-bed of the Multiphor II system was covered with 1 ml of low viscosity silicon oil (Fluka). The gel was then positioned onto the flat-bed corresponding to the anodic site. Then, cathodic and anodic SDS buffer strips were carefully applied along the corresponding sides of the gel. When the second equilibration step was completed, the strip was placed with the gel side facing down onto the SDS gel as close as possible to the cathodic buffer strip.

Subsequently, the IEF electrode holder was placed on the electrophoresis unit and the electrodes were aligned with the centre of the buffer strips. Proteins were separated for 45 min at 1000 V; 20 mA and 40 W. After that the IGP DryStripe was removed and the cathodic buffer strip was moved forward to cover the area of the removed Drystrip. The position of the electrode was adjusted and the gel was run for further 195 min at 1000 V; 40 mA and 40 W.

2.8.4 Western blot analysis of lactoferrin-binding proteins

Following separation by SDS-PAGE, proteins were immediately transferred to nitrocellulose membranes using XCell II™ Blot Module (Invitrogen). Briefly, the top left corner of each membrane was removed to aid post-transfer orientation. Nitrocellulose membrane and two pieces of Whatman™ 3MM filter paper were pre-soaked in Tris-glycine transfer buffer (**Appendix 1**), then the membrane and gel were placed in-between the two pieces of filter paper and two sponges. Using the Invitrogen XCell II™ Mini-Cell apparatus, the gel was clamped into the assembly with the gel closest to the cathode core. The blotting module was filled with 1× Tris-glycine transfer buffer and the outer chamber was filled with ddH20 to serve as a coolant, prior to running at 30 V for 1hr.

Following transfer, the nitrocellulose membrane to which the proteins were bound was blocked in 1× phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 (Sigma). The blocking solution was then poured off and membranes washed 3 times with 1× PBS containing 0.01% (v/v) Tween 20 (PBST) for 15 min. After the wash step the blot was probed for 1 hr at RT with 25 ml of 25 µg/ml of bovine lactoferrin (Sigma, UK) in PBST. Subsequently, the blot was washed as previously, and incubated for 1 hr at RT on a rotary shaker with the primary rabbit-anti lactoferrin antibody (Sigma) diluted 1:500 in PBST. Following this, the membrane was washed prior to incubation with a 1:10,000 horseradish peroxidase conjugated sheep polyclonal anti-rabbit IgG antibody (Sigma) in 15 ml of PBST for 1 h. Membranes were washed twice as before and subsequently the HRP-labelled proteins were detected by an ImageQuant LAS 4000 luminescent image analyser (GE Healthcare), using Pierce® ECL Western Blotting Substrate (Thermo Scientific).

2.9 Polyacrylamide Gel staining

2.9.1 Colloidal Coomassie blue staining

Resolved proteins for analysis by LC-ESI-MS/MS or MALDI were first visualized using SimplyBlue SafeStain (Invitrogen, Paisley, UK) or colloidal Coomassie blue stain (Genomic Solutions, MI USA). Molecular weight standards were included routinely on SDS-PAGE gels. Electrophoresed proteins for LC-ESI-MS/MS analysis were stained with SimplyBlue SafeStain. After electrophoretic separation, the gels were washed for 15 min with ddH_20 by gently shaking on a rotary shaker. Then, gels were stained in 25 ml of SimplyBlue SafeStain for 1 hr and de-stained in ddH_20 for at least 1 hr.

Protein spots on 2D gels were fixed for at least 30 min in 10% acetic acid, 40% ethanol. The fix solution was decanted and 500 ml of freshly prepared colloidal stain (**Appendix 1**) was added and left shaking gently until spots became visible. Prior to gel examination, residual stain was removed by rinsing the gels with water.

2.9.2 Silver staining

To enhance staining sensivity, silver staining of protein gels was carried out using the SilverQuest™ Silver staining kit (Invitrogen), following the manufacturer's guidelines. Each gel was rinsed with ddH_20 and placed into a plastic tub containing 100 ml of fixing solution (40% (v/v) ethanol, 10% (v/v) acetic acid). The gel was microwaved on full power for 30 sec, and immediately agitated at room temperature for 5 min on a rotary shaker. The fixation solution was removed, and the gel was placed in 30% (v/v) ethanol and microwaved on high power for further 30 sec and agitated again for 5 min at room temperature. The ethanol was decanted and 100 ml of sensitizing solution (30 ml of 100% ethanol, 10 ml sensitizer and 60 ml ddH_20) was added to the tub containing the washed gel. The gel was then subjected to further microwaving as before and agitated for 2 min. Afterwards, the sensitizing solution was decanted and the gel was washed twice in 100 ml ddH_20; each wash step included 30 sec microwaving and 2 min agitation at room temperature. Following this the gel was subsequently placed into 100 ml of staining

solution (1 ml stainer and 99 ml of ddH_20) microwaved on high power for 30 s and agitated at room temperature for 5 min.

2.10 Protein identification

2.10.1 Protein spot quantification and identification

In order to identify and quantify protein spots, digital images were acquired of 2D gels, which were then analysed using the ImageMaster 2D Platinum 7 software (GE Healthcare, UK). Prior to spot detection, the 2D maps of the iron-replete and iron-restricted groups were compared and matched by selecting one reference image from the iron-replete group exhibiting the best quality spots. Two landmarks were chosen within the reference map, which were also present in all of the other gels, covering both the X and Y axes to align gels sufficiently. Once gels had been matched, spots were detected and analysed for differential expression. Spots were considered only when they were present in 2 out of the 3 biological replicates. One way analysis of variance (ANOVA) was performed to find statistically significant differences ($p < 0.05$) in expression of proteins between iron-replete and iron-restricted groups. Differentially expressed proteins were accepted when they were present in all of the three biological replicates within a group with a p value of 0.05 or less. The selected spots were then filtered based on an average expression level change of at least 1.5-fold. Afterwards proteins of interest were manually excised from the gels and submitted for identification by MALDI-ToF mass spectrometry (**2.10.3.**). True matches of protein identity were affirmed by Molecular weight search (MOWSE) scores (Pappin *et al.*, 1993) and by comparing predicted versus observed pI and molecular mass.

2.10.2 Liquid chromatography-electrospray ionization-mass/mass spectrometry (LC-ESI-MS/MS)

Following first-dimensional separation, outer-membrane proteins were excised from gels by cutting each lane into 25 horizontal slices. Each individual slice was transferred to a 1.5 ml microcentrifuge tube before performing a standard in-gel destaining, reduction, alkylation and trypsinolysis procedure (Shevchenko *et al.*, 1996). The samples were transferred to a high performance liquid chromatography (HPLC) sample vial and stored at

+4°C until required for LC-ESI-MS/MS analysis. Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex UK, Camberley, UK) comprising a WPS-3000 well-plate microautosampler, an FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump, and an SRD-3600 solvent rack controlled by Chromeleon chromatography software (Dionex). A micropump flow rate of 246 µl/min was used in combination with a cap-flow splitter cartridge, affording a 1/82 flow split and a final flow rate of 3 µl/min through a 5 cm by 200 µm inside diameter monolithic reversed phase column (Dionex-LC Packings) maintained at 50°C. Samples of 4 µl were applied to the column by direct injection. Peptides were eluted by the application of a 15-min linear gradient from 8 to 45% solvent B (80% acetonitrile, 0.1% [v/v] formic acid) and directed through a 3 nl UV detector flow cell. LC was interfaced directly with a three-dimensional high-capacity ion trap mass spectrometer (Esquire HCTplus; Bruker Daltonics, Bremen, Germany) via a low-volume (50 µl/min maximum) stainless steel nebulizer (Agilent, catalog no. G1 946-20260) and ESI. Parameters for tandem MS analysis were set as previously described. (Batycka *et al.*, 2006).

Deconvoluted MS/MS data were imported into Proteinscape V2.1 (Bruker Daltonics, Bremen, Germany) proteomic data analysis software and searched against the annotated draft genomes of *B. hyodysenteriae* P8544 and P7455 genomic database using the MASCOT V2.2 9 Matrix Science, London) search algorithm (Perkins *et al.*, 1999). The protein content of individual gel slices was established using the "protein search" feature of ProteinScape, while non-redundant lists of all proteins contained in all 25 gel slices of each of the three biological replicates were produced using the "protein extractor" feature of the software. The presentation and interpretation of MS/MS data was performed in accordance with published guidelines (Taylor & Goodlett, 2005) and to this end, the fixed and variable modifications selected were carbamidomethyl (C) and oxidation (M), respectively, and mass tolerance values for MS and MS/MS were set at 1.5 Da and 0.5 Da, respectively. MOWSE scores attained for individual protein identifications were inspected manually and considered significant only if (i) two peptides were matched for each protein, (ii) each peptide contained an unbroken "b" or "y" ion series of a minimum of four amino acid residues, (iii) a sequence coverage ≥5% and present in at least two out of three biological replicates.

2.10.3 Matrix-assisted laser desorption/ionisation-time of flight (MALDi-TOF) mass spectrometry

Protein spots on 2D-gels were identified by Matrix-assisted laser desorption/ionisation-time of flight (MALDi-TOF) mass spectrometry.

Spots of interest were cut from 2D gels under sterile conditions and placed into sterile tubes. Gel pieces were washed in 400µl 50% acetonitrile, in 25mM ammonium bicarbonate pH8.0 for 15 minutes 3 times. Gel pieces were washed in 100% acetonitrile for 5 minutes before being dried in a speed-Vac for 30 minutes.

Gel pieces were covered with trypsin solution (10µg/ml trypsin (Promega), 25mM ammonium bicarbonate, pH8.0) and allowed to rehydrate for 10 mins. After 10 min if all the trypsin solution was absorbed a further 5 µl was added. The samples were then incubated overnight at 37ºC (16-24 hrs). After incubation, 10 µl of 50% Acetonitrile (Fluka) was added and the sample was vortexed for 30 min. For MALDI analysis, 0.5 µl of the supernatant was mixed with the 0.5 µl of matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA) and spotted onto a MALDI sample plate. Following this, 1 µl of Pepmix 1 standards (Bruker Daltonics) were mixed with 10µl matrix solution and 0. 4µl was spotted adjacent to each sample spot. Spots were allowed to dry completely before being analysed using an Ultraflex II MALDI-ToF-ToF mass spectrometer. Data for Peptide Mass Fingerprint (PMF) analysis was accumulated from 10 × 100-shot acquisitions. Each sample was calibrated using the adjacent standard. Where required, further analysis was carried out by obtaining ion fragmentation on individual peptides (MS/MS).

Data was processed using Flex analysis software and compiled into mass lists. Searches were performed using a Mascot^{TM} search engine, searching against a database compiled from the draft genomes of *B. hyodysenteriae* P8544 and P7455. Mass tolerances were set at 50 ppm for PMF and 0.5 Da for MS/MS data.

2.11 Gene prediction and annotation

2.11.1 Automated gene prediction and annotation

The *B. hyodysenteriae* P7455 and P8544 genomes were annotated using the xBASE bacterial genome annotation service (Chaudhuri *et al.*, 2008), which employs an automated suite of bioinformatic programmes. Via xBASE, gene prediction was conducted using Glimmer (Delcher *et al.*, 2007), with user-defined settings to detect a minimum gene length of 90 bp and a maximum gene overlap of 50 bp; tRNA genes were identified using tRNAScan-SE (Lowe *et al.*, 2007); rRNA genes were identified using RNAmmer v.1.2 (Lagesen *et al.*, 2007), via the RNAmmer server (http://www.cbs.dtu.dk/services/RNAmmer/). In addition, Protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied to the translations of predicted coding sequences against a manually-selected reference sequences (in this case, *B. hyodysenteriae* WA1, accession # NC_02225 and accession # NC_02226). The best result for each BLAST search was then imported as the gene annotation (according to user-defined E-value cut-off), and the resulting annotated genomes were in GenBank file format to facilitate viewing.

2.11.2 Prediction of signal peptides and lipoproteins

Secretion signal peptide and lipoprotein encoding motifs within ORFs were predicted using three open access, web-based software programs. SignalP (Bendtsen *et al.*, 2004) was accessed via the Centre for Biological Sequence Analysis (CBS) SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/), and was used to predict the presence and location of secretion signal peptide cleavage sites in prokaryotic and eukaryotic sequences. The LipoP 1.0 server (Juncker *et al.*, 2003) was used to predict lipoprotein coding sequences and discriminate between lipoprotein signal peptides and other signal peptides in Gram-negative bacteria. Additional software, SpLip (Setubal *et al.*, 2006), was employed to specifically identify spirochaete lipoproteins and was kindly provided for use by David Haake (University of California, David Geffen School of Medicine, CA, USA). SpLip employs a position-specific scoring matrix, also known as weight matrix (WM) (Durbin, 1998), which uses a training set (TS) consisting of 28 experimentally-verified

lipidated spirochaetal lipoproteins. Moreover, the TS includes 26 sequences described by Haake (Haake, 2000), as well as the Lipl21 (Cullen *et al.*, 2003a) and LigB (Matsunaga *et al.*, 2003) from *Leptospira interrogans.* Since *L. interrogans* is the closest relative to *B. hyodysenteriae,* the TS of *L. Interrogans copenhaenii* and *L. interrogans serovar lai* were used to predict lipoproteins in WA1, P8544 and P7455.

2.11.3 Functional analyses

The function of predicted unique proteins in the genomes of *B. hyodysenteriae* P8544 and P7455 were inferred using several Web-based software programs, as described below.

2.11.3.1 *Basic Local Alignment Search Tool*

Translated proteins were identified by comparison with sequences resident in the National Centre for Biotechnology Information (NCBI) non-redundant, SWISS-PROT and Trembl databases, using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

2.11.3.2 *InterProScan*

InterProScan was used to predict the function of putative unique proteins encoded by ORFs detected in the genomes of *B. hyodysenteriae* P8544 and P7455. InterProScan (Zdobnov & Apweiler, 2001) is a computational tool that scans a given protein sequence against the protein signatures of the member of the InterProScan database. This program uses a collaboration of various databases to give a unique, non-redundant characterization of a given protein family, domain or functional site (Zdobnov & Apweiler, 2001). The program was installed locally and the gene sequences of interest were submitted in FASTA format.

2.11.3.3 *Prediction of outer-membrane proteins*

Gram-negative cells possess an outer-membrane containing integral membrane proteins. Integral outer-membrane proteins generally present a β-structure and form transmembrane β-barrel proteins containing between 8-22 transmembrane β-strands (Tamm *et al.*, 2001). The commercially available software 'integral β-barrel outer membrane protein predictor' (BOMP; Berven *et al.*, 2004) (http://services.cbu.uib.no/tools/bomp) was used to identify β-barrel outer membrane proteins among the sets of proteins detected in the outer-membrane enrichment fractions of *B. hyodysenteriae* (**Chapter 6**) in order to predict their localization (**Appendix 5**).

2.12 Genome comparison

In order to compare the two genomes sequenced in this project, different bioinformatics software programmes were used to identify regions/genes which were shared and distinct for each genome.

2.12.1 ACT comparison

Comparison of the genomes was carried out using Artemis Comparison Tool (ACT) Release 9 (Carver *et al.*, 2005) (Sanger Pathogen Sequencing Unit).The publicly available genome sequence of *B. hyodysenteriae* WA1, Acc.# NC_012225 (chromosome) and Acc.# NC_012226 (plasmid) were also included as a query sequences also and were downloaded from the NCBI online database. In order to use ACT the programme Double ACT, (http://www.hpa-bioinfotools.org.uk/pise/double_act.html) was used to generate a comparison file for comparing genomes within ACT. A BLAST search for the whole genome was carried out by performing the tblastx option which searches a translated nucleotide sequence database using a translated nucleotide query. Following this, the comparison file could be uploaded into ACT. The sequences were aligned to the query sequence, showing the features at the nucleotide and amino acid levels, with intensity of colour being used to visualise the similarity between regions.

2.12.2 Panseq 2.0

Panseq determines the core and accessory regions among a collection of genomic sequences based on user-defined parameters (Laing *et al.*, 2010). In the current project Panseq 2.0 was used to identify unique regions in the two draft genomes of *B. hyodysenteriae* using the Novel Region Finder (NRF) module (**Appendix 4**). The NRF module compares an input sequence(s) using a FASTA file containing the assembled contigs of the draft genomes to a database containing the reference sequence. Contiguous regions not present in the database but present in the input sequences are extracted, accomplished by the MUMmer alignment program (Kurtz *et al.*, 2004).

2.12.3 Circular genome comparison

The BLAST Ring Image Generator (BRIG) software (http://sourceforge.net/projects/brig/) was used to generate circular genome comparison images, to visualise genotypic similarities and differences between the reference genome WA1 and the two draft genomes of *B. hyodysentyeriae* as a set of concentric rings coloured according to BLAST match (Alikhan *et al.*, 2011).

2.13 Statistical analyses

Data were analysed and graphs were drawn using GraphPad Prism 5. Significant differences were assessed by Fishers's exact two tailed method (**Chapter 3** and **Chapter 4**), the non-parametric Mann-Whitney U-test (Glover & Mitchell, 2008) (**Chapter 5**), and one-way analysis of variance (ANOVA) (Glover & Mitchell, 2008) (**Chapter 6**). All statistical methods were performed using the GenStat® edition 12 software (GeneStat™, UK). In this study, a p-value of <0.05 was considered to be statistically significant (Fisher, 1925). Statistical advice was provided by Biomathematics and Statistics Scotland (BioSS; University of Edinburgh, Kings Building, Edinburgh, Scotland).

Chapter 3: General characterization of virulent and avirulent *B. hyodysenteriae*

3.1 Introduction

Many attempts have been made to differentiate and classify species of *Brachyspira* (**Chapter 1**).The discrimination of all *Brachyspira* spp. is complicated due to their share many common characteristics. Moreover the established phenotypic and molecular diagnostic methods seem to hamper the identification of novel *Brachyspira* spp. and therefore their detection and diagnosis. Molecular techniques like PCR provide a sensitive means of diagnosis, but the specifity of these assays depends on the conservation of these target genes within a species and do not provide reliability in the occurrence of strain diversity and/or mutation. Recently, a *Brachyspira* spp. strain isolated from pigs and mallard ducks showed typical features of *B. hyodysenteriae* including strong haemolysis and indolee positive but failed to be classified as *B. hyodysenteriae* by PCR and was thus described as *B. suanatina* (Råsbäck *et al.*, 2007). Additionally, potentially novel species of *Brachyspira* named *B. sp*. SASK3044 and *B. hampsonii* have been identified which were isolated from multiple pigs with typical sign of SD in the US (Chander *et al.*, 2012; Harding *et al.*, 2010). However, despite the common features of being strongly haemolytic and causing SD in pigs the isolate could not be classified into group I (*B. hyodysenteriae*) by PCR, suggesting that the existing diagnostic methods are not sufficiently robust for classification of *Brachyspira* spp. and lead to misinterpretation especially, in the identification of unknown species. The regular detection of atypical *B. hyodysenteriae* strains during the last years indicates that possible exchange of genetic information driven by the prophage-like transfer agent contributes to strain diversity and novel isolates among *B. hyodysenteriae*. It also emphasizes the need for improved classifications methods for diagnosis of *Brachyspira* spp.

Mildly-pathogenic *B. hyodysenteriae* isolates seem to be another atypical form of *B. hyodysentriae* which have been recognized worldwide. However, to-date only one preliminary and limited study of pathogenic and putative non-pathogenic *B. hyodysenteriae* strains has been reported (Walker, 2001). The study by Walker (2001), suggested that the absence of an *mglB* homologue might be responsible for the existence of milder or non-pathogenic isolates. Interestingly, the study demonstrated that a *mglB* homologue was amplified from 13 of 17 (76 %) pathogenic field isolates tested. In comparison a very weak product was amplified from only 4 of 29 (14%) of mild/non-pathogenic isolates. This result showed a statistically significant difference between

virulent and avirulent *B. hyodysenteriae* with respect to amplification of the *mglB* gene, indicating that a higher percentage of pathogenic *B. hyodysenteriae* (76 %) have an *mglB* homologue compared to only 14% of mild/non-pathogenic strains. However, the study was carried out in only a small population of virulent and avirulent *B. hyodysenteriae* isolates and the percentage distribution of the *mglB* might substantial differ when analysed in a wider panel. Nevertheless, the presence of a *mglB* homologue in other spirochaetes like *T. pallidum*, *T. denticola* (Becker *et al.*, 1994a; Porcella *et al.*, 1996), *B. piloscol*, *B. innocens* and *B. intermedia* (Zhang *et al.*, 2000) have been also demonstrated. Similar to findings by Walker (2000), other studies have shown that the *mglB* gene was absent in a closely related non-pathogenic *T. pallidum* isolates (Becker *et al.*, 1994a). The glucose/galactose-binding protein MglB is known to be involved in chemotaxis and transport of glucose and galactose in *E.coli* (Boos, 1969; Kalckar, 1976). Dual function of MglB in *Brachyspira* remains to be determined but might be similar. However, enhanced motility of *B. hyodysenteriae* in highly viscous material as well as chemotaxis towards mucin appear to be key factors in mucosal localization (Milner & Sellwood, 1994). Pig colonic mucin consists of complex glycoproteins incorporating sugars such as galactose, galactosamine and glucosamine (Marshall & Allen, 1978). *In-vitro* studies showed that *B. hyodysenteriae* is able to utilize D-galactose, D-glucose, N-acetyl-D-glucosamine and D-glucosamine (Trott *et al.*, 1996) indicating that mucin offers an excess of suitable metabolites to restore energy which would be needed to maintain chemotxis and motility. Therefore, *B. hyodysenteriae* isolates lacking the *mglB* homologue might be disadvantaged in respect to mucosal localization and thus in establishing SD. Further screening studies of the *mglB* gene in a wider panel of virulent and avirulent isolates would answer the questions if the *mglB* gene of *B. hyodysenteriae* could have potential as a marker which could be useful for differentiation.

The first aim of this chapter was the confirmation and validation of the *Brachyspira* field isolates. A physical and genetic map of pathogenic *B. hyodysenteriae* $B78^T$ became available in 1994 which served as basis for genetic analysis of that pathogen (Zuerner & Stanton, 1994) followed by the complete genome sequence of pathogenic strain WA1 (Bellgard *et al.*, 2009). However, at the first stage of this study no genome sequence of an avirulent *B. hyodysenteriae* strain was publically available which could have been used to facilitate a genome comparative analysis of pathogenic and non-pathogenic *B. hyodysenteriae*. Therefore, a general phenotype comparison of the virulent and avirulent

isolates was conducted including screening a panel of *B. hyodysenteriae* isolates, compromising virulent and putatively avirulent strains by PCR to determine the presence/absence of different target genes associated with virulence as well as comparison of the growth kinetic between virulent and avirulent strains.

3.2 Results

3.2.1 Identification and verification of *B. hyodysenteriae* isolates

Faecal samples can contain a mixture of *Brachyspira* species. Although the cells of different species differ in size it is not possible to distinguish between the species under the light microscope. In order to confirm that the entire obtained field isolates were *B. hyodysenteriae* the following differentiating methods were conducted prior to further experiments.

3.2.2 Biochemical test

The biochemical tests were conducted originally by the SAC diagnostic service. The results are summarized in **Table 3.1**. All field isolates were characterized as *B. hyodysenteriae* (Group 1). The majority of the tested isolates shared an identical biochemical profile including strong haemolysis, positive for indole, α and β galactosidase activity and lack of hippurate hydrolysis. The occurrence of isolates being indole negative was not significantly different among virulent and avirulent isolates (P= 0.359).

3.2.3 23S rRNA RFLP-PCR

The Restriction Fragment Length Polymorphism (RFLP) patterns of the 23S rRNA amplicons from all field isolates were compared to the RFLP patterns obtained from reference strains of *B. hyodysenteriae*, *B. piloscoli* and *B. innocens.* The amplification of the highly conserved 23S rRNA region of each *B. hyodysenteriae* isolate resulted in an expected PCR product of a size of 555 bp (data not shown). Subsequently, each of the amplification products obtained for all isolates were digested using the restriction enzyme *Hph1,* producing 3 unique fragments of 298 bp, 201 bp and 51 bp which are typical for *B. hyodysenteriae* (**Figure 3.1**) (Barcellos *et al.*, 2000).

Table 3.1: Biochemical features of the *B. hyodysenteriae* isolates. The panel includes 26 virulent and 23 avirulent *B. hyodysenteriae* isolates. The biochemical tests were carried out by the SAC.

Strain	Putative Virulence	β-Haemolysis	Indole production	Hippurate hydrolysis	α-Glucosidase activity	β-Glucosidase activity	Group
P8544*	V	Strong	Positive	Negative	Positive	Positive	1
P8226/7*	V	Strong	Positive	Negative	Positive	Positive	1
QCR1*	V	Strong	Positive	Negative	Positive	Positive	1
P7455*	A	Strong	Positive	Negative	Positive	Positive	1
P7377/3*	A	Strong	Positive	Negative	Positive	Positive	1
QCR2	V	Strong	Positive	Negative	Positive	Positive	1
P8404	V	Strong	Positive	Negative	Positive	Positive	1
P5943/07	V	Strong	Positive	Negative	Positive	Positive	1
P6129/2/08	V	Strong	Positive	Negative	Positive	Positive	1
P6812/1/08	V	Strong	Positive	Negative	Positive	Positive	1
P6858/08	V	Strong	Positive	Negative	Positive	Positive	1
P7124/08	V	Strong	Positive	Negative	Positive	Positive	1
P7210/1/08	V	Strong	Positive	Negative	Positive	Positive	1
P7286/1/08	V	Strong	Positive	Negative	Positive	Positive	1
P7309/1/08	V	Strong	Positive	Negative	Positive	Positive	1
P7271/1/08	V	Strong	Positive	Negative	Positive	Positive	1
P7346/08	V	Strong	Positive	Negative	Positive	Positive	1
P7343/4/08	V	Strong	Negative	Negative	Positive	Positive	1
P7381/2/08	V	Strong	Positive	Negative	Positive	Positive	1
P7458/1/08	V	Strong	Positive	Negative	Positive	Positive	1
P7458/2/08	V	Strong	Positive	Negative	Positive	Positive	1
P5683/07	V	Strong	Negative	Negative	Negative	Negative	u
P7645/09	V	Strong	Positive	Negative	Positive	Positive	1
P7624/1/09	V	Strong	Positive	Negative	Positive	Positive	1
P7620/1/09	V	Strong	Positive	Negative	Positive	Positive	1
P7563/3/08	V	Strong	Negative	Negative	Positive	Positive	1
P7486/2/08	V	Strong	Positive	Negative	Positive	Positive	1
P1093/6/01	A	Strong	Negative	Negative	Positive	Positive	1
P949/4/00	A	Strong	Positive	Negative	Positive	Positive	1
P949/5/00	A	Strong	Positive	Negative	Positive	Positive	1
P949/9/00	A	Strong	Positive	Negative	Positive	Positive	1
P278/97	A	Strong	Positive	Negative	Positive	Positive	1
P944/14/00 †	A	Strong	Positive	Negative	Positive	Positive	1
P271/97	A	Strong	Positive	Negative	Positive	Positive	1
P246/1/97	A	Strong	Positive	Negative	Positive	Positive	1
P265/97	A	Strong	Positive	Negative	Positive	Positive	1
P246/2/97	A	Strong	Positive	Negative	Positive	Positive	1
P935/LI/00 †	A	Strong	Positive	Negative	Positive	Positive	1
P949/3/LI/00	A	Strong	Positive	Negative	Positive	Positive	1
P354/2/97	A	Strong	Positive	Negative	Positive	Positive	1
P264/97	A	Strong	Positive	Negative	Positive	Positive	1
P257/97 †	A	Strong	Positive	Negative	Positive	Positive	1
P252/A/97	A	Strong	Positive	Negative	Positive	Positive	1
P252/B/97 †	A	Strong	Positive	Negative	Positive	Positive	1
P935/15/00	A	Strong	Positive	Negative	Positive	Positive	1
P935/2/00	A	Strong	Positive	Negative	Positive	Positive	1
P944/15/00 †	A	Strong	Positive	Negative	Positive	Positive	1
P7649/2/09	A	Strong	Positive	Negative	Positive	Positive	1
WA1	A	Strong	Positive	Negative	Positive	Positive	1

*these isolates were routinely used in this study

† these isolates have been challenged in a pig model and their non-pathogenicity was confirmed

A= avirulent *B. hyodysenteriae*. Pigs did not show any clinical signs of SD

V= virulent *B. hyodysenteriae*. Pigs showed typical signs of SD

1= isolates were characterized as *B. hyodysenteriae* (Group 1) according to the biochemical profile described in Table 1.3

U= unclassified. These isolate did not match the parameters of the biochemical group 1 of *B. hyodysenteriae* or any other biochemical group of *Brachyspira* spp.

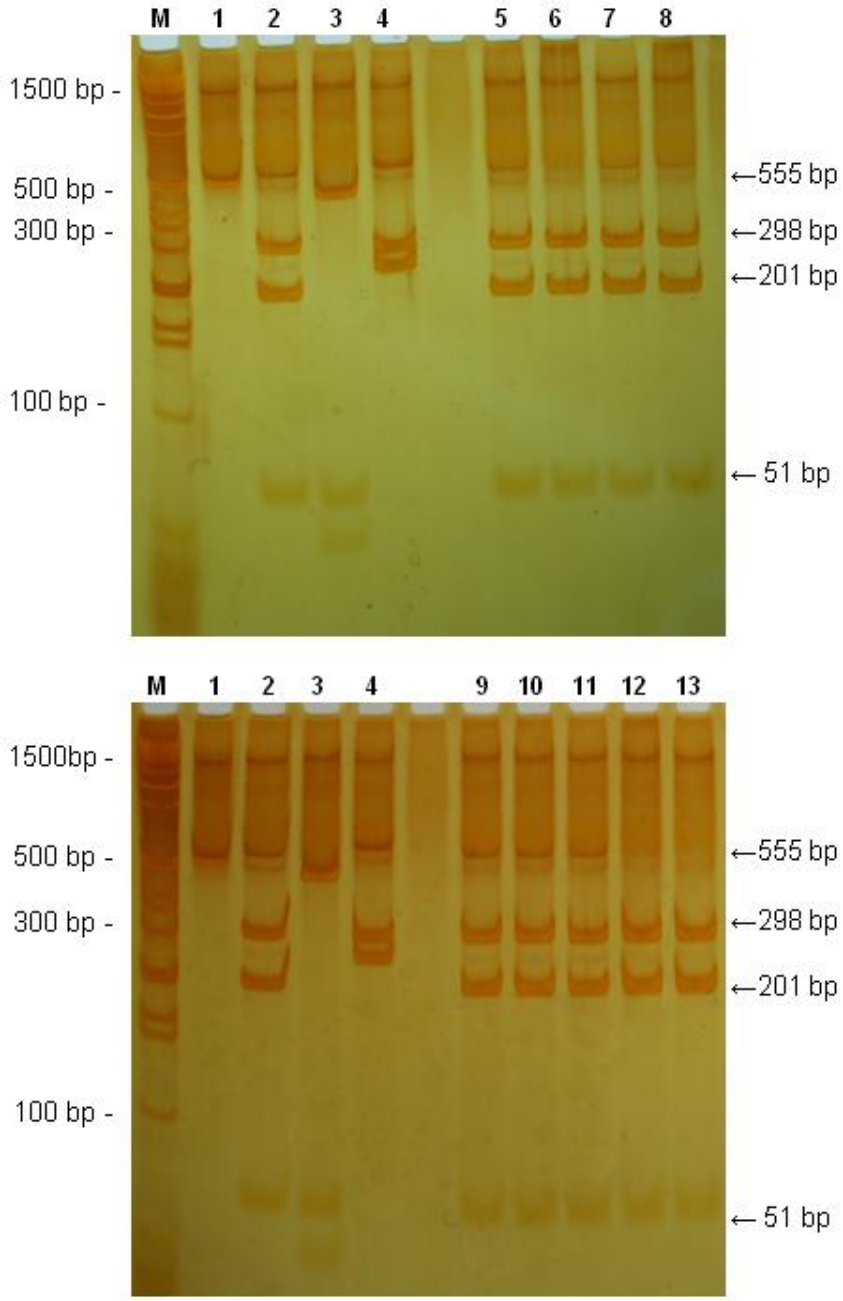


Figure 3.1: 23S rRNA analysis of representative *B. hyodysenteriae* field isolates The 23s rRNA amplicon of each *B. hyodysenteriae* isolate was digested with *Hph1*. Digested products were separated by a 10 % (w/v) polyacrylamide gel and visualised by silver staining. Lanes correspond to 100 bp marker (M), *B. hyodysenteriae* WA1 undigested (1), *B. hyodysenteriae WA1* (2), *B. piloscoli* (3), *B. innocens* (4), *B. hyodysenteriae* 8544 (5, 6), *B. hyodysenteriae* 7455 (7, 8), *B. hyodysenteriae* QCR1 (9), QCR2 (10), 8408 (11), 8226/7 (12) and 7377/3 (13). The images are representative of the entire panel of tested *B. hyodysenteriae* field isolates.

3.3 Growth characteristics of *B. hyodysenteriae*

Routinely, *B. hyodysenteriae* were grown in Brain Heart Infusion broth (BHI). This medium is known to be very rich and provides all necessary nutrients for fastidious growing bacteria. The growth was assessed from all available and cultivable isolates, including the three pathogenic isolates P8544, P8226/7 and QCR1 as well as the two putative avirulent isolates P7455 and P7377/3. Typical growth of these isolates in filter sterilized BHI supplemented with 5% FCS is shown in **Figure 3.2**. The isolates P8544 and P7455 grew quicker than the other tested isolates with reaching an OD_{600nm} maximum of 1.0 within 22-24 hrs. In contrast, the isolates P8226/7, P7377/3 and QCR1 reached stationary phase after 30 hrs of growth with an OD_{600nm} maximum ranging from 0.919 and 0.987. The curves obtained from P7455 and P8544 were used as the standard level of growth to compare the growth of *B. hyodysenteriae* under different conditions (**Chapter 5**).

Additionally, viable cells were counted via a modified Miles and Misra method. For each isolate 1 ml of culture was taken at the four time points: 0 hrs, 16, 24 hrs and 40 hrs. The samples were diluted up to 10^{10} in 1 × PBS and subsequently 10 µl of sample were spotted onto BA No 2 plates and incubated in the anaerobic workstation for 8 days. Three biological replicates of each isolate and time point were serially diluted as described above and are summarized in **Table 3.2**. Accurate cell count was at times difficult to determine as it was sometimes not clear whether colonies were already merged together. Therefore, the highest culture dilution yielding in detectable growth after 8 days should be acknowledged as the main parameter for growth rather than the average number of counted colonies.

Generally, start cultures of the five tested isolates (t=0) yielded in noticeable growth up to dilutions varying between 10^2-10^3containing approximately 3.4×10^4 up to 9.4×10^4 CFU/ml and proved to be to sufficient for initiation of growth. The cell numbers of each isolates increased over time up to cell counts of 7.2×10^9 up to 4.5×10^9 CFU/ml. The reduced doubling of P8544 and P7455 observed by monitoring the OD over time conforms with the viable cell count as both isolates yielded in a higher cell counts after 16 hrs and onwards compared to the isolates P8226/7, P7377/3 and QCR1. Thus, the optical density readings compared favourably with the total cell counts. Regularly purity checks confirmed that the cultures were free from contaminants in the broth and on plates.

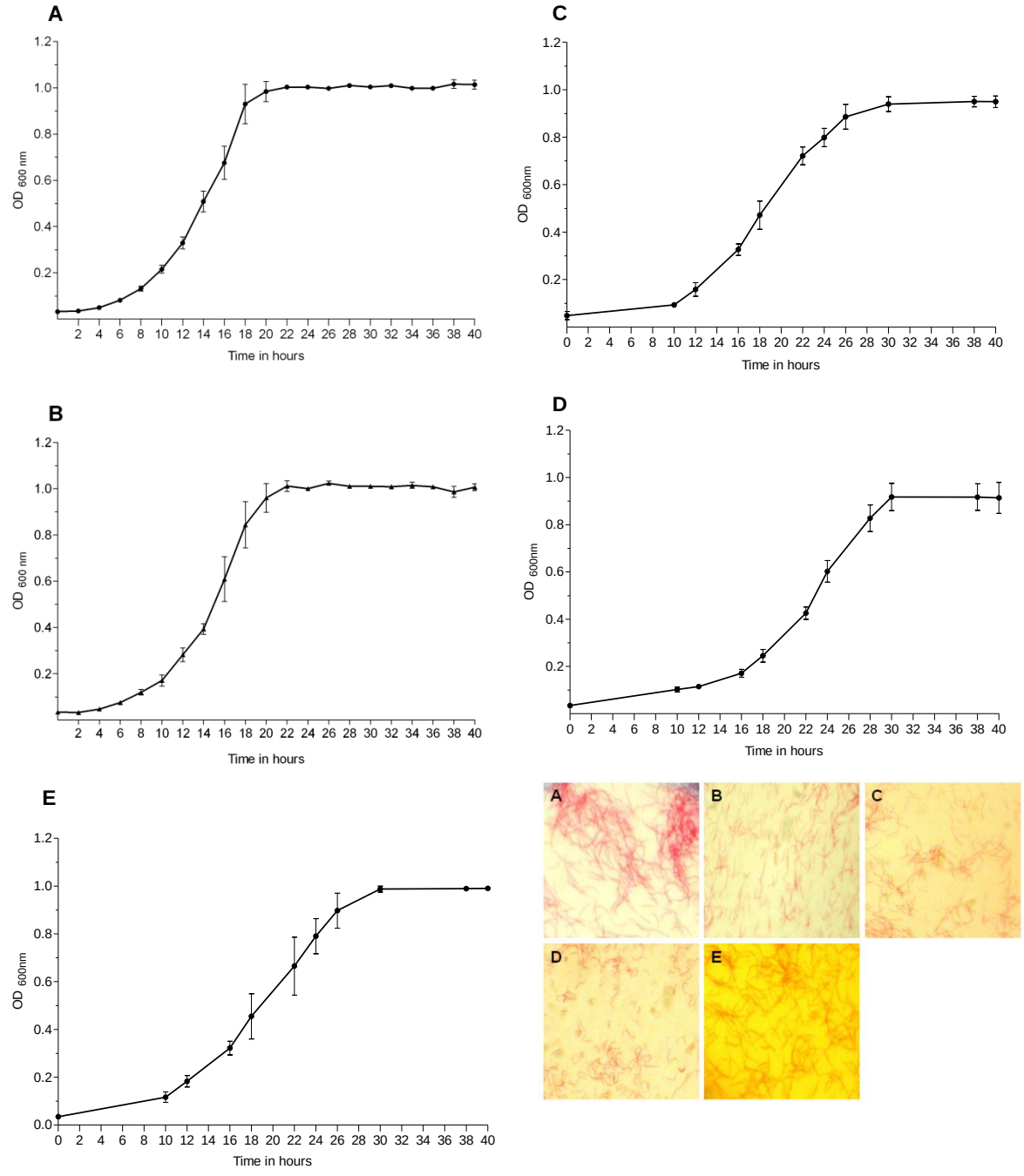


Figure 3.2: Typical growth curves of *B. hyodysenteriae* field isolates. Cells of pathogenic P8544 (A), avirulent P7455 (B), avirulent P7377/3 (C), pathogenic P8226/7 (D) and pathogenic QCR1 (E) were grown in 50 ml of BHI supplemented with 5% FCS at 37°C with constant stirring under anaerobic conditions using the Don Whitley anaerobic workstation for 40 hrs. At regular time intervals, the OD_{600nm} was measured using a Novaspec II visible spectrophotometer. The data represents the mean of 5 biological replicates for P8544 and P7455 while the curves of QCR1, P8226/7 and P7377/3 represent 3 biological replicates on different days. The error bars represent the standard deviation (SD). Gram-stain was carried out after time points 16, 24 and 40 hrs to check the purity of cultures. Examples of pure cultures from all isolates after 40 hrs are shown.

Table 3.2: Viable cell count of *B. hyodysenteriae*. Cultures of the isolates P8544, P7455, P8226/7, P7377/3 and QCR1, containing a volume of 1 ml, were taken at time points 0, 16, 24 and 40 hrs. Samples were serial diluted in 1× PBS up to 10^{10} and spotted onto BA plates in triplicate 10 µl aliquots. After 8 days of incubation in the anaerobic work cabinet, the highest dilution yielding in growth was determined. Colonies were calculated by counting detectable colonies in of the highest dilution and the mean of the three technical replicates was taken. The experiment was carried out in three biological replicates of each isolate and time point. The average CFU/ml per isolate and time point was calculated from the mean of the three biological replicates. Cells numbers were increasing in all tested *B. hyodysenteriae* isolates over a time period of 40 hrs ranging from 3.4×10^{4} CFU/ml up to a maximum of 4.0×10^{10} CFU/ml.

Isolate	**t [hrs]**	**OD_{600nm}[1]**	**highest dilution yielding growth after 8 days**			**Colony count**			**CFU/ml[3]**
			A	**B**	**C**	**a[2]**	**b[2]**	**c[2]**	
P8544	0	0.032	10^{3}	10^{3}	10^{2}	1	1	2	7.3×10^{4}
P8544	16	0.675	10^{7}	10^{6}	10^{6}	3	1.6	1.6	1.1×10^{9}
P8544	24	1.05	10^{8}	10^{8}	10^{9}	2	1.6	1	4.5×10^{10}
P8544	40	0.99	10^{9}	10^{8}	10^{7}	2	1	2.3	4.0×10^{10}
P7455	0	0.034	10^{2}	10^{3}	10^{3}	1.6	1	1	7.2×10^{4}
P7455	16	0.608	10^{7}	10^{6}	10^{7}	2	3	1	1.1×10^{9}
P7455	24	1.02	10^{8}	10^{9}	10^{8}	3	1	1	1.3×10^{10}
P7455	40	1.0	10^{8}	10^{8}	10^{7}	2	1	2	1.0×10^{10}
P7377/3	0	0.037	10^{2}	10^{3}	10^{2}	1.6	1	1.6	3.4×10^{4}
P7377/3	16	0.326	10^{6}	10^{5}	10^{5}	1	2.3	3	5.1×10^{7}
P7377/3	24	0.798	10^{8}	10^{7}	10^{7}	1	1.6	1	4.2×10^{9}
P7377/3	40	0.949	10^{8}	10^{7}	10^{8}	1	2	1	7.3×10^{9}
P8226/7	0	0.037	10^{2}	10^{3}	10^{2}	1.6	1	1	4.4×10^{4}
P8226/7	16	0.164	10^{4}	10^{5}	10^{4}	2	1	2.3	4.7×10^{6}
P8226/7	24	0.602	10^{7}	10^{6}	10^{6}	1	4	1.3	5.1×10^{8}
P8226/7	40	0.913	10^{8}	10^{8}	10^{7}	1	1	1.6	7.2×10^{9}
QCR1	0	0.032	10^{2}	10^{3}	10^{3}	2.3	1.6	1	9.4×10^{4}
QCR1	16	0.322	10^{5}	10^{5}	10^{6}	2	1.6	1	4.5×10^{7}
QCR1	24	0.79	10^{7}	10^{7}	10^{6}	1	2	3	1.1×10^{9}
QCR1	40	0.99	10^{8}	10^{7}	10^{8}	1	2	1	7.3×10^{9}

[1]Average OD of three biological replicates as shown in Figure 3.2.

[2]Average colony count of three technical replicates detected in the highest dilution.

[3]Average CFU/ml calculated of the three biological replicates.

3.4 PCR screening

In order to detect possible differences between virulent and putative avirulent isolates of *B. hyodysenteriae* a set of ten genes, which are recognized as potential virulence factors involved in adhesion and/or surface proteins, cell motility and host cell membrane degradation (**Chapter 1**) were chosen to verify the absence and/ or presence of these targets in the panel of the 49 field isolates of *B. hyodysenteriae*. Additionally, a gene which was confirmed on the plasmid sequence of *B. hyodysenteriae* WA1 (Bellgard *et al.*, 2009) was included to determine the presence of the plasmid within that panel. Genes for virulence life-style (VL-S) factors were chosen as potential targets, since they might occur in a considerable number of pathogenic strains and therefore might be absent in mildly- or non-pathogenic isolates and give the reason for not causing typical SD in pig.

The results of the analysis of the 49 isolates are summarized in **Table 3.2-Table 3.4**. Most of the isolates produced a single clear product of the expected size for each of the chosen targets genes, suggesting that all tested target genes are highly conserved among different *B. hyodysenteriae* strains (between 70 and 100%). The detection of all 10 genes in *B. hyodysenteriae* P8544 and P7455 is visualized in **Figure 3.3**. The image is also representative for all other PCR products by the *B. hyodysenteriae* panel.

Based on the presence/absence of the 10 genes, 7 different patterns were detected in the panel defined as avirulent, including 14 (60.86 %) isolates amplifying all tested target genes, 1 isolate negative for *mViN* and *sppA*, 4 isolates negative for *bitC*, 1 isolate negative for *flgG*, 1 isolate negative for *bitC*, *arp* and *vspI*, two isolates negative for *bitC* and *vspI* and one isolate negative for *arp*, *vspI* and *mviN*. In the virulent panel, 6 gene patterns were recognized showing 19 isolates (73.1 %) which amplified a product for all of the target genes, 2 isolates negative for the *bitC* genes, 1 isolates negative for *vspI*, 1 isolates negative for *bitC*, *vspI* and *mViN*, 1 was negative for *sppA* and 1 isolate was negative for the genes *vspI* and *mViN*.

All 49 isolates were positive for the genes *mglB, bhlp29.7, clpX and pBWA1*. The gene *bitC* showed the most variation in carriage between all isolates. While 79% of total isolates were positive for *bitC*, 88.5% of virulent strains were observed to carry the gene whereas only 69.5% of the avirulent strains carried the gene.

Table 3.3: Detection of the 10 target genes in the 26 virulent *B. hyodysenteriae* isolates.

Virulent strains		Virulence life-style genes									
		Chemotaxis Motility		*Iron*	*Surface proteins/ adhesion*				*Protease*		*Plasmid Related*
		flgG	***mglB***	***bitC***	***arp***	***bhlp29.7*[1]**	***vspI*[2]**	***mViN***	***clpX***	***sppA***	***pBWA1***
WA1 ATCC	**A**										
QCR1	**E**										
QCR2	**E**										
P8544	**E**										
P8226/7	**EU**										
P8404	**E**										
P5943/07	**EU**										
P6129/2/08	**S**										
P6812/1/08	**E**										
P6858/08	**E**										
P7124/08	**E**										
P7210/1/08	**S**										
P7286/1/08	**S**										
P7309/1/08	**E**										
P7271/1/08	**E**										
P7346/08	**E**										
P7343/4/08	**E**										
P7381/2/08	**E (16)**										
P7458/1/08	**E (16)**										
P7458/2/08	**E**										
P5683/07	**EU**										
P7645/09	**E**										
P7624/1/09	**S**										
P7620/1/09	**S**										
P7563/3/08	**NE**										
P7486/2/08	**E**										
Present		26/26	26/26	23/26	26/26	26/26	23/26	24/26	26/26	24/26	26/26

red indicates that the PCR product was amplified successfully whereas yellow indicates that no PCR product was obtained.

The number in brackets indicates the units from which strains were isolated.

[1] the gene was formerly known as *bmpB*.

[2] the gene was formerly known and annotated as *vspH*.

Capital letters are representative for the geographical regions were isolates were obtained: **A** Australia; **E** England, **EU** European Union; **S** Scotland; **NE** Non-EU.

Table 3.4: Detection of the 10 target genes in the 23 putative avirulent *B. hyodysenteriae* isolates.

avirulent strains	Virulence life-style genes									
	Chemotaxis/ Motility		*Iron*	*membrane/ adhesion*				*Protease*		*Plasmid Related*
	flgG	***mglB***	***bitC***	***arp***	***bhlp29.7***	***vspI***	***mViN***	***clpX***	***sppA***	***pBWA1***
P7455 **E (32)**										
P7377/3 **E (32)**										
P1093/6/01 **S**										
P949/4/00 **S (2)**										
P949/5/00 **S (2)**										
P949/9/00 **S (2)**										
P278/97 **S**										
P944/14/00† **E(3)**										
P271/97 **S**										
P246/1/97 **S**										
P265/97 **S**										
P271/98 **S**										
P246/2/97 **S (22)**										
P935/LI/00†**E(23)**										
P949/3/LI/00**E(2)**										
P354/2/97 **E**										
P264/97 **S**										
P257/97 † **S**										
P252/A/97 **S (22)**										
P252/B/97† **S(22)**										
P944/15/00† **E(3)**										
P7649/2/09 **E**										
P935/2/00 **E (23)**										
Present	22/23	23/23	16/23	21/23	23/23	19/23	21/23	23/23	21/23	23/23

† isolates confirmed as avirulent by the SAC in a pig model to confirm their mild pathogenicity

red indicates that PCR product was amplified whereas yellow indicates that no PCR product was obtained.

Capital letters are representative for the geographical regions were isolates were obtained: **E** England; **S** Scotland.

Table 3.5: Overview of the percentage distribution of the 10 targets in virulent and avirulent *B. hyodysenteriae* isolates.

Strains	Virulence life-style genes									
	arp	***bitC***	***bhlp29.7***	***clpX***	***flgG***	***mglB***	***mViN***	***sppA***	***vspI***	***pBWA1***
virulent [%]	100	88.5	100	100	100	100	92.3	92.3	88.5	100
avirulent [%]	91.3	69.5	100	100	95.6	100	91.3	91.3	82.6	100
In total [%]	95.6	79	100	100	97.8	100	91.8	91.8	85.6	100

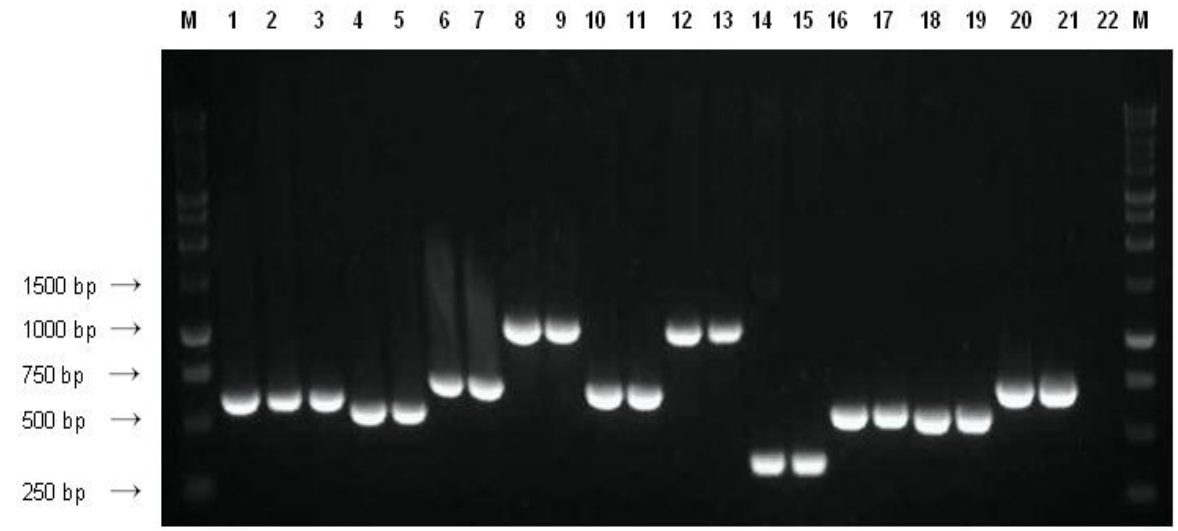


Figure 3.3: Detection of the 10 target genes in the virulent *B. hyodysenteriae* P8544 and in the putative avirulent *B. hyodysenteriae* P7455 isolates by using PCR. Samples correspond to 1kb DNA ladder (M), the *flgG* product of WA1 served as a positive control (1), P8544 (2) and P7455 (3), The *sppA* PCR product of P8544 (4) and P7455 (5), the *pBWA1* PCR product of P8544 (6) and P7455 (7), the *arp* PCR product of P8544 (8) and P7455 (9), the *mViN* PCR product of P8544 (10) and P7455 (11), the *ClpX* PCR product of P8544 (12) and P7455 (13), the *bitC* PCR product of P8544 (14) and P7455 (15), the *vspI* PCR product of P8544 (16) and P7455 (17), the *bhlp 29.7* PCR product of P8544 (18) and P7455 (19), the *mglB* PCR product of P8544 (20) and P7455 (21), negative control (22).

The prevalence of virulent isolates carrying the *bitC* gene was not statistically different to the proportion of avirulent isolates being positive for *bitC* with a *P* value >0.05.

The PCR screening in respect to the ten studied genes also showed diversity of field isolates which were recovered from the same unit. For instance, the avirulent isolates P944/14/00 and P944/15/00 were recovered from unit three but showed different gene patterns indicating that more than one *B. hyodysenteriae* isolate was present in that unit. Different patterns in multiple isolates from the same unit could also been observed in P953/Li/00 and P935/2/00 while other strains did not reveal distinctive gene pattern e.g. isolates from unit 22, unit 16 and unit 32. It is not possible to make a statement whether these isolates are identical or not just on the basis of ten genes. Further typing methods such as PFGE or Box-PCR or even full genome sequencing are required in order to make appropriate conclusions about the relationship of these tested field isolates.

Diversity of 10/49 isolates within 7 housekeeping genes could also been shown by MLST. A population of 150 virulent isolates of *B. hyodysenteriae* originated from different countries were analysed by MLST (**Appendix 2**). The MLST project was funded by BPEX and the data was analysed and kindly provided by Richard Ellis and Ben Strugnell from Animal Health and Veterinary Laboratories Agency (AHVLA) (Weybridge, Surrey). Seven MLST loci previously described for *B. hyodysenteriae* (La *et al.*, 2009) were used, these being the genes encoding alcohol dehydrogenase (*adh*), alkaline phosphatise (*alp*), esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*plp*K), phosphoglucomutase (*pgm*) and acetyl-CoA acetyltransferase (*thi*). The population of the 150 *B. hyodysenteriae* isolates included ten virulent isolates which were used in this study. The relative relationship of the ten virulent isolates is shown in **Figure 3.4**. Diversity between the ten strains was observed with P5943/07 (EU) forming a separate clade. Additionally the ten isolates were divided into nine sequence types (STs) with five newly described types. The corresponding allele numbers assigned to all STs are shown in **Table 3.6**. Differences in the allelic profile of these isolates suggest that the entire DNA panel used in this study is representative for diverse group of *B. hyodysentriae* isolates.

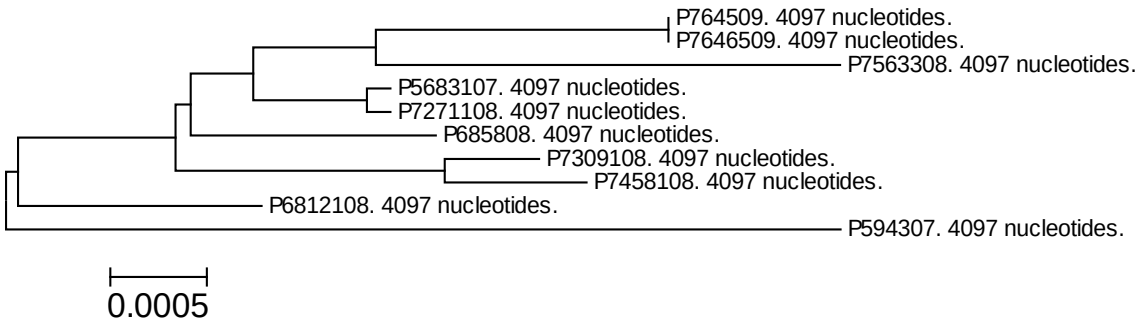


Figure 3.4: UPGMA dendogram based on concatenated nucleotide sequences from seven loci from a population of 10 pathogenic *B. hyodysenteriae* field isolates. The data was analysed and provided by Richard Ellis and Ben Strugnell from Animal Health and Veterinary Laboratories Agency (AHVLA) (Weybridge, Surrey). The tree was generated from concatenated DNA sequences obtained from the seven MLST loci *pgm*, *adh*, *alp*, *est*, *glpk*, *gdh* and *thi*. The scale bar shows the distance equivalent to three substitutions per 1,000 nucleotide positions.

Table 3.6: Allelic profile of the ten virulent *B. hyodysenteriae* isolates.

Isolate	seven MLST loci							ST*
	adh	***alp***	***est***	***gdh***	***glpk***	***pgm***	***thi***	
P5683-1-07	2	111	7	5	10	2	3	New
P5943-07	2	3	3	10	4	3	16	78
P6812-1-08	2	11	107	10	7	3	3	New
P6858-08	2	11	8	1	10	2	6	New
P7271-1-08	2	11	7	5	10	2	7	New
P7309-1-08	2	18	8	5	6	2	11	New
P7458-2-08	2	21	3	20	6	11	11	88
P7563-3-08	1	11	3	6	21	2	3	New
P7645-09	2	13	3	6	23	2	21	87
P7646-5-09	2	13	3	6	23	2	21	87

*Sequence type.

new: These STs are not recorded in the PubMLST site at the time of writing.

3.5 Sequencing of the *mglB* gene_02552

Due to the findings obtained previously (Walker, 2001, unpublished data) indicating that a higher percentage of presumably avirulent isolates are lacking one *mglB* homologue (**3.1**), primers (*mglB*-F and *mglB*-R) were designed to amplify a 689 bp region of the *mglB*_02552. The amplified product contained the 256 bp region which was target of the original screening PCR described by Walker (2001) using primer F1/F2 (**Chapter 2**) specific for the *mglB* sequence of *B. piloscoli* AF200741.

As shown in **Table 3.2** and **Table 3.3**, a product was successfully amplified from all tested strains using the primers *mglB*-F and *mglB*-R. Amplicons from 9 *B. hyodysenteriae* isolates (five avirulent and four virulent) were sequenced and aligned against the *B. hyodysenteriae* WA1 *mglb*_02552 sequence. The sequence of the *B. piloscoli mglB* sequence AF200741 was also included to highlight the 256 bp region to be able to compare possible differences in the sequences. The **Figure 3.5** shows the sequenced 689 bp region of the *mglb* gene including the 256 bp region of *B. piloscoli* indicating by red arrows. All sequenced strains share high homology over the entire sequence. Interestingly, no obvious sequence variation was observed for isolates from which no PCR product was amplified by using primer F1/F2 and *Taq* Polymerase (P944/15/00 and P7286/1/08), isolates from which a weak product was amplified (P721/1/97, P278/79, P944/14/00, P7271/08 and P6858/1/08) and isolates from which an abundant fragment was amplified (P8544 and P7455).

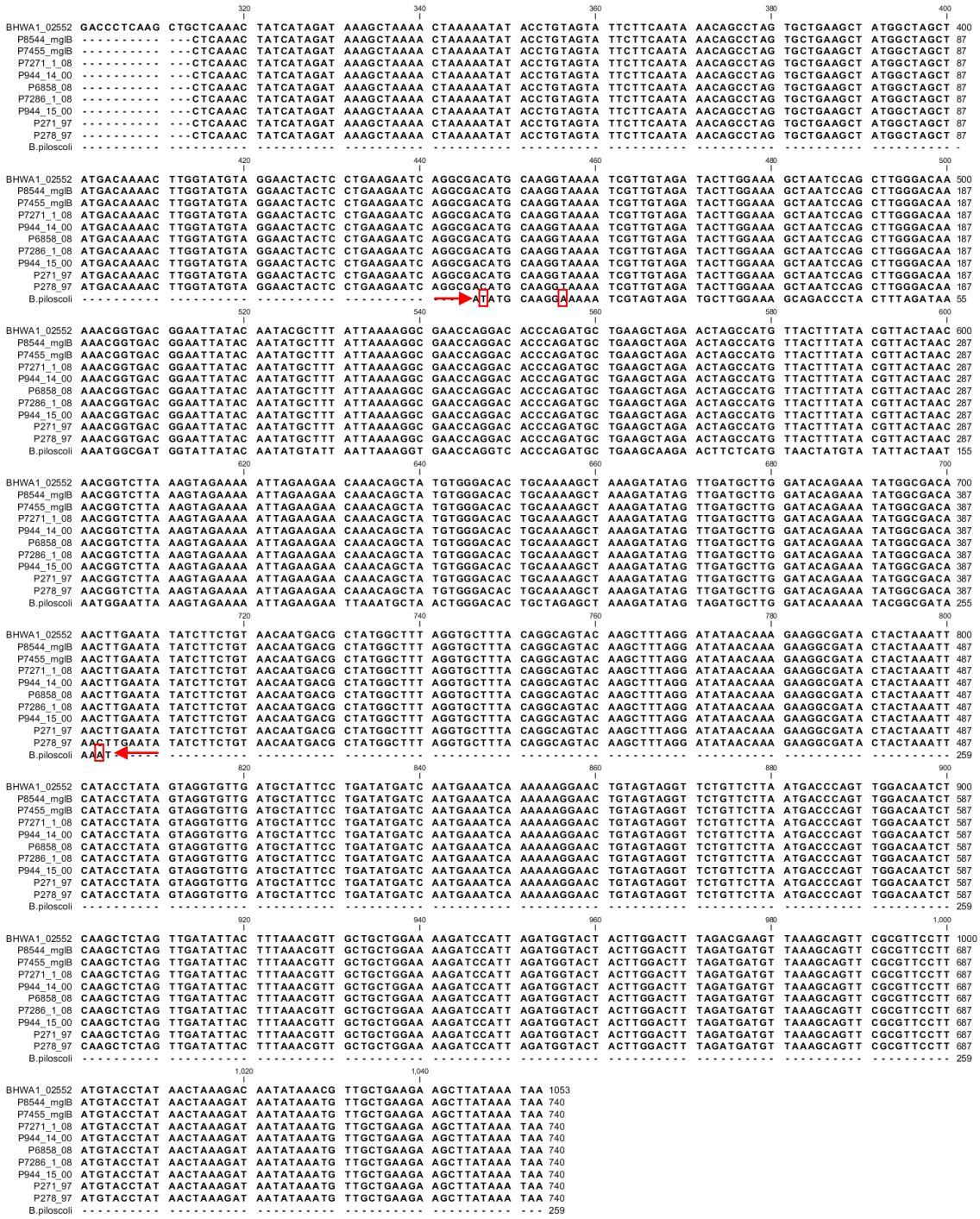


Figure 3.5: Comparison of the amplified *mglB*_02552 sequences of 4 virulent and 5 avirulent *B. hyodysenteriae* isolates against the reference strain WA1 and the *mglB* gene sequence of *B. piloscoli*. PCR was carried out using KOD Polymerase (Novagen).The red arrows are indicating the start and end of the *mglB* sequence of *B. piloscoli.* Sequence variations are highlighted in red boxes.

3.6 Discussion

Brachyspira spp. is differentiated on phenotypic criteria using biochemical assays and RFLP-PCR. However, identification of *Brachyspira* spp. is hampered due to the presence of strains with atypical biochemical and genotypic characteristics (Burrough *et al.*, 2012; Ohya *et al.*, 2008). Therefore, a combination of biochemical features and PCR-RFLP is used in routine diagnostic for precise identification.

3.6.1 Phenotypic characteristics of virulent and avirulent *B. hyodysenteriae* isolates

Today mildly-pathogenic *B. hyodysenteriae* have been recognized but no deeper investigation of these isolates has been conducted so far. In the first instance of the project a general analysis of the virulent and avirulent strains has been carried out by confirmation and validation of all isolates using the routine diagnostic methods. The biochemical tests and 23S rRNA PCR/RFLP confirmed that the five isolates routinely used in this study were *B. hyodysenteriae* (group 1). The results of both test systems were in complete agreement and no obvious difference in the biochemical profiles could be observed within a panel of 23 avirulent and 26 virulent *B. hyodysenteriae* isolates. Nevertheless, a few isolates could be identified to be indole negative (11.53 % in the virulent panel and 4.34 % in the avirulent panel), indicating that these strains lacking the enzyme tryptophanase which hydrolyses the amino acid tryptophan to indole, pyruvic acid and ammonia. *Brachyspira hyodysenteriae* isolates lacking indole production seem to occur frequently within tested strains and have been recognized earlier (Hommez *et al.*, 1998). Thus, indole negative and indole positive *B. hyodysenteriae* isolates are categorized in group 1 of the biochemical profile (Fellström *et al.*, 1999). However, the majority of tested isolates was able to produce indole suggesting that the gene *tnaA* (BHWA1_01919) which encodes for the enzyme tryptophanase is highly conserved in *B. hyodysenteriae*. The virulent strain P5683/07 isolated from the EU was strongly β-haemolytic but it was identified to be negative for all other tested biochemical features. Therefore, this isolate remains unclassified as it was not possible to categorize it into any of the known groups. Nevertheless, the 23S rRNA RFLP-PCR confirmed the strain to be *B. hyodysenteriae* (personal communication with the SAC). Besides, the strain possessed all 10 target genes as determined by PCR, confirming that it belongs to the species *B. hyodysenteriae*. The

results of this atypical strain do not seem to be novel as the existence of strongly β-haemolytic, atypical *B. hyodysenteriae* strains lacking the common phenotypic and genotypic diagnostic characteristics have been reported elsewhere suggesting the existence of diverse *B. hyodysenteriae* strains (Burrough *et al.*, 2012). This emphasises that the bacterium is in a continuous adaptation phase by gaining and losing genes, complicating the sensitivity of the diagnostic assays and detection of the pathogen in general. Indeed, polymorphic deletions or insertions of nucleotides in CDS will contribute to the variability among *B. hyodysenteriae*. Due to the recognized diversity between *B. hyodysenteriae* strains, the common biochemical and genotypic methods would require regular updating with probable restructuring of the current diagnostic methods to cover potentially novel species in the future. However, the known diagnostic methods seem to be still a reliable tool for detection of the majority of *Brachyspira* spp. but should be taken with care.

In this chapter growth curves of three virulent and two putative avirulent strain of *B. hyodysenteriae* were carried out to study possible differences in their growth characteristics using routinely used media. In most laboratories, *B. hyodysenteriae* is cultured in either Brain Heart Infusion broth or Trypticase Soy broth supplemented with 2-10% FCS (known as Kunkles broth) at a temperature range of 37-42°C (Calderaro *et al.*, 2005; Kunkle *et al.*, 1986). Kunkle *et al.* (1986) reported the first growth curve of *B. hyodysenteriae* by comparing the growth rate of that organism in three different broths containing a combination of two components of FCS, cholesterol and pig faeces extract over a period of 50 hours (Kunkle *et al.*, 1986). Although all of the tested media resulted in similar growth rates, Kunkels broth supplemented with 2% FCS and 1% cholesterol at 38°C permitted quicker growth than the other tested media and cells reached stationary phase after 37 hrs with a total cell number of 4×10^9 /ml. In the current study the stationary phase of the five tested isolates was reached between 22 and 30 hours with maximal cell counts of 7.2×10^9 up to 4.5×10^{10} CFU/ml. Generally, *B. hyodysenteriae* is known as a fastidious organism. Studies reported that cells of the type strains B204 and $B78^T$ were grown for 3-5 days in BHI supplemented with 20% FCS at 37°C (Gommel *et al.*, 2013), while other studies demonstrated that the strain $B78^T$ reached end of log phase within 48 hrs with a total cell count of 5.0×10^8 CFU/ml (Karlsson *et al.*, 2003) and thus approximately 10 hrs later than cells grown by Kunkel *et al*. (1986). Therefore, different observation regarding the growth kinetic of *B. hyodysenteriae* stains have been made, however, none of the published studies were able to demonstrate a similar accelerated

growth behaviour of *B. hyodysenteriae* than shown in this study. Due to regularly purity checks, it can be assured that cultures were pure and the quicker doubling time was not caused by contaminants. It could be also possible that the isolates used are well lab-adapted as the number of passaging are unknown when the project started. Continuous subculturing of bacteria can lead to genetic and phenotypic changes (Brooke *et al.*, 2003; Kim *et al.*, 2002) which might affect growth behaviour. The main reason for the surprisingly quicker growth rate of *B. hyodysenteriae* cells compared to other studies remains unclear as same media and conditions were used as described elsewhere. However, differences in the growth profile of this study and Kunkel growth experiment might be due to the composition of the medium ingredients, medium batch, the concentration of FCS and the temperature. Besides the growth curve experiments were carried out in an anaerobic workstation (**2.3.3**) whereby Kunkel *et al.* (1986) cultivated *B. hyodysenteriae* in sealed anaerobic jars. Studies reported significant differences in the transcript profiles (John *et al.*, 2011) and variations in number and size of colonies (Haines *et al.*, 2011) of cells of *C. jejuni* grown in a microaerophilic atmosphere created by a controlled work cabinet and sealed jars. Therefore, variations in the yield of growth and doubling time of *B. hyodysenteriae* cells using the two different systems can be expected. Although anaerobic workstations have been used for culturing *B. hyodysenteriae* cells (Durmic *et al.*, 2000) and the gas mixtures appeared to be similar than used in this study. Another difference was that BHI was filter-sterilized while the literature describes BHI generally to be autoclaved. Enhancement of growth using filter-sterilized media has not been reported elsewhere but was observed in our laboratory experience when working with *Streptococcus* (personal communication with M.C. Fontaine, Moredun Research Institute).

The generally slow and fastidious nature of *B. hyodysenteriae* makes it easy for contaminations to take over. In particular, members of the *Enterobacteriaceae* have been shown to grow rapidly in broth (Fujikawa & Morozumi, 2005). Since Kunkels first report other attempts have been made to improve and support the recovery rate of *B. hyodysenteriae* especially after isolation from pig faeces (Calderaro *et al.*, 2005; Lugsomya *et al.*, 2012). At the beginning of this project nine field isolates were chosen for routine culturing and experimental studies. However, only five out nine of these isolates were pure whereby two out of the four contaminated isolates did not grow at all. The other two isolates were tried to get pure by a combination of antimicrobials like spectinomycin 400 µg/ml, rifampicin 30 µg/ml as well as serial dilutions. However, none of these methods

resulted in pure cultures. Previously primary pre-enrichments steps using a blood agar medium media containing colistin (C), 25-100 U/ml, rifampicin (R) 30 µg/ml and spectinomycin (S) 400 µg/ml (BAM-CRS) were recommended and has been successful (Calderaro *et al.*, 2005). Nevertheless other studies indicated that the pre-enrichment step did not inhibit the growth of other microorganism using the earlier described media (Lugsomya *et al.*, 2012). Antimicrobial resistance amongst bacteria of the intestinal microflora has dramatically increased due to high usage of antimicrobials in livestock (Hanson *et al.*, 2002; Prapasarakul *et al.*, 2010) which also limits the sensivity in *B. hyodysenteriae* to certain drugs. The field isolates used in this study have not been tested for any minimal inhibition concentrations (MICs) which would have been useful in order to obtain uncontaminated cultures. Due to time constraints the project was continued with three virulent and two avirulent strains of *B. hyodysenteriae* for which pure cultures were obtained **(Table 2.1)**.

3.6.2 Virulence genes in virulent and avirulent *B. hyodysenteriae* isolates

A panel of 49 DNA samples of virulent and avirulent *B. hyodysenteriae* isolates was screened for carriage of 10 target genes linked with virulence.

The genes *mglB, clpX, bhlp29.7 and pBWA1* were observed in all of the tested isolates. Some avirulent strains of *B. hyodysenteriae* have been shown to lack a homologue of the *mglB* gene and may have potential as a marker (Walker *et al.*, 2002; Walker, 2001). However, this study was carried out before the complete genome sequence of *B. hyodysenteriae* WA1 became available and primers were designed upon the *mglB* gene sequence of *B. piloscoli* (Genbank accession number # AF200741) due to lack of data. Therefore, it was decided to further the analysis of the *mglB* as a potential marker. The previously sequenced *B. hyodysenteriae* strain WA1 possesses three *mglB* genes which lie within a single chromosomal locus. The 256 bp region which was target of the original screening PCR lies with the gene *mglB* BHWA1_02552. PCR analysis using alternative PCR primers mglb-F and mglB-R allowed the amplification of a 689 bp region of the mglB_02552 gene from all tested *B. hyodysenteriae* isolates as shown in **Table 3.2** and **Table 3.3**. Furthermore, the same primers (F2/F1) as used in the study of Walker were used to attempt to amplify the 256 bp target region from within the *mglB*_02552 (data not shown). Interestingly, PCR was carried out using KOD Polymerase (Novagen) instead of

Taq Polymerase which was able to produce products corresponding to the 256 bp *mglB* gene region from strains which had previously appeared negative or very weak by PCR. Sequencing of the weak PCR products was not conducted in the previous study due to low DNA abundance. Therefore, to determine whether there was a difference in the sequence within the 256 bp region, associated with the different PCR results, the whole 689 bp region of the *mglB*_02552 gene was sequenced to allow further investigation. Sequence conservation across the whole of the sequenced region was very high, and no differences were observed whether within the 256 bp region that gave rise to variable PCR results or the region of 689 bp amplified by the primers F2/F1. Moreover, the sequence conservation of the whole *mglB* was later confirmed by the genome comparison of *B. hyodysenteriae* P7455 and P8544 (**Chapter 4**). Therefore, it is likely that failure of the PCR procedure, itself in addition to primer annealing due to nucleotide differences in the primer-binding site, rather than actual differences between strains is likely the reason for apparent variability in *mglB* sequence, which is in agreement with the sequence results and genome sequencing for the *mglB* gene. Moreover, the MglB homologues seem to be ubiquitous among *Brachyspira* spp. (Zhang *et al.*, 2000) and other spirochaetes including *T. denticola* (Becker *et al.*, 1994b).

The gene BHWA1_02682, which encodes a radical SAM protein on the plasmid sequence of *B. hyodysenteriae* WA1, could be amplified from all isolates, suggesting that all isolates are likely to contain the ~ 36 kb plasmid. Interestingly, it has been suggested that the plasmid-encoded genes are involved in the pathogenicity of *B. hyodysentriae* and strains lacking the plasmid showed reduced virulence (La *et al.*, 2011). So far, only two single Australian isolates (A1 and WA400) have been suggested to lack the plasmid indicating that these isolates may exists in pig herds but that the majority of isolates contain the plasmid. La *et al.* (2011) examined 3 plasmid-specific genes in 264 Australian field isolates and detected heterogeneity in the plasmid sequence, as 87.5% (231/264) of the isolates amplified a product for tested genes these being DNA primase-like protein (BHWA1_02687), putative replicative DNA helicase (BHWA1_02686) and integrase (BHWA1_02688) while only 6.43% (17/264) were positive for putative replicative DNA helicase and integrase and 2.27% (6/264) produced a product of DNA primase-like protein and putative replicative DNA helicase. These isolates may be originated from different geographical regions within Australia responsible for the sequence diversity among the plasmids, however, further information about origins of these 264 field isolates and the two

avirulent strains was not available. The two predicted avirulent Australian isolates failed to amplify any of the tested genes. Moreover, no plasmid DNA band could be observed in A1 and WA400 using Pulsed-field-gelelectrophoresis. The WA1 plasmid contains a cluster of *rfb* genes encoding the rhamnose biosynthesis pathway which are involved in LOS biosynthesis (Boels *et al.*, 2004) and therefore recognized as a potential virulence factor. It had been suggested that the absence of this gene cluster results in a different LOS structure which might affect the ability of *B. hyodysenteriae* to colonize the colon (Bellgard *et al.*, 2009). The results of the PCR screening of the 49 field isolates is in agreement with the further studies indicating that the plasmid seems to be ubiquitous in *B. hyodysenteriae*. Nevertheless, the current study cannot confirm that the absence of the plasmid might be an indicator for non-pathogenic isolates, as all avirulent isolates amplified the plasmid-related genes BHWA1_02682.

The outer-membrane proteins, in particular those that are surface-exposed, play a crucial role in virulence of bacterial pathogens. The Vsp proteins constitute the major part of the outer-membrane of *B. hyodysenteriae*. Previous studies discovered eight copies of a *vsp* gene in *B. hyodysenteriae* B204 and recently two further *vsp* genes called *vspI* and *vspJ* have been identified in *B. hyodysenteriae* B204, WA1 and X576 (Witchell *et al.*, 2011). The previously discovered eight genes are in two unlinked four-membered gene clusters (*vsp a-d* and *vsp e-h*) and seemed to have occurred by duplication events in the *B. hyodysenteriae* genome. Duplication of linked genes have been described amongst others in *C. jejuni* (Wassenaar *et al.*, 1995) and *Borrelia hermsii* (Kitten *et al.*, 1993) and are believed to play a role in escaping the immunological response of the host or to adapt to stress conditions which are caused by the environment and might be therefore important in the pathogenic potential of *B. hyodysenteriae*. However, the gene *vspI* was present in 88.5% of virulent and 82.6% of avirulent isolates, and was therefore not a target of interest regarding the identification of genetic marker. Studies have shown that the protein sequences of the *vsp* genes are highly conserved between strains, but indicate that the number of *vsp* genes can differ as the *vspG* gene was absent in *B. hyodysenteriae* X576 and WA1 (Witchell *et al.*, 2011). Nevertheless, due its potential impact on virulence, it would be interesting to compare the arrangement and number of *vsp* genes in virulent and avirulent strains in order to identify possible differences.

La *et al.* (2005) were able to detect the gene, *bhlp27.9*, encoding an outer-membrane protein called Bhlp27.9 in a panel of 48 Australian *B. hyodysentriae* isolates by PCR (La *et al.*, 2005). Thus, the screening is in agreement with previous studies indicating that the gene *bhlp29.7* is ubiquitous in *B. hyodysenteriae* and therefore present in virulent and as well as avirulent isolates. Nevertheless, a study of 71 German field isolates showed that only 58% of the tested isolates carried that gene. Previous investigations suggested that the Bhlp29.7 protein might be a target for serological tests for virulent *B. hyodysenteriae* (La *et al.*, 2009). However, it was shown that Bhlp29.7 cross reacts with *B. innocens*. Therefore, *bhlp27.9* is not a reliable tool for general detection of *B. hyodysenteriae* or to distinguish between virulent or avirulent strains, as the genes seems not to be well conserved in different geographical locations and is present in different *Brachyspira* spp.

Flagella motility is crucial to *B. hyodysenteriae* and influences host colonization by promoting migration through viscous milieus such as gastrointestinal mucus. Due to the observation that presumptive avirulent strains are frequently less present within the crypt as virulent strains (J.R. Thomson, personal communication) as well as the fact that knockouts in flagella assembly genes reduced the virulence of *B. hyodysenteriae*, it was thought that non-pathogenic strains may lack flagella genes which would impact on their motility and therefore colonization ability. The *flgG* gene encodes a protein which is a component of the proximal and distal rods of the *B. hyodysenteriae* flagellum. However, only one avirulent strain could be detected lacking the *flgG* gene. Indeed, PCR detection of further flagella assembly genes like fliW would be necessary to investigate possible differences within the avirulent and virulent panel. Due to the ever decreasing stock of genomic DNA from the panel of test strains it was decided that further PCR studies would be on held until a comparative genome analysis of both types was completed, thus informing appropriate targets for further studies.

Iron-acquisition systems are important factors for the virulence of pathogens. The Bit system of *B. hyodysenteriae* contains typical features of a periplasmic iron transport system. It consists of three periplasmic binding proteins encoded by three genes, *bitA, bitB and bitC*, which are supposed to be iron-binding proteins (Dugourd *et al.*, 1999). These lipoproteins have been shown to share similarity with the periplasmic iron-binding protein AfuA of the afuABC operon of *A. Pleuropneumoniae* (Chin *et al.*, 1996) *and* HitA of the *H. influenza* HitABC periplasmic iron import operon (Sanders *et al.*, 1994). Phylogenetic

analysis revealed that BitB and BitC were distant but still clustered with other known periplasmic iron proteins. Thus, it is believed that the BIT system of *B. hyodysenteriae* has evolved differently, but seems to share functions with other periplasmic iron transport systems.

Since the BIT system may contribute to pathogenicity, the distribution of the *bitC* gene among virulent and avirulent *B. hyodysenteriae* isolates was investigated. Screening showed that the *bitC* gene was carried by 88.5% of virulent and 69.5% of avirulent isolates of *B. hyodysenteriae*. Interestingly, the *bitC* gene seemed to be absent in 50% of the German field isolates (Barth *et al.*, 2012). The former investigations showed that the BIT-system could only be detected in *B. hyodysenteriae* and *B. intermedia* but seems to be absent in *B. innocens* and *B. piloscoli* (Dugourd *et al.*, 1999). In addition, Dugourd *et al.* found that *bitC* and *bitB* are involved in iron acquisition. Thus, these findings indicate that the BIT-system might be associated with the pathogenicity of *B. hyodysenteriae*, which could explain why more virulent isolates possess *bitC* gene than avirulent strains. Therefore, this gene may be suitable for further studies. Further studies related to iron-acquisition by *B. hyodysenteriae* are discussed in **Chapter 5**.

A haemolysin is considered to be a major virulence factor of *B. hyodysenteriae* and is one key phenotypic factor to distinguish *B. hyodysenteriae* from other *Brachspira* spp. Previous screening experiments of the same DNA panel used in this study have shown that the four haemolysin genes *tylA*, *tylB*, *tylC* and *hlyA* were present in the non-pathogenic strains of *B. hyodysenteriae* (Walker, 2001). The conservation of these genes among *B. hyodysenter*iae isolates may not be unusual as the panel showed strong haemolysis regardless of pathogenic or non-pathogenic isolates.

Generally, the PCR screening revealed that non-pathogenic of *B. hyodysenteriae* isolates is not a consequence of absence of known virulence determinants and other factors need to be considered. It is known that pathogens acquire virulence factors to access new niches along with new metabolic pathways to persist in the new captures environment (Schmidt & Hensel, 2004). Genes which are associated with these newly acquired metabolic pathways in pathogenic bacteria may be absent in their less virulent counterparts. For instance, the nickel transporter NixA has been demonstrated to contribute to urease activity which is essential for *H. pylori* to colonize the stomach and cause gastritis (Yoshiyama &

Nakazawa, 2000). Further studies revealed that *nixA* is exclusively found in gastritis-causing *Helicobacter* spp., and absent in the genomes of other *Helicobacter* spp., which colonizes different environments suggesting that *nixA* is crucial for *H. pylori* virulence and is likely acquired by horizontal gene transfer. Since metabolism is closely tied to the genotype of an organism, its physiology and its environment, metabolomics offers a unique possibility to look at genotype-phenotype as well as genotype and envirotype relationships. Therefore, subtle differences in avirulent *B. hyodysenteriae* isolates affecting the activity and/or substrate affinity of essential metabolic enzymes and/or transporters might be the reason for their lack of adaptation within the host resulting in not fully definitive form of SD in pigs. However, this hypothesis requires clearly intensive metabolic and metabolomics studies.

For further investigations in the current project, one isolate from each group (virulent and avirulent) was selected as a representative in order to perform comparative genomics and proteomics analysis regarding the identification of a potential target gene/protein. Sequencing of all five available isolates and preferable more strains would have been ideal but was not feasible due to a restricted budget and timeframe. The choice of selection for representatives was very small, however, the isolates P8544 (virulent) and P7455 (avirulent) were chosen as they exhibited the most phenotypically similarities especially in respect to their growth kinetics and also in their geographical origin and carriage of the tested target genes.

The studies in this chapter did not detect any obvious phenotypic or genotypic differences between virulent and avirulent *B. hyodysenteriae* isolates. The screening showed that known virulence genes of *B. hyodysenteriae* were found in the majority of the tested panel suggesting that these tested target genes are highly conserved among pathogenic and non-pathogenic isolates and thus not useful as distinctive marker. Therefore, genome sequencing of both types of *B. hyodysenteriae* may give a deeper insight in genetic differences to identify possible target genes which will be discussed in **Chapter 4**. As PCR and comparative genome analysis is only assessing the presence of a sequence but does not provide information about gene expression additional expression analysis of different targets would enhance the understanding of the virulence in *B. hyodysenteriae* and will be further discussed in **Chapter 5** and **Chapter 6.**

Chapter 4: Comparative genome analysis of *B. hyodysenteriae*

4.1 Introduction

Whole genome sequencing is the process of deciphering the order of nucleotide bases in a complete DNA sequence of an organism's genome; in the case of bacteria, this entails sequencing chromosomal DNA as well as any non-chromosomal nucleic acids the organism may possess. Comparative genomic analyses of whole genome sequences of many different pathogenic and commensal microorganisms provides insight into adaption mechanisms and enhances the understanding of pathogenicity. It is becoming evident that distinct genomic regions have a significant impact on pathogenic potential, such as colonization and fitness. Therefore, analysis of the genomes of microorganisms provides the basis for understanding infections in livestock and humans and is therefore fundamental to the development of diagnostics, vaccines and therapeutic drugs.

DNA sequencing uses several methods to establish the order of nucleotide bases in a given molecule of DNA. The automated Sanger method has dominated the market for two decades; however, due to the high demand of large datasets, efforts have been made towards the improvement of new sequencing technologies. Currently, next-generation sequencing platforms have become the premier tool for genomic analysis. There are three techniques that dominate the commercial market: Roche 454 Genome Sequencer, the Illumina (SOLEXA) Genome Analyzer and the Life Technologies SOLiD System. The Solexa platform uses a sequencing synthesis principal, which results in considerably shorter reads of up to 100 bases. This technology is the most widely used platform and also has the capability to produce paired-end reads (Metzker, 2010). The progress in next generation sequencing technology provides an inexpensive and rapid way to produce large datasets of many hundreds of genomes. In 1995 the first completed prokaryotic genome sequence of *Haemophilus influenza* was published (Fleischmann *et al.*, 1995). Since then, the number of publicly-available genome sequences has increased drastically, primarily due to the improvement in the sequencing technology and the concurrent reduction in costs. In October 2012, the GOLD database (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi) listed 24,691 genome projects, of which 15,331 were focussed on bacteria, of which 3,412 were reported to be completed and 11,910 in draft form.

The constant increase in the availability of genome sequences data has led to a reliance on automatic annotation tools to minimize the time consuming procedure of manual annotation of the entire genome. Genome annotation is the process of predicting open-

reading frames (ORF) in a DNA sequence and assigning a biological function to it. Most automatic annotation pipelines, e.g. Rapid Annotation using Subsystem Technology (RAST) server (Aziz *et al.*, 2008), the Bacterial Annotation System (BASys) (Van Domselaar *et al.*, 2005) and Xbase (Chaudhuri *et al.*, 2008) use a closely-related reference genome to predict genes and assign protein function to the new genome. However, the accuracy of automatic annotation software has been controversially, as the pipelines are known to introduce and propagate poor annotations and errors which have an impact on further (downstream) comparative genome analyses. For example, the genome of *Halorhabdus utahensis* was annotated using three different automatic annotation tools in order to evaluate the effectiveness of each service, the conclusion being that they all differed considerably in their prediction of genes and features (Bakke *et al.*, 2009). Recently, misannotation of the *vspG* and *vspH* genes in the available *B. hyodysenteriae* X576 and WA1 genome sequences has been reported (Witchell *et al.*, 2011). Manual annotation revealed that the translated products of the two genes share a higher sequence identity (>96%) with the newly-identified protein sequences of VspI and VspJ. Therefore, there is recognition that errors in annotation need to be minimized, since these errors are propagated in downstream studies.

In 2009 the first complete genome sequence of an Australian *B. hyodysenteriae* strain, termed WA1, was published and the whole genome made accessible in NCBI (Acc.# NC_012225 (chromosome) and Acc.# NC_012226 (plasmid)). The genome consists of a single circular *ca.* 3 Mb chromosome, and a 35,940 bp circular plasmid. Compared to the other three genera of sequenced spirochaetes, the genome of *B. hyodysenteriae* WA1 is larger than *Borrelia* and *Treponema* (0.9 to 2.8 Mb) and smaller than *Leptospira* (3.6 to 4.7 Mb), but is more closely related to *Leptospira* spp. (Paster *et al.*, 1991) as *Borrelia* spp. are generally distinct from other spirochaetes by their having a linear chromosome and a circular plasmid (Fraser *et al.*, 1997). In addition, the genome sequences of *B. piloscoli* 95/1000 *B. intermedia* PWS/^{T} and the incomplete genome sequence of *B. murdochii* 56-150 ^{T} became available. Comparison of these four *Brachyspira* genomes showed that *B. intermedia* PWS/^{T} is the largest sequenced species (3,304,788 bp) followed by *B. murdochii* 56_150^{T}, whereas *B. piloscoli* 95/1000 is slightly smaller than *B. hyodysenteriae*. Recently, two further *B. piloscoli* strains were sequenced, one, B2904, isolated from an avian host and one, WesB, a human isolate (Mappley *et al.*, 2012). The results have shown that all sequenced *B. piloscoli* isolates share high similarity although the genomes of B2904 and WesB were larger than that of *B. piloscoli* 95/1000. Preliminary

studies by Mappley *et al.* (2012) proposed that *B. aalborgi* 513^T is 2.5 Mb (unpublished data) and thus the smallest sequenced *Brachyspira* spp. next to *B. piloscoli* 95/1000.

In general it is thought that the size of the genome mirrors the specialisation of the pathogen. The smaller genome sizes of *B. hyodysenteriae*, *B. aalborgi* and *B. piloscoli* are suggestive that reductive evolution has taken place (Andersson & Kurland, 1998). Such a phenomena has been reported in diverse bacterial groups, including *T. denticola* (Seshadri *et al.*, 2004) and *Chlamydia* (Horn *et al.*, 2004) showing that bacteria undergo genome-reduction by gene deletions and rearrangements when they transit from a free-living state to a constantly host-restricted state. Reduction of the genome size of host-adapted pathogens is mainly driven by the reduced selective pressure to maintain accessory genes which are not essential for survival beyond the host (Rio *et al.*, 2003). Comparative genome comparison of all four *Brachyspira* genomes has shown that *B. intermedia* had the greatest number of unique genes (269) followed by *B. murdochii* (212), whereas *B. hyodysenteriae* (116) and *B. piloscoli* (131) contains less accessory genetic material (Hafstrom *et al.*, 2011). The lower number of accessory genes in *B. hyodysenteriae* and *B. piloscoli* is associated with higher specialization in both species, indicating that they are more niche-specific pathogens and therefore well-adapted to survive in their chosen environment. Loss of genes with function that might be replaced by uptake of host metabolites has been noted (Fraser *et al.*, 1995).Therefore, it might not be surprising that COG analysis of *Brachyspira* spp. has shown that *B. piloscoli* has the lowest number of genes classed associated with inorganic transport and metabolism, while genes of *B. murdochii* were 5-10 times more abundant in the COG category "Amino acid transport and metabolism" than in all the other *Brachyspira* spp. (Hafstrom *et al.*, 2011). Compared to *B. hyodysenteriae*, *B. piloscoli* has a much broader host-range, indicating that these species should have distinct life strategies. Hafstrom *et al.* (2011) reported that *B. hyodysenteriae* and *B. piloscoli* had the least number of shared genes among *Brachyspira* spp., reflecting the fact that the two species developed and specialized independently. Global genome comparison has shown that that *B. murdochii* and *B. piloscoli* seem to have a higher degree of overall metabolic flexibility compared to *B. hyodysenteriae* enabling them to colonize a broader host-range. For instance, *B. hyodysenteriae* lacks the glycine reductase (*grd)* complex, suggesting that this pathogen is, unlike *B. piloscoli* and *B. murdochhii*, unable to utilise glycine as a carbon and energy source. In addition, the absence of the *grd* cluster is also associated with the fact that *B. hyodysenteriae* is less able to withstand oxidative stress than *B. piloscoli* (Boye *et al.*, 2001); taken together, these observations help us understand

how *B.piloscoli* is better adapted to surviving in varying environmental conditions, and thus different host species.

On the basis of the available sequences, *B. hyodysenteriae* and *B. intermedia* appear to be the only *Brachyspira* species that contain a plasmid. Compared to *B. hyodysenteriae* WA1, the plasmid of *B. intermedia PWS/*^{T} is only 3,260 bp in size and contains only three putative genes encoding products of unknown function (Hafstrom *et al.*, 2011; Wanchanthuek *et al.*, 2010). The plasmid of *B. hyodysenteriae* WA1 is ~ 36 kbp in size and contains 31 genes including those encoding 5 hypothetical proteins, 6 Rfb proteins predicted to be involved in the rhamnose biosynthesis, and glycosyltransferase proteins (Bellgard *et al.*, 2009). It has been assumed that the plasmid has a low copy number (La *et al.*, 2011). A study by La *et al.* (2011) suggested that the plasmid contributes to virulence in *B. hyodysenteriae* as plasmid-free isolates were not able to cause SD in pigs; however, the main contribution of the plasmid-encoded proteins and their impact on pathogenicity still remains to be determined. Besides, heterogeneity among different plasmid sequences has been recognized by PCR (La *et al.*, 2011) but no deeper plasmid analyses have been carried out so far.

In the previous chapter, representative *B. hyodysenteriae* strains were chosen for further investigation; one virulent strain (P8544) and one avirulent strain (P7455). Consequently, the purpose of the work described in this chapter was to undertake the analysis of the genomes of these strains. Sequence data was obtained by SOLEXA sequencing, and was then used to perform a comparative sequence analysis of these draft genomes with the complete, published genome sequence of *B. hyodysenteriae* WA1; the primary purpose being to investigate genetic differences between pathogenic and non-pathogenic isolates and to identify possible target genes which might be useful as a diagnostic test(s).

4.2 Results

4.2.1 Genome structure and general features of P8455 and P7455

The genomes of *B. hyodysenteriae* P8544 and P7455 each comprise a single circular chromosome and a single circular plasmid (**Figures 4.1** and **4.2**). The general features are presented in **Table 4.1**. Both genomes have an average G+C content of 27 % which is similar to the published genome of *B. hyodysenteriae* WA1. The plasmid sequences of P8544 and P7455 have an average G+C-content of 22 % which is slightly higher than that of pBWA1. In addition one copy of the rRNA operon (23S-16S-5S) was identified by RNAmmer and 33 tRNA were predicted in each chromosome by tRNA Scan-RE. Compared to the complete *B. hyodysenteriae* WA1 genome it seems that the Tyrosine tRNA gene (BHWA1_00943) is missing in both newly sequenced isolates which is possibly due to missing regions among the assembled contigs. In addition, a considerable number of hypothetical proteins have been identified (858 in P8544 and 847 in P7455) emphasising that the encoded products of over 30% of the predicted coding sequences in *B. hyodysenteriae* are of unknown function.

The sequence reads for P8544 were assembled into 199 contigs (smallest contig 112 bp, largest contig 322,303 bp) using VELVET 0.6 (Zerbino &Birney, 2008) with a total length of 3,036,471 bp. Contigs were scaffold against the *B. hyodysenteriae* WA1 genome using Mauve. Following automatic annotation using Xbase, 2,743 ORFS, corresponding to a coding density of 86.5%, were identified. However, further analysis of the P8544 genome revealed that some of the genes appeared to have been identified on more than one occasion. This was due to a failure to assemble some sequences into intact coding sequences, due to the presence (for example) of short sequence repeats, or due to the absence of some sequences through sequencing failure. The coding sequences which could not be clustered within other contigs were clustered into an artificial contig of 7,101 bp, containing 36 predicted CDS. These 36 CDS were identified during Xbase annotation. However, BLASTn comparison of these sequences with other nucleotide sequences within the P8544 genome revealed that they all appeared to correspond to genes present elsewhere within the genome, but were truncated, missing sections of coding sequence at either their 5'- or 3'-ends. Consequently, because the 7,101 bp region did not contain any coding sequences which were absent from any of the other assembled contigs, these 36 truncated coding sequences were excluded from further analyses.

Table 4.1: General genomic features predicted for *B. hyodysenteriae* P8544 and P7455 in comparison with *B. hyodysenteriae* WA1.

Feature	*B. hyodysenteriae* WA1	*B. hyodysenteriae* P8544	*B. hyodysenteriae* P7455
Genome			
Size (bp)	3,036,634	3,036.471 (3,043,572)*	3,009,934 (3,017,280)*
Number of ORFs	2,669	2,743	2,724
Number of contigs	-	199	205
N50	-	112	112
Largest contig size (bp)	-	322,303	454,500
Genes assigned to KO	1094 (40.98%)	1082 (39.45%)	1085 (39.83%)
;;Genes not assigned to KO	1575 (59.01%)	1639 (60.55%)	1661 (60.55%)
Proteins assigned with a Lipoprotein signal peptide	179	174	179
††	3,000,694	3,005,002	2,975,112
Chromosome			
Size (bp)			
G+C content (%)	27.06	27.00	27.03
Number of ORF	2,611	2,714	2,692
Coding percentage	86.8	86.7%	86.7
Hypothetical/ proteins	878	858	847
Unique genes predicted †	-	35	27
tRNA	34	33	33
rRNA	3	3	3
Plasmid			
Size (bp)	35,940	31,469	34,822
G+C content	21,82	22.16	22.9
Number of ORFs	31	29	32

*original genome size including the 7,101-7,321 bp region containing truncated genes as described in **4.1.2**

† genes for which no homologue was identified in *Brachyspira hyodysenteriae* WA1

†† lipoproteins were predicted within each genome using SpliP with an *L. interrogans copenhageni* Training matrix

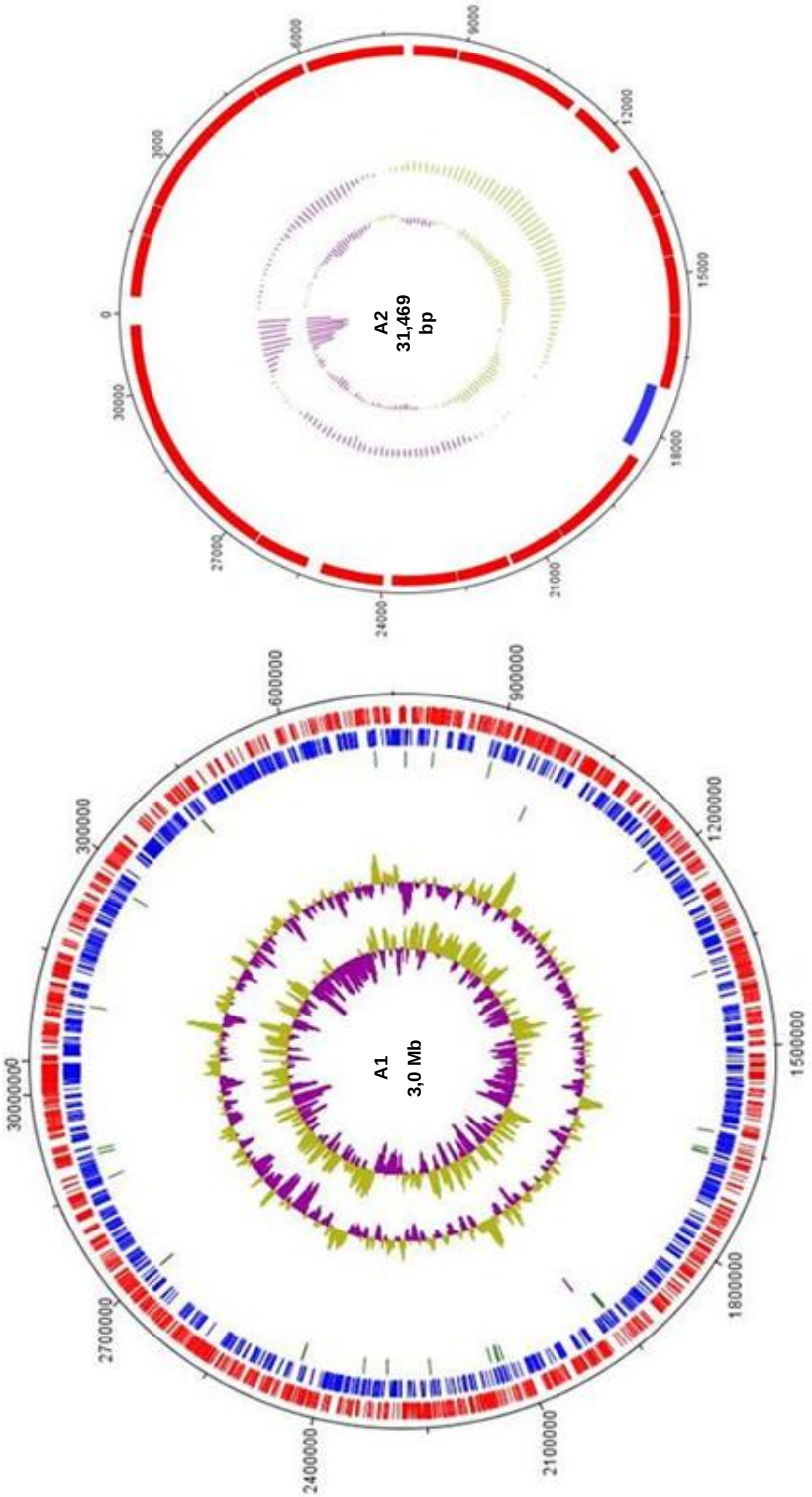


Figure 4.1: Circular representation of the draft genome of *B. hyodysenteriae* P8544 with annotated genes using DNAPlotter. A1: the chromosomeof P8544. A2: plasmid of P8544. From the outside in, the outer two tracks show the open reading frame orientated in the forward (red) and reverse (blue) directions respectively. The third track marks the tRNA genes (green) and the fourth track shows the rRNA operons (pink). The fifth track shows the GC skew as negative values (purple) or positive values (olive). The innermost track shows the G+C% content. plot.

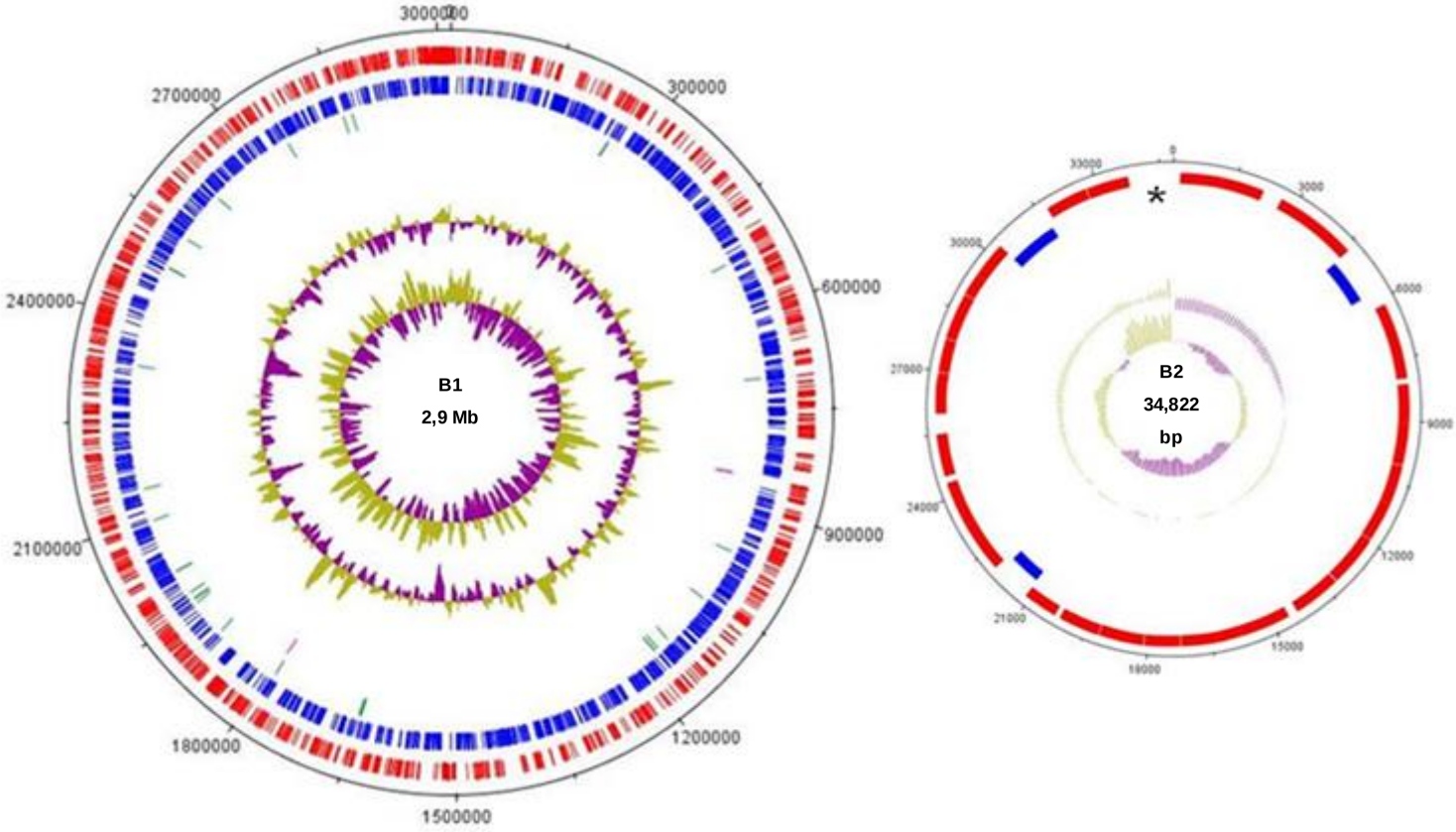


Figure 4.2: Circular representation of the draft genome of *B. hyodysenteriae* P7455 with annotated genes using DNAPlotter. B1: the chromosome of P7455. B2: the plasmid of P7455. From the outside in, the outer two tracks show the open reading frame orientated in the forward (red) and reverse (blue) directions respectively. The third track marks the tRNA genes (green) and the fourth track shows the rRNA operons (pink). The fifth track shows the GC skew, purple indicating negative values and olive positive values. The innermost track shows the G+C% content plot. (*)The gene Bhyoa7455_01 could not been displayed by DNAPlotter as the gene sequence was truncated by the linear input file of contig 31. The gene was predicted to be present by CloneManager version 9.

The plasmid sequence of P8544 (contig 22) comprises 31,469 bp, containing, 29 predicted ORFS. The P8544 plasmid is smaller than the plasmids of WA1 and P7455.

Sequence reads for P7455 were assembled into 205 contigs with a total length of 3,009,934 bp. The largest contig was 454,500 bp long, while the shortest contig was just 112 bp in length. Prior to automatic annotation the contigs were re-ordered as mentioned previously. The automatic annotation predicted 2,724 genes, corresponding to a coding density of 86.7 %. Similar to the P8544 genome, a region spanning 7,321 bp was not assembled into one of the contigs, containing 38 truncated genes of *B. hyodysenteriae* which matched missed sections of genes present in the other 205 contigs. The plasmid sequence of P7455 (contig 31) comprised 34,822 bp containing 32 predicted ORFs.

Similar numbers of proteins (179 for WA1 and P7455 and 174 for P8544) were assigned a lipoprotein signal peptide using the SpLip software. The three genomes were searched against three databases, LipoP 1.0, SignalP 4.0 and SpLip, as described in **2.10.2**. and results are summarized in **Appendix 5**. Due to discrepancy in the lipobox sequence of spirochaetes compared to other Gram-negative bacteria, only lipoprotein signal peptides predicted via the SpLip software were listed and further discussed in this study. Further detail regarding prediction of signal peptides will be presented in **Chapter 6**.

The size of the whole draft genomes of P8544 and P7455 are slightly smaller (7,000 bp to 19,000 bp) compared to the closed genome of WA1, which could in part be due to incomplete coverage of these genomes during assembly. In addition, the prediction of higher numbers of ORFs (74 and 55) in the two English genomes of *B. hyodysenteriae* than in WA1 is likely an artefact of automated annotation and the presence of truncated ORFs through incomplete sequencing or sequencing errors. Therefore, to draw a definite conclusion about the correct size and number of genes, the genomes of P8544 and P7455 needed to be closed.

For the purpose of submission to GenBank, it was necessary to re-annotate the genomes of *B. hyodysenteriae* P8544 and P7455 using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (Pruitt *et al.*, 2009). Subsequently, the genome sequences were deposited in the GenBank repository under accession number PRJNA175262 for *B. hyodysenteriae* P7455 and PRJNA175260 for *B. hyodysenteriae* P8544. However,

annotation of the genomes conducted in this chapter was utilised by Xbase as described earlier and no attempt has yet been made to compare the two different annotation systems.

4.2.2 Comparative genome comparison

Brachyspira hyodysenteriae WA1 was the only strain of this species whose whole genome is sequenced and available (from NCBI) prior to this work commencing. To allow a more meaningful comparison of the avirulent and virulent strains included in this study, the genome of WA1 was also included in analyses.

The general features of the genomes summarized in **Table 4.1** indicate that all three *B. hyodysenteriae* genomes share high similarity. The high sequence homology between the genomes was also confirmed by BRIG (**Figure 4.3**). The matches in **Figure 4.3** are calculated from the reference genome WA1. Consequently, genomic regions which are present in P8544 and P7455 but absent in WA1 are not displayed in the image. Novel regions in the genome sequences of P8544 and P7455 are discussed in **4.2.4**.

4.2.2.1 *Functional genome comparison*

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) is a database resource to improve understanding of high-level functions and utilities of biological systems from molecular-level information, such as large-scale molecular datasets generated by genome sequencing approaches. The KEGG Automatic Annotation Server, KAAS (Moriya *et al.*, 2007) was utilised to assign function to each predicted protein in the genomes of WA1, P8544 and P7455, by conducting BLAST comparisons against the KEGG GENE database in order to identify orthologous proteins (**Figure 4.4)**. KAAS searches both the manually curated KEGG GENES database (which includes data from 1358 bacteria, 151 eukaryotes and 114 archaea) and the KEGG Orthology (KO) database (containing 15,173 groups).

Of the proteins encoded by the 2,743 predicted genes in the P8544 genome, 39.44% (n=1,082) were assigned KEGG orthologues (KO) by KAAS. Similarly, 39.83% (n=1,085of the proteins encoded by the 2,742 predicted genes in the P7455 genome were assigned KO, as well as 40.98% (n=1,049) of the proteins encoded by genes in the genome of WA1. The large majority of proteins assigned KO in all genomes were observed to have a metabolic function and more specifically this group was dominated by proteins involved

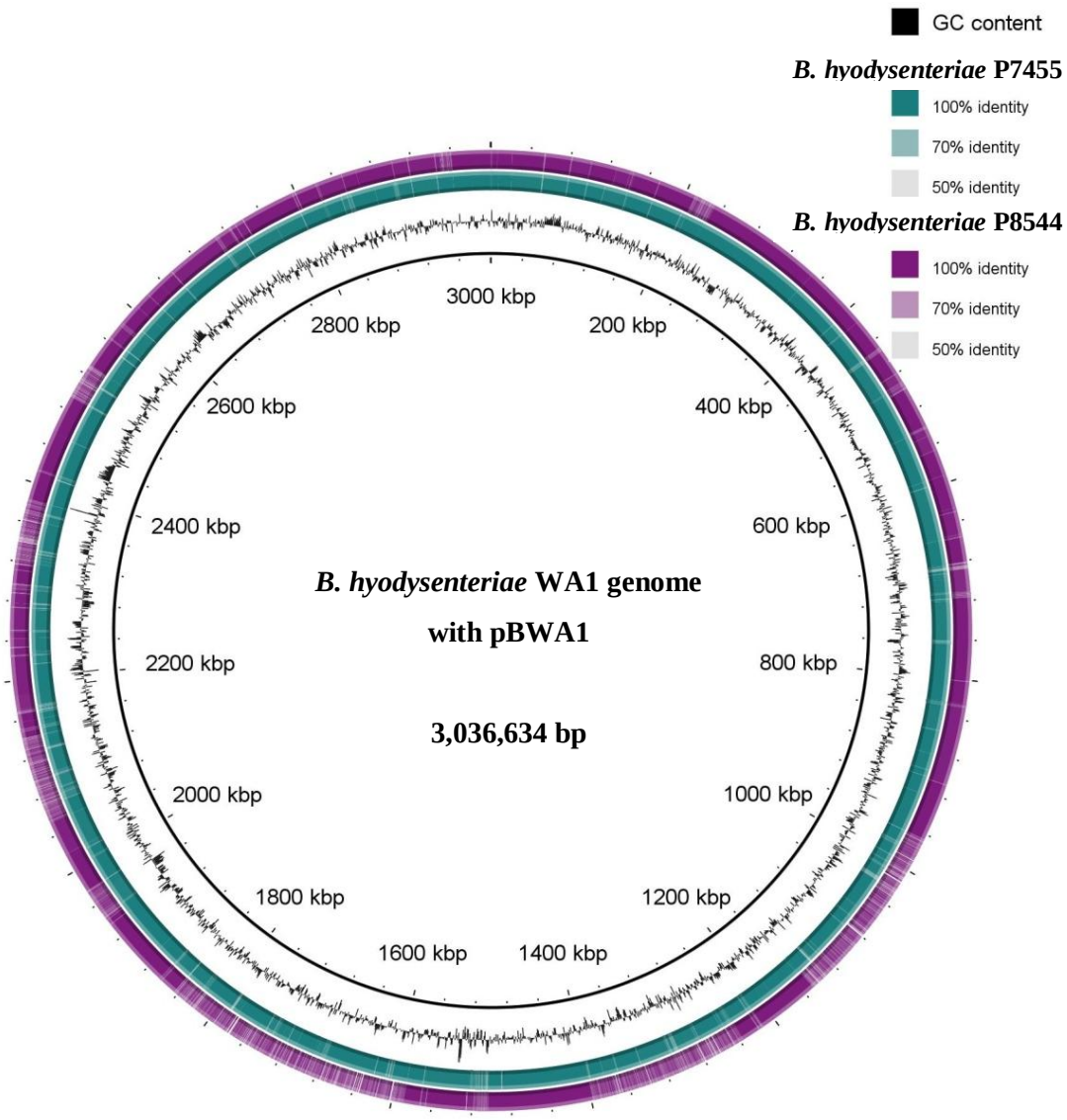
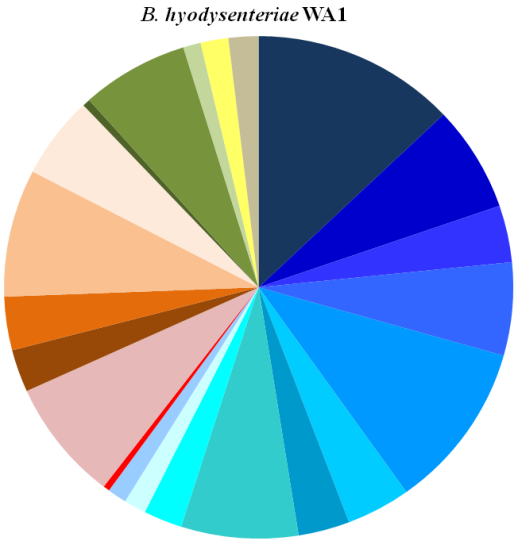
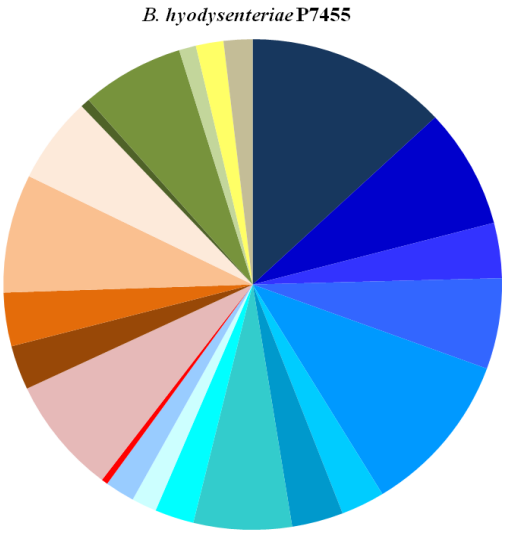


Figure 4.3: BRIG image of *B. hyodysenteriae* WA1 vs. the draft genomes of *B. hyodysenteriae* P8544 and P7455. The innermost ring represents the whole genome sequence of the published strain WA1, followed by the GC content (black).The remaining outer rings show a BLAST comparison of the two draft genomes of *B. hyodysenteriae* P7455 (green) and P8544 (purple) against the reference genome, WA1. The varying colour intensity of the rings indicates a BLAST match of a particular percentage identity, as shown in the legend. Most of the genome sequences of P8544 and P7455 appear in dark colour indicating 100% sequence similarity with WA1.

Legend:

KEGG function	P8544	P7455 (%)	WA1
Metabolism			
Carbohydrate Metabolism	5.32	4.74	4.76
Energy Metabolism	2.88	2.86	2.51
Lipid Metabolism	1.31	1.32	1.35
Nucleotide metabolism	2.15	2.17	2.21
Amino acid Metabolism	3.79	3.89	3.97
Metabolism of other Amino acids	1.02	1.03	1.5
Glycan Biosynthesis and Metabolism	1.2	1.21	1.2
Metabolism of Cofactors, Vitamins	2.26	2.31	2.74
Metabolism of Trepenoide, Polypeptide	0.91	0.92	0.9
Biosynthesis of secondary metabolites	0.58	0.59	0.52
Xenobiotics Biodegradation and Metabolism	0.69	0.7	0.45
Genetic Information Processing			
Transcription	0.15	0.15	0.15
Translation	2.81	2.79	2.88
Environmental Information Processing			
Folding, Sorting and Degradation	1.06	1.06	1.01
Replication and repair	1.28	1.28	1.27
Membrane transport	2.84	2.83	3.03
Signal Transduction	2.08	2.06	1.95
Cellular process			
Transport and catabolism	0.18	0.22	0.18
Cell motility	2.41	2.42	2.51
Cell growth and death	0.4	0.4	0.41
Organismal Systems	0.68	0.65	0.7
Human disease	0.67	0.64	0.65





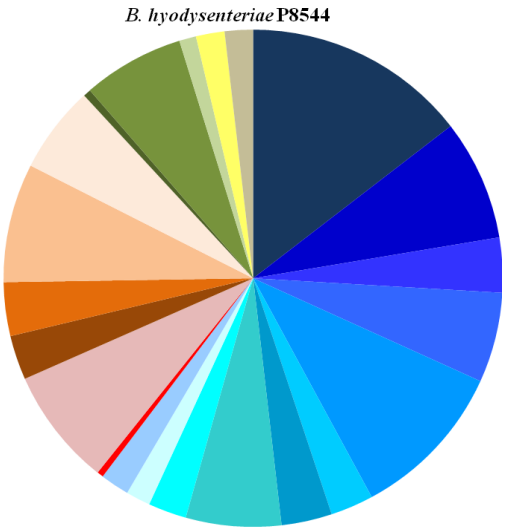


Figure 4.4: Percentage distribution of KEGG classification categories in *B. hyodysenteriae* WA1, P8544 and P7455. Of the predicted proteomes, 39.45- 40.98% have orthologues in the KO database, whereas 59-60.55% of proteins had no orthologue in the KO database. The majority of KEGG-characterised proteins in the three genomes have carbohydrate metabolism and amino acid metabolism functions.

in carbohydrate metabolism (5.23% in P8544, 4.74% in P7455, and 4.76% in WA1), amino acid metabolism (3.79% in P8544, 3.89% P7455, and 3.97% in WA1) and energy metabolism (2.88% in P8544, 2.86% in P7455, and 1.72% in WA1). However, there a considerable number of proteins classified in membrane transport were also identified (2.84% in P8544, 2.83% in P7455, and 3.03% in WA1), cell motility (2.41% in P8544, 2.42% in P7455, and 2.51% in WA1) as well as translation (2.81% in P8544, 2.79% in P7455, and 4.23% in WA1). The high percentage similarity of KOs assigned to each genome reflects the close relationship between these isolates.

4.2.2.2 *Presence/ absence of genes in the three B. hyodysenteriae genomes*

Global genome comparison of the three genomes revealed that 15 ORFs which were found to be shared among the genomes of P8544 and P7455 were absent in WA1. Furthermore, 46 genes present in WA1 were absent in P8544, while 39 genes of WA1 were not detected in P7455. However, whether these genes are truly absent from each of the genomes sequenced in the course of this study, or whether they are present but not represented in the incomplete sequences remains unclear and will require further analysis to confirm.

A considerable number of ORFs could be identified in each of the new sequenced *B. hyodysenteriae* genomes which did not show any homologue to *B. hyodysenteriae* WA1 and were therefore considered to be novel. In total, 35 ORFs were predicted to be novel in P8544 and 27 novel ORFs were found in P7455; these genes are discussed in more detail in section **4.2.3.1**. Additionally, the commercially available software, Panseq 2.0, was used to detect unique regions in both genomes by comparing each of the three genomes against each other. The results revealed that the majority of these genes were located in novel genomic regions which were recognized by Panseq, confirming that they are exclusively found in the isolates P8544 and P7455. Indeed, depending on the stringency of the settings of the programme, Panseq identified further unique regions for each genome (as shown in **Appendix 4**). However, only the regions containing the 27-35 novel genes are summarized in **Table 4.2**. Of those proteins < 200 aa in size, the majority could not be recognized as novel regions by Panseq using the default settings described in **Chapter 2**.

Table 4.2: Presence and absence of genes in the genomes of *B. hyodysenteriae* WA1, P8544 and P7455. Genes which were absent in at least one of the three genomes were summarized. In total, 27-35 genes were detected in the genomes of *B. hyodysenteriae* P8544 and P7455which had no homologues in the genome of WA1. These predicted ORFs were further characterized using BLASTp and the closest match was displayed. The novel region finder Panseq confirmed that these ORFs occurred in genomic regions which were unique to the sequence of P8544 and P7455 using WA1 as a query.

***B. hyodysenteriae* WA1**		***B. hyodysenteriae* P8544**					***B. hyodysenteriae* P7455**				
Locus_ID	**Product**	**Locus_ID**	**Product**	**Panseq***			**Locus_ID**	**Product**	**Panseq***		
				C[1]	**S[2]**	**E[3]**			**C[1]**	**S[2]**	**E[3]**
BHWA1_00001	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00022	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00128	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00405	type II restriction-modification enzyme	x	x	x	x	x	x	x	x	x	x
BHWA1_00406	type I restriction-modification enzyme	x	x	x	x	x	x	x	x	x	x
BHWA1_00437	Nucleotidyltransferase substrate binding protein	x	x	x	x	x	Bhyoa7455_0141	Nucleotidyltransferase substrate binding protein	N/A		
BHWA1_00438	restriction modification system DNA specificity domain protein	x	x	x	x	x	Bhyoa7455_0139	restriction modification system DNA specificity domain protein	N/A		
BHWA1_00439	type I site-specific deoxyribonuclease, HsdR family	x	x	x	x	x	Bhyoa7455_0138	type I site-specific deoxyribonuclease, HsdR family	N/A		
BHWA1_00479	ankyrin repeat-containing protein	Bhyov8544_1628	ankyrin repeat-containing protein	N/A			Bhyoa7455_0079	ankyrin repeat-containing protein	N/A		
BHWA1_00508	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00595	ankyrin repeat-containing protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00637	hypothetical protein	Bhyov8544_1442	hypothetical protein	N/A			unkown*	hypothetical protein	N/A		
BHWA1_00638	hypothetical protein	Bhyov8544_1443	hypothetical protein	N/A			unkown*	hypothetical protein	N/A		
BHWA1_00698	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00722	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00723	hypothetical protein	Bhyov8544_0808	hypothetical protein	N/A			unknown*	hypothetical protein	N/A		
BHWA1_00825	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00826	glycosyl transferase, family 8	x	x	x	x	x	x	x	x	x	x
BHWA1_00830	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_00839	hypothetical protein	x	x	x	x	x	x	x	x	x	x
BHWA1_01035	hypothetical protein	x	x	x	x	x	x	x	x	x	x

BHWA1_01096	ankyrin repeat-containing protein	x	x	x	x	x	Bhyoa7455_2112	ankyrin repeat-containing protein	N/A			
BHWA1_01103	hypothetical protein	x	x	x	x	x	Bhyoa7455_2231	hypothetical protein	N/A			
BHWA1_01133	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01160	hypothetical protein	x	x	x	x	x	Bhyoa7455_2340	hypothetical protein	N/A			
BHWA1_01185	hypothetical protein	x	x	x	x	x	Bhyoa7455_2314	hypothetical protein	N/A			
BHWA1_01220	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01260	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01309	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01310	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01311	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01312	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01314	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01315	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01319	Appr-1-p processing enzyme family protein	Bhyov8544_1746	Appr-1-p processing enzyme family protein	N/A			unknown*	Appr-1-p processing enzyme family protein	N/A			
BHWA1_01320	hypothetical protein	Bhyov8544_1747	hypothetical protein	N/A			unknown*	hypothetical protein	N/A			
BHWA1_01359	hypothetical protein	Bhyov8544_1786	hypothetical protein	N/A			Bhyoa7455_0704	hypothetical protein	N/A			
BHWA1_01520	hypothetical protein	x	x	x	x	x	X	X	x	x	x	
BHWA1_01820	TPR domain-containing protein	x	x	x	x	x	Bhyoa7455_1667	TPR domain-containing protein	N/A			
BHWA1_01847	OrfC	x	x	x	x	x	Bhyoa7455_2542	OrfC	N/A			
BHWA1_01864	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01936	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_01953	hypothetical protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_02005	Predicted endonuclease	x	x	x	x	x	x	x	x	x	x	
BHWA1_02031	nucleotidyltransferase substrate binding protein	x	x	x	x	x	x	x	x	x	x	
BHWA1_02035	putative ankyrin repeat-containing protein	Bhyov8544_1786	putative ankyrin repeat-containing protein	N/A			Bhyoa7455_2263	putative ankyrin repeat-containing protein	N/A			
BHWA1_02259	YcfA-like protein	x	x	x	x	x	Bhyoa7455_0655	YcfA-like protein	N/A			
BHWA1_02276	hypothetical protein	x	x	x	x	x	Bhyoa7455_0635	hypothetical protein	N/A			
BHWA1_02574	Ycfa like protein	Bhyov8544_1786	Ycfa like protein	N/A			unkown*	Ycfa like protein	N/A			
BHWA1_02672	putative hydrolase (HAD) superfamily	x	x	x	x	x	pBhyo7455_05	putative hydrolase (HAD) superfamily	N/A			
BHWA1_02673	hydrolase (HAD superfamily)protein-like protein	x	x	x	x	x	pBhyo7455_04	hydrolase (HAD superfamily)protein-like protein	N/A			
BHWA1_02674	Fe-S oxireductase containing radical SAM	x	x	x	x	x	pBhyo7455_03	Fe-S oxireductase containing radical SAM	N/A			

BHWA1_02676	Fe-S oxireductase containing radical SAM	x	x	x	x	x	pBhyo7455_01	Fe-S oxireductase containing radical SAM	N/A			
BHWA1_02666	Glycosyltransferase	x	x	x	x	X	pBhyo7455_11	Glycosyltransferase	N/A			
BHWA1_02689	putative alpha-1,2-fucosyltransferase; glycosyl transferase	pBhyo8544_08	putative alpha-1,2-fucosyltransferase; glycosyl transferase	N/A			x	x	x	x	x	
x	x	Bhyov8544_0171	short chain dehydrogenase, putative [*Aspergillus flavus*]	N/A			Bhyoa7455_0510	short chain dehydrogenase, putative [*Aspergillus flavus*]	N/A			
x	x	Bhyov8544_0303	hypothetical protein XF0398 [*Xylella fastidiosa* 9a5c]	304	1	2661	x	x	x	x	x	
x	x	Bhyov8544_0559	hypothetical protein [*Clostridium difficile* 002-P50-2011]	63	1	8593	x	x	x	x	x	
x	x	Bhyov8544_0632	unnamed protein product [*Brachyspira murdochii* DSM 12563]_1538		93023	95223	x	x	x	x	x	
x	x	Bhyov8544_0633	unnamed protein product [*Brachyspira murdochii* DSM 12563]				x	x	x	x	x	
x	x	Bhyov8544_0646	hypothetical protein Bint_2751 [*Brachyspira intermedia* PWS/A]				x	x	x	x	x	
x	x	Bhyov8544_0683	Abi family protein [*Nitrosococcus watsonii* C-113]	N/A			Bhyoa7455_1716	Abi family protein [*Nitrosococcus watsonii* C-113]	N/A			
x	x	Bhyov8544_0795	hypothetical protein Bint_2640 [*Brachyspira intermedia* PWS/A]	104#	-	-	x	x	x	x	x	
x	x	Bhyov8544_0799	hypothetical protein Cflav_PD1273 [bacterium Ellin514]	N/A			Bhyoa7455_0979	hypothetical protein Cflav_PD1273 [bacterium Ellin514]	N/A			
x	x	Bhyov8544_0980	hypothetical protein Swol_0219 [*Syntrophomonas wolfei subsp. wolfei str. Goettingen*]	29#	-	-	x	x	x	x	x	
x	x											
x	x	Bhyov8544_1064	hypothetical protein [*Bacteroides cellulosilyticu*]	N/A			Bhyoa7455_0832	hypothetical protein [*Bacteroides cellulosilyticus*]	N/A			
x	x	Bhyov8544_1094	DNA methyltransferase [*Brachyspira murdochii* DSM 12563] Bmur_1744	N/A			Bhyoa7455_1978	DNA methyltransferase [*Brachyspira murdochii* DSM 12563] Bmur_1744	N/A			
x	x	Bhyov8544_1095	DNA methyltransferase [*Brachyspira murdochii* DSM 12563] Bmur_1744	N/A								
x	x	Bhyov8544_1096	DNA methyltransferase [Brachyspira murdochii DSM 12563] Bmur_1744	N/A								
x	x	Bhyov8544_1649	transporter, MFS superfamily [*Brachyspira pilosicoli* 95/1000_0041	N/A			Bhyoa7455_0076	transporter, MFS superfamily [*Brachyspira pilosicoli* 95/1000_0041	N/A			

x	x	Bhyov8544_1650	conserved hypothetical protein [*Brachyspira pilosicoli* 95/1000_0042	N/A			Bhyoa7455_0075	conserved hypothetical protein [*Brachyspira pilosicoli* 95/1000_0042	N/A		
x	x	Bhyov8544_1743	conserved hypothetical protein [*Brachyspira pilosicoli* 95/1000_0177	42	401	3394	x	x	x	X	x
x	x	Bhyov8544_1756	hypothetical protein SPAP_0864 [*Streptococcus pneumoniae* AP200]				x	x	x	X	x
x	x	Bhyov8544_1818	phosphoribosylaminoimidazole carboxylase ATPase subunit [*Actinobacillus*]	N/A			Bhyoa7455_1118	phosphoribosylaminoimidazole carboxylase ATPase subunit [*Actinobacillus*]	N/A		
x	x	Bhyov8544_1898	anti-sigma-factor antagonist [*Brachyspira intermedia* PWS/A]	N/A			Bhyoa7455_1745	anti-sigma-factor antagonist [*Brachyspira intermedia* PWS/A]	N/A		
x	x	Bhyov8544_2013	hypothetical protein Bmur_2524 [*Brachyspira murdochii* DSM 12563	19	40471	42422	x	x	x	x	x
x	x	Bhyov8544_2086	hypothetical protein Bint_0182 [*Brachyspira intermedia* PWS/A	N/A			Bhyoa7455_2355	hypothetical protein Bint_0182 [*Brachyspira intermedia* PWS/A	N/A		
x	x	Bhyov8544_2093	hypothetical protein BP951000_1514 [*Brachyspira pilosicoli* 95/1000]	159#	-	-	x	x	x	x	x
x	x	Bhyov8544_2106	hypothetical protein Bmur_1645 [*Brachyspira murdochii* DSM 12563]	N/A			Bhyoa7455_2106	hypothetical protein Bmur_1645 [*Brachyspira murdochii* DSM 12563]	N/A		
x	x	Bhyov8544_2249	conserved hypothetical protein [*Brachyspira intermedia* PWS/A Bint_2186]	23#			x	x	x	x	x
x	x	Bhyov8544_2255	truncated Lex2A [*Haemophilus influenzae*]				x	x	x	x	x
x	x	Bhyov8544_2303	conserved hypothetical protein [*Fusobacterium sp*. 1_1_41FAA]	22	19539	25349	x	x	x	x	x
x	x	Bhyov8544_2311	NAD-dependent epimerase/dehydratase [*Butyrivibrio proteoclasticus* B316]				x	x	x	x	x
x	x	Bhyov8544_2313	conserved hypothetical protein [*Sulfurovum sp. NBC37-1*]				x	x	x	x	x
x	x	Bhyov8544_2369	dolichyl-phosphate beta-D-mannosyltransferase [*Brachyspira pilosicoli* 95/1000]	26	20957	26039	x	x	x	x	x
x	x	Bhyov8544_2370	Methyltransferase domain. [*Synergistetes bacterium* SGP1]				x	x	x	x	x
x	x	Bhyov8544_2401	ankyrin repeat-containing protein [*Brachyspira intermedia* PWS/A	10	25111	27565	x	x	x	x	x
x	x	Bhyov8544_2427	conserved hypothetical protein [*Enterococcus faecalis* HH22				x	x	x	x	x

x	x	Bhyov8544_2430	ankyrin [*Brachyspira murdochii* DSM 12563] Bmur_2481				x	x	x	x	x	
x	x	Bhyov8544_2460	CDP-glycerophosphotransferase family [*Treponema azotonutricium* ZAS-9	18	3266	6850	x	x	x	x	x	
x	x	Bhyov8544_2461	hypothetical protein [*Candidatus Nanosalinarum* sp. J07AB56]				x	x	x	x	x	
x	x	Bhyov8544_2483	hypothetical protein Bmur_2348 [*Brachyspira murdochii* DSM 12563	N/A			Bhyoa7455_1812	hypothetical protein Bmur_2348 [*Brachyspira murdochii* DSM 12563	N/A			
x	x	Bhyov8544_2484	conserved hypothetical protein [*Brachyspira murdochii* DSM 12563 Bmur_2349	N/A			Bhyoa7455_1811	conserved hypothetical protein [*Brachyspira murdochii* DSM 12563 Bmur_2349	N/A			
x	x	Bhyov8544_2490	ABC transporter ATP-binding protein [*Polaribacter irgensii* 23-P]	N/A			Bhyoa7455_1805	ABC transporter ATP-binding protein [*Polaribacter irgensii* 23-P]	N/A			
x	x	Bhyov8544_2531	DNA polymerase beta domain protein region [*Brachyspira murdochii* Bmur_1349	73	15045	17069	x	x	x	x	x	
x	x	Bhyov8544_2544	propionyl-CoA carboxylase, beta subunit [*Roseobacter* sp. CCS2]				x	x	x	x	x	
x	x	Bhyov8544_2545	hypothetical protein NH8B_1950 [*Pseudogulbenkiania* sp. NH8B				x	x	x	x	x	
x	x	Bhyov8544_2546	hypothetical protein SI859A1_00926 [*Aurantimonas manganoxydans* SI85-9A1]	143	548	16857	x	x	x	x	x	
x	x	Bhyov8544_2597	hypothetical protein APT_2250 [*Acetobacter pasteurianus* NBRC	4#	-	-	x	x	x	x	x	
x	x	Bhyov8544_2581	RES domain protein [*Brachyspira murdochii* DSM 12563] Bmur_1895	68	723	3476	x	x	x	x	x	
x	x	Bhyov8544_2585	hypothetical protein [*Oribacterium* sp. oral taxon 078 str. F0262]		6032	8341	x	x	x	x	x	
x	x	Bhyov8544_2587	putative KAP NTPase P-loop domain-containing protein [*Brachyspira pilosicoli* 95/1000]		10267	13103	x	x	x	x	x	
x	x	Bhyov8544_2676	UDP-galactopyranose mutase [*Campylobacter jejuni subsp. jejuni* 327	59	1	2471	x	x	x	x	x	
x	x	x	x	x	x	x	Bhyoa7455_0003	hypothetical protein [*Harpegnathos saltator*]	120	1	454324	
x	x	x	x	x	x	x	Bhyoa7455_0608	hypothetical protein Bint_2375 [*Brachyspira intermedia* PWS/A	21	32215	34537	
x	x	x	x	x	x	x	Bhyoa7455_0626	Abortive infection bacteriophage resistance protein [*Brachyspira intermedia* PWS/A] _02390				

x	x	x	x	x	x	x	Bhyoa7455_0652	adenine-specific DNA methylase [*Helicobacter cinaedi* CCUG 18818]			
x	x	x	x	x	x	x	Bhyoa7455_0654	predicted protein [*Helicobacter bilis* ATCC 43879]			
x	x	x	x	x	x	x	Bhyoa7455_0742	conserved hypothetical protein [*Escherichia coli* E110019]	23	1	89244
x	x	x	x	x	x	x	Bhyoa7455_0743	D12 class N6 adenine-specific DNA methyltransferase [*Streptococcus mitis* SK1080]			
x	x	x	x	x	x	x	Bhyoa7455_0746	hypothetical protein Bmur_1901 [*Brachyspira murdochii* DSM 12563]			
x	x	x	x	x	x	x	Bhyoa7455_0747	type I site-specific deoxyribonuclease, HsdR family [*Methanococcus voltae* A3]			
x	x	x	x	x	x	x	Bhyoa7455_0748	serine/threonine protein kinase [*Rhodopirellula baltica* WH47]			
x	x	x	x	x	x	x	Bhyoa7455_0749	hypothetical protein [*Archaeoglobus veneficus* SNP6]			
x	x	x	x	x	x	x	Bhyoa7455_0888	hypothetical protein [*Clostridium clostridioforme* 2_1_49FAA]	11	1	159659
x	x	x	x	x	x	x	Bhyoa7455_0955	hypothetical protein Bint_1171 [*Brachyspira intermedia* PWS/A]			
x	x	x	x	x	x	x	Bhyoa7455_1266	transposase [*Thiorhodococcus drewsii* AZ1]	235	1	70812
x	x	x	x	x	x	x	Bhyoa7455_1267	conserved hypothetical protein [*Helicobacter cinaedi* CCUG 18818]			
x	x	x	x	x	x	x	Bhyoa7455_1341	methyltransferase type 11 [*Desulfarculus baarsii* DSM 2075]	1	1	85109
x	x	x	x	x	x	x	Bhyoa7455_1378	helicase [*Bacteroides fragilis* YCH46]	282	1	66687
x	x	x	x	x	x	x	Bhyoa7455_1715	hypothetical protein [*Synechococcus* sp. PCC 7002]	32	8410	21882
x	x	x	x	x	x	x	Bhyoa7455_1835	hypothetical protein Bint_2751 [*Brachyspira intermedia* PWS/A]	14	1	60500
x	x	x	x	x	x	x	Bhyoa7455_1976	hypothetical protein [*Paramecium tetraurelia* strain d4-2]	6	1	42840
x	x	x	x	x	x	x	Bhyoa7455_2005	hypothetical protein [*Mariprofundus ferrooxydans* PV-1]	28	1	41747
x	x	x	x	x	x	x	Bhyoa7455_2084	hypothetical protein [*Haemophilus influenzae* F3047]	17	1	36454

x	x	x	x	x	x	x	pBhyoa7455_20	Methyltransferase type 11 [*Paludibacter propionicigenes* WB4]	31	14334	34822
x	x	x	x	x	x	x	pBhyoa7455_25	hypothetical protein [*Fusobacterium nucleatum subsp. polymorphum* F0401			
x	x	x	x	x	x	x	Bhyoa7455_2334	conserved hypothetical protein [*Brachyspira murdochii* DSM 12563] Bmur_1153	33	1	24523
x	x	x	x	x	x	x	Bhyoa7455_2601	hypothetical protein [*Chlorobium phaeobacteroides* BS1	30	1	8714
x	x	x	x	x	x	x	Bhyoa7455_2638	hypothetical protein Bint_1219 [*Brachyspira intermedia* PWS/A]	69	367	6776

C[1] contig number

S[2] start of the novel region predicted within that contig

E[3] end of the novel region predicted within that contig

x no homologue found

N/A non-applicable

Cursive locus_ID indicates that predicted ORFs are shorter than 50 bp.

*These genes were predicted to be absent in the genome but were confirmed to be present by PCR in section **4.2.4.**

These sequences were not detected by Panseq 2.0.

4.2.2.3 *Distribution of potential virulence genes among virulent and putatively avirulent B. hyodysenteriae isolates*

The presence of previously-described putative virulence genes of *B. hyodysenteriae* WA1 (**Chapter 1**) were determined within the genomes of P8544 and P7455, in order to further expand on the preliminary study conducted by PCR (**Chapter 3**).

Due to the observation that possible avirulent isolates of *B. hyodysentriae* were less frequently found in the colonic crypt than virulent isolates, it was hypothesised that flagella motility might differ between virulent and avirulent strains. The genes involved in flagella assembly were compared between P8544 and P7455 and the type strain WA1. The bacterial flagella system, consisting of 22 core structural genes, present in WA1 were also identified in the genomes of both English field isolates (**Table 4.3**), suggesting that if flagella are associated with the difference between virulent and avirulent phenotypes, then this difference would need to lie at the level of flagella expression rather than presence/absence.

No discrepancies in haemolytic capability could be discovered, as the seven haemolysin genes identified in WA1 were also found in the genomes of P7455 and P8544, these being: *tlyA* (Bhyoa7455_0332 ,Bhyov8544_0517), *tlyB* (Bhyoa7455_0830, Bhyov8544_1062), *tlyC* (Bhyoa7455_1894 , Bhyov8544_2045), *hylA* (Bhyoa7455_2063, Bhyov8544_1232), genes encoding putative haemolysin III (Bhyoa7455_0129, Bhyoa7455_1503, Bhyov8544_0317, Bhyov8544_0563) and a putative haemolysin CBS domain containing protein (Bhyoa7455_2510, Bhyov8544_1555) (**Table 4.3**).

The gene encoding the galactose/glucose-binding protein (*mglB*) has been suggested to be a potential marker for avirulent *B. hyodysenteriae* isolates. However, the presence of the *mglB* gene (BHWA1_022552) in the panel of virulent and putatively avirulent isolates was already shown by PCR and sequencing (reported in **Chapter 3**). Additionally, the presence of three copies of the *mglB* gene (Bhyov8544_1388-Bhyov8544_1390; Bhyoa7455_1360, Bhyoa7455_1359, Bhyoa7455_0367) was confirmed by comparative genome analysis of P8544 and P7455 proving that this gene is not a candidate for diagnostics as it appears to be conserved among pathogenic and non-pathogenic *B. hyodysenteriae* strains.

Table 4.3: Distribution of known virulence genes in the three genomes of *B. hyodysenteriae.*

WA1	Gene	Product	P8544	P7455
Flagella assembly				
BHWA1_00702	*fliD*	flagella hook-associated protein FliD	Bhyov8544_0829	Bhyoa7455_0950
BHWA1_01652	*flgK*	hook filament junction	Bhyov8544_1133	Bhyoa7455_1311
BHWA1_01419	*flgE*	flagella hook protein FlgE	Bhyov8544_2037	Bhyoa7455_1886
BHWA1_02001	*fliK*	flagella hook-length control domain containing protein	Bhyov8544_0700	Bhyoa7455_2286
BHWA1_02000	*flgD*	flagella hook capping protein	Bhyov8544_0699	Bhyoa7455_2287
BHWA1_00916	*flgG*	flagella basal body rod protein	Bhyov8544_2482	Bhyoa7455_1457
BHWA1_00842	*fliE*	putative flagella hook-basal body protein FliE	Bhyov8544_2135	Bhyoa7455_1278
BHWA1_00844	*flgB*	flagella basal body rod protein FlgB	Bhyov8544_2137	Bhyoa7455_1280
BHWA1_00843	*flgC*	flagella basal body rod protein FlgC	Bhyov8544_2136	Bhyoa7455_1279
BHWA1_00669	*fliF*	flagella MS-ring protein	Bhyov8544_1473	Bhyoa7455_0918
BHWA1_00668	*fliG*	flagella motor switch protein G	Bhyov8544_1472	Bhyoa7455_0917
BHWA1_01392	*fliM*	flagella motor switch protein FliM	Bhyov8544_2550	Bhyoa7455_2476
BHWA1_01391	*fliN*	flagella motor switch protein	Bhyov8544_2551	Bhyoa7455_2475
BHWA1_01417	*motA*	flagella Motor Protein	Bhyov8544_2035	Bhyoa7455_1884
BHWA1_01416	*motB*	putative flagella motor protein	Bhyov8544_2034	Bhyoa7455_1883
BHWA1_00347	*flhA*	flagella biosynthesis protein A	Bhyov8544_0411	Bhyoa7455_0226
BHWA1_00346	*flhB*	flagella biosynthesis protein FlhB	Bhyov8544_0412	Bhyoa7455_0227
BHWA1_00667	*fliH*	flagella assembly protein H	Bhyov8544_1471	Bhyoa7455_0916
BHWA1_01393	*fliL*	putative flagella basal body-associated protein	Bhyov8544_1470	Bhyoa7455_0915
BHWA1_00344	*fliQ*	flagella biosynthetic protein (fliQ)	Bhyov8544_0414	Bhyoa7455_0229
BHWA1_00343	*fliP*	flagella biosynthesis protein FliP	Bhyov8544_0415	Bhyoa7455_0230
BHWA1_00345	*fliR*	flagella biosynthetic protein fliR	Bhyov8544_0413	Bhyoa7455_0228
BHWA1_00020	*fliw*	flagella assembly protein	Bhyov8544_1192	Bhyoa7455_1954
BHWA1_00350	*fleN*	flagella synthesis regulator FleN	Bhyov8544_0408	Bhyoa7455_0223
BHWA1_01244	*flaA*	flagella filament outer layer protein FlaA	Bhyov8544_0107	Bhyoa7455_0573
BHWA1_02338	*flaA*	flagella filament outer layer protein FlaA	Bhyov8544_0108	Bhyoa7455_0574
BHWA1_02337	*flaA*	flagella filament outer layer protein FlaA	Bhyov8544_1046	Bhyoa7455_0814
BHWA1_02626	*fliS*	putative Flagella protein FliS	Bhyov8544_1248	Bhyoa7455_2079
BHWA1_01501	*flaB*	flagella filament core protein flaB2	Bhyov8544_2088	Bhyoa7455_2357
BHWA1_01452	*flaB3*	periplasmic flagella filament protein FlaB3	Bhyov8544_2069	Bhyoa7455_1918
BHWA1_00842	*fliE*	putative flagella hook-basal body protein FliE	Bhyov8544_2135	Bhyoa7455_1278
heamolytic genes				
BHWA1_00238	*tlyA*	hemolysin protein	Bhyov8544_0517	Bhyoa7455_0332
BHWA1_01228	*tlyB*	hemolysin B	Bhyov8544_1062	Bhyoa7455_0830
BHWA1_01427	*tlyC*	Hemolysin C	Bhyov8544_2045	Bhyoa7455_1894
BHWA1_02643	*acpP*	cyl carrier protein (ACP) contains:Beta-hemolysin	Bhyov8544_1232	Bhyoa7455_2063
BHWA1_00448		putative hemolysin III	Bhyov8544_0317	Bhyoa7455_0129
BHWA1_01870		putative hemolysin III	Bhyov8544_0563	Bhyoa7455_1503
BHWA1_00587		putative hemolysin CBS domain containing protein	Bhyov8544_1555	Bhyoa7455_2510
BIT-system				
BHWA1_00867	*bitA*	periplasm c-iron-binding protein BitA	Bhyov8544_2669	Bhyoa7455_2669
BHWA1_00866	*bitB*	periplasm c-iron-binding protein BitB	Bhyov8544_2672	Bhyoa7455_2668
BHWA1_00868	*bitB*	periplasm c-iron-binding protein BitB	Bhyov8544_2669	Bhyoa7455_2670
BHWA1_02570	*bitB*	periplasm c-iron-binding protein BitB	Bhyov8544_1373	Bhyoa7455_1375
BHWA1_02571	*bitB*	periplasm c-iron-binding protein BitB	Bhyov8544_1372	Bhyoa7455_1376
BHWA1_00869	*bitC*	periplasm c-iron-binding protein BitC	Bhyov8544_2669	Bhyoa7455_2671
BHWA1_00870	*bitD*	periplasm c-iron-binding protein BitD	Bhyov8544_2193	Bhyoa7455_1764
BHWA1_00871	*bitE*	periplasm c-iron-binding protein BitE	Bhyov8544_2191	Bhyoa7455_1765
mglB-locus				
BHWA1_02552	*mglB*	galactose/glucose-binding protein	Bhyov8544_1388	Bhyoa7455_1360
BHWA1_02553	*mglB*	galactose/glucose-binding protein	Bhyov8544_1389	Bhyoa7455_1359
BHWA1_02554	*mglB*	galactose/glucose-binding protein	Bhyov8544_1390	Bhyoa7455_0367
Oxidative stress				
BHWA1_02018	*nox*	coenzyme A disuLfide reductase	Bhyov8544_0716	Bhyoa7455_2270
BHWA1_02490	*nox*	DH oxidase	Bhyov8544_0254	Bhyoa7455_0424

VSH-1				
BHWA1_01838	*hvp45*	Hvp 45 VSH-1 capsid protein	Bhyov8544_2521	Bhyoa7455_1652
BHWA1_01839	*hvp19*	Hvp 19/Hvp 22 VSH-1 associated protein 1	Bhyov8544_2522	Bhyoa7455_1651
BHWA1_01840	*hvp13*	Hvp 13 VSH-1 associated protein 2	Bhyov8544_2523	Bhyoa7455_1650
BHWA1_01841	*hvp38*	Hvp 38 VSH-1 associated protein 3	Bhyov8544_2524	Bhyoa7455_1649
BHWA1_01842		hypothetical protein	hyov8544_2525	Bhyoa7455_1648
BHWA1_01843	*orfB*	orfB	Bhyov8544_2526	Bhyoa7455_1647
BHWA1_01844	*hvp24*	Hvp 24 VSH-1 capsid protein	Bhyov8544_2527	Bhyoa7455_1646
BHWA1_01845		hypothetical protein	Bhyov8544_2528	Bhyoa7455_1645
BHWA1_01846	*Hvp53*	Hvp 53 VSH-1 major tail protein	Bhyov8544_2629	Bhyoa7455_2543
BHWA1_01847	*orfC*	orfC	-	Bhyoa7455_2542
BHWA1_01848	*orfE*	orfE	Bhyov8544_2628	Bhyoa7455_2541
BHWA1_01849		hypothetical protein	Bhyov8544_2627	Bhyoa7455_2540
BHWA1_01850	*hvp32*	Hvp 32 VSH-1 tail protein	Bhyov8544_2626	Bhyoa7455_2539
BHWA1_01851	*hvp101*	Hvp 101 VSH-1 tail protein	Bhyov8544_2625	Bhyoa7455_2538
BHWA1_01852	*hvp28*	Hvp 28 VSH-1 tail protein	Bhyov8544_2624	Bhyoa7455_2537
BHWA1_01853	*Lys*	Lys - endolysin; glycoside hydrolase	Bhyov8544_2623	Bhyoa7455_2536
BHWA1_01854	*orF*	orF	Bhyov8544_2622	Bhyoa7455_2535
BHWA1_01855	*hol*	Hol	Bhyov8544_2621	Bhyoa7455_2534
BHWA1_01856	*orfG*	orfG	Bhyov8544_2620	Bhyoa7455_2533
variable surface protein				
BHWA1_00889	*vspA*	variable surface protein –VspA	Bhyov8544_2173	Bhyoa7455_1783
BHWA1_01455	*vspD*	variable surface protein- VspD	Bhyov8544_2712	Bhyoa7455_1921
BHWA1_01456	*vspC*	variable surface protein – VspC	Bhyov8544_2718	Bhyoa7455_2702
BHWA1_01457	*vspB*	variable surface protein – VspB	Bhyov8544_2721	Bhyoa7455_2721
BHWA1_01458	*vspD*	variable surface protein – VspD	-	-
BHWA1_01459	*vspA*	variable surface protein – VspA	Bhyov8544_2649	Bhyoa7455_2619
BHWA1_01600	*vspE*	variable surface protein – VspE	Bhyov8544_1973	Bhyoa7455_2361
BHWA1_01601	*vspF*	variable surface protein – VspF	Bhyov8544_2680	Bhyoa7455_2651
BHWA1_02382	*vspI*	variable surface protein – VspI	Bhyov8544_0150	Bhyoa7455_0531
Proteases				
BHWA1_00080		intracellular protease	Bhyov8544_1831	Bhyoa7455_1105
BHWA1_00443		inactive metal-dependent protease, putative molecular chaperone	Bhyov8544_0322	Bhyoa7455_0134
BHWA1_00583	*ClpB*	ATP-dependent Clp protease, ATP-binding subunit CpB	Bhyov8544_1559	Bhyoa7455_2514
BHWA1_00767	*sppA*	sppA, Periplasmic serine proteases (ClpP class)	Bhyov8544_0760	Bhyoa7455_2306
BHWA1_01228	*hlyB*	hemolysin B; ClpA, ATPases with chaperone activity, ATP-binding subunit	Bhyov8544_1062	Bhyoa7455_0830
BHWA1_01231	*clpX*	membrane-associated ATP-dependent Clp protease ATP-binding subunit	Bhyov8544_1059	Bhyoa7455_0827
BHWA1_01345	*ftsH*	ATP-dependent metalloprotease FtsH	Bhyov8544_1772	Bhyoa7455_0718
BHWA1_01454		Zn dependant metalloprotease	Bhyov8544_2071	Bhyoa7455_1920
BHWA1_01477		serine endoprotease	Bhyov8544_2499	Bhyoa7455_2444
BHWA1_01721	*Ion*	ATP-dependent protease La	Bhyov8544_1730	Bhyoa7455_1445
BHWA1_01763	*nfeD*	NfeD, Membrane-bound serine protease (ClpP class)	Bhyov8544_1687	Bhyoa7455_1488
BHWA1_01867		peptidase/protease	Bhyov8544_0560	Bhyoa7455_1500
BHWA1_01876		carboxyl-terminal protease	Bhyov8544_0569	Bhyoa7455_1509
BHWA1_02105		putative membrane associated zinc metalloprotease	Bhyov8544_0886	Bhyoa7455_1594
BHWA1_02165	*hslV*	ATP-dependent protease peptidase subunit	Bhyov8544_0947	Bhyoa7455_2030
BHWA1_02166	*hslU*	ATP-dependent protease HslVU (ClpYQ),ATPase subunit	Bhyov8544_0948	Bhyoa7455_2029
BHWA1_02221	*clpP*	ATP-dependent Clp protease proteolytic subunit ClpP	Bhyov8544_2606	Bhyoa7455_2549
BHWA1_02222	*clpX*	ATP-dependent protease ATP-binding subunit ClpX	Bhyov8544_2607	Bhyoa7455_2550
BHWA1_02356		CAAX amino terminal protease family protein	Bhyov8544_0124	Bhyoa7455_0557
Chemotaxis				
BHWA1_00489	*cheA*	chemotaxis histidine kinase CheA	Bhyov8544_1638	Bhyoa7455_0087
BHWA1_02542	*cheA*	chemotaxis histidine kinase CheA	Bhyov8544_1400	Bhyoa7455_0377
BHWA1_00493	*cheB*	response regulator receiver modulated CheB methylesterase	Bhyov8544_1642	Bhyoa7455_0083
BHWA1_02169	*cheB*	putative chemotaxis response regulator CheB	Bhyov8544_0951	Bhyoa7455_2026
BHWA1_01397	*cheC*	chemotaxis protein CheC	Bhyov8544_2598	Bhyoa7455_2557
BHWA1_00492	*cheD*	chemotaxis protein CheD	Bhyov8544_1641	Bhyoa7455_0084
BHWA1_01642	*cheD*	methyl-accepting chemotaxis protein CheD	Bhyov8544_1123	Bhyoa7455_1301
BHWA1_00585	*cheX*	chemotaxis response regulator CheX	Bhyov8544_1557	Bhyoa7455_2512
BHWA1_01332	*cheX*	chemotaxis response regulator CheX	Bhyov8544_1759	Bhyoa7455_0731
BHWA1_00490	*cheW*	putative chemotaxis signal transduction protein	Bhyov8544_1639	Bhyoa7455_0086
BHWA1_00715	*cheW*	putative chemotaxis signal transduction protein	Bhyov8544_0816	Bhyoa7455_0964
BHWA1_02278	*cheW*	putative chemotaxis signal transduction protein	Bhyov8544_0052	Bhyoa7455_0631
BHWA1_01000	*cheY*	chemotaxis response regulator CheY	Bhyov8544_1308	Bhyoa7455_1153
BHWA1_00488	*cheY*	chemotaxis response regulator CheY	Bhyov8544_1637	Bhyoa7455_0088
BHWA1_01333	*cheY*	chemotaxis response regulator CheY	Bhyov8544_1760	Bhyoa7455_0730
BHWA1_00491	*cheR*	chemotaxis protein methyltransferase CheR	Bhyov8544_1640	Bhyoa7455_0085
BHWA1_01334	*cheR*	chemotaxis protein methyltransferase CheR	Bhyov8544_1761	Bhyoa7455_0729

The prophage-like agent VSH-1 was also observed to be integrated into the chromosome of P8544 and P7455. However, different regions of the prophage were assembled in different contigs of these new *B. hyodysenteriae* genomes. In P8544 the head region is part of two contigs (67 and 73), whereas the tail and lysis region is assembled into contigs 25 and 1. In P7455 the head of the prophage was identified in contig 163 whereby the tail and lysis were assembled into contigs 44 and 42. Although the different prophage regions could not be properly re-ordered to obtain the entire 16 kbp VSH-1 region, the comparison of the single genes revealed that all 19 VSH-1-associated genes identified in WA1 were present in P7455, whereas the gene, *orfC*, seemed to be missing from the P8544 sequence. However, at this stage it remains unclear if P8544 lacks the *orfC* gene due to incomplete sequence coverage, and amplification of the target gene would thus be required to make a conclusion. Nevertheless, amplification was not performed, as *orfC* was present in the putatively avirulent strain, P7455, and virulent strain, WA1, and was therefore not a potential target of further interest in this study. Regardless, it can be assumed that horizontal transfer of genes occurs via the same mechanism in virulent and avirulent *B. hyodysenteriae* strains.

The entire ABC transporter cassette of the BIT-system found in *B. hyodysenteriae* WA1 could be also identified in the genomes of P8544 and P7455, indicating that the one known iron-acquisition system is present in representatives of virulent and avirulent *B. hyodysenteriae* strains, even though the data presented in **Chapter 3** showed that the *bitC* gene was only present in 79 % of the entire test panel of strains. Therefore, it may be that this gene does not play a major role in the functionality of the BIT system, as assumed by other researchers, due to the frequent occurrence of *B. hyodysenteriae* isolates lacking *bitC*.

Chemotaxis genes identified in the genome of *B. hyodysenteriae* WA1 were also present in the two field isolates. Seventeen copies of the chemosensory transducer (*che)* genes were identified in P8544 and P7455. In addition, a total of 55 genes encoding methyl-accepting chemotaxis proteins (MCPs) in P8544 and P7455 were found (data not shown). In contrast, in WA1, only 46 MCPs have been detected (Bellgard *et al.*, 2009). However, further investigation revealed that 9 copies of a gene encoding methyl-accepting chemotaxis protein McpB (BHWA1_01857) were found in different lengths and different contigs within the genome sequence of P8544 and P7455, showing that the same number of MCPs identified in WA1 were also present in both genomes of virulent and avirulent *B.*

hyodysenteriae. Assembly errors are well known throughout various assemblers. Particularly, the assembly software Velvet which has been used in this study has been shown to have the largest scaffold misjoin compared to other assemblers (Salzberg *et al.*, 2012) which could result in truncated genes as described above.

The *vsp* genes have been suggested to play a role in immunogenic variation within *B. hyodysenteriae* strains, and these were also present in the genomes of P8544 and P7455 with the exception of the second copy of *vspD* (BHWA1_01458). The lack of *vspD* might be due missing sequence data as mentioned earlier. Besides a total number of 15 copies of the *vspF* gene were predicted in both genomes of P8544 and P7455 varying in size and occurring in several contigs as described above.

Proteases are known to be involved in the destruction of host tissue by spirochaetes. The same genes annotated as proteases in WA1 were found in the field isolates, indicating that avirulent and virulent do not seem to differ in their number of proteolytic capacity genes.
In addition, the ability to withstand oxidative stress has been shown to play an important role in the pathogenicity in *B. hyodysenteriae*; however, the two *nox* genes (BHWA1_02018, BHWA1_02490) responsible for oxidase activity were identified in the genomes of both P8544 and P7455.

The *rfbABCD* regions coding for the rhamnose pathway have been associated with virulence and were detected in the plasmid sequence of P8544 and P7455. These genes are listed in **Table 4.4** and **Table 4.5** and will be discussed further in section **4.2.3**.

4.2.3 The plasmid of *B. hyodysenteriae*

During assembly and annotation of the *B. hyodysenteriae* P7455 and P8544 genomes, a contig was identified in each genome which was completely absent from the chromosome of WA1, indicating that these contigs may be part of the plasmid. Therefore, these contigs were analysed separately from the remainder of the chromosome-associated contigs, and were compared directly with the plasmid sequence of *B. hyodysenteriae* WA1 (NC_012226).

The annotated genes of each plasmid contig are summarized in **Table 4.4** and **Table 4.5**. In addition, due to the manageable size, ORFs of each contig (in a closed, circular form) were

Table 4.4: Overview of the annotated genes in the plasmid sequence of the virulent *B. hyodysenteriae* P8544.

Locus tag	matching locus tag	location	gene	protein	e-value	Length
pBhyov8544_02	BHWA1_02695	339..1526		hypothetical protein	0.0	395
pBhyov8544_03	BHWA1_02694	1534..2112	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase	3.79e-111	192
pBhyov8544_04	BHWA1_02693	2118..2999	*rfbD*	dTDP-4-keto-L-rhamnose reductase	1.21e-149	293
pBhyov8544_05	BHWA1_02692	2996..3859	*rfbA*	glucose-1-phosphate thymidylyltransferase	1.85e-166	287
pBhyov8544_06	BHWA1_02691	3873..4943	*rfbB*	dTDP-glucose 4,6-dehydratase	0.0	356
pBhyov8544_07	BHWA1_02690	4951..5931		lipopolysaccharide biosynthesis protein-like protein	1.50e-149	326
pBhyov8544_08	BHWA1_02689	6015..7844		putative alpha-1,2-fucosyltransferase; glycosyl transferase, family 11	0.0	609
pBhyov8544_09	BHWA1_02688	8023..8832		integrase	3.45e-136	269
pBhyov8544_10	BHWA1_02687	8885..10096		DNA primase-like protein	0.0	403
pBhyov8544_11	BHWA1_02686	10093..11187		putative replicative DNA helicase	5.34e-97	364
pBhyov8544_12	BHWA1_02685	11348..12358		hypothetical protein	0.0	336
pBhyov8544_13	BHWA1_02684	12827..13570	*cdsM*	plasmid partition protein	7.79e-126	247
pBhyov8544_14*	HMPREF9369-00085	13572..13808		hypothetical protein [Fusobacterium nucleatum subsp]	0.47	78
pBhyov8544_15	BHWA1_02683	13818..14603	*rfbF*	glucose-1-phosphate cytidylyltransferase	4.63e-154	261
pBhyov8544_16	BHWA1_02682	14622..15725		Radical SAM domain protein	0.0	367
pBhyov8544_17	BHWA1_02681	15746..16279	*rfbB*	dTDP-4-dehydrorhamnose 3,5-epimerase	2.17e-104	177
pBhyov8544_18	BHWA1_02680	16282..17142		NAD dependent epimerase/dehydratase family superfamily protein	7.17e-151	286
pBhyov8544_19	BHWA1_02679	c17139.18404		glycosyl transferase, group 1-like protein	0.0	421
pBhyov8544_20	BHWA1_02678	18545..19564		Fe-S oxidoreductase containing radical SAM domain"	0.0	339
pBhyov8544_21	BHWA1_02675	19548..20537		radical SAM protein	1.05e-62"	329
pBhyov8544_22	BHWA1_01692	20551..21579		UDP-glucose 4-epimerase	3.19e-11	342
pBhyov8544_23	BHWA1_02675	21651..22634		radical SAM protein	9.71e-81	327
pBhyov8544_24*	Sun_2139	22655..23851		Conserved hypothetical protein [SuLfurovum sp. NBC37-1]	1e-43	398
pBhyov8544_25*	Bmur_2571	23995..25179		FkbM family methyltransferase [Brachyspira Murdochii DSM]	8e-107	394
pBhyov8544_26	BHWA1_02670	25444..26472		putative glycosyl transferase, group 1	3.04e-149	342
pBhyov8544_27	BHWA1_02669	26481..27482		glycosyl transferase, group 1-like protein	4.79e-154	333
pBhyov8544_28	BHWA1_02668	27489..28427		glycosyl transferase, family 2	4.84e-162	312
pBhyov8544_29	BHWA1_02667	28424..29386		NAD-dependent epimerase/dehydratase	2.84e-164	320
pBhyov8544_30	BHWA1_02671	29383..31275		putative glycosyltransferase	5.58e-150	630

* Unique genes in the plasmid sequence

Table 4.5: Overview of the genes annotated in the plasmid sequence of the putative avirulent *B. hyodysenteriae* P7455.

Locus tag	matching locus tag	location	gene	protein	e-value	Length
pBhyoa7455_01 †	BHWA1_02676	33754..44		Radical SAM superfamily		371
pBhyoa7455_02	BHWA1_02675	181..1173		radical SAM protein	7.78e-138	330
pBhyoa7455_03	BHWA1_02674	1184..2188		Fe-S oxidoreductase containing radical SAM domain	0.0	334
pBhyoa7455_04	BHWA1_02673	2643..4637		hydrolase (HAD superfamily) protein-like protein	0.0	664
pBhyoa7455_05	BHWA1_02672	C 4640..5797		putative hydrolase (HAD superfamily) protein	0.0	385
pBhyoa7455_06	BHWA1_02671	6143..7972		putative glycosyltransferase	0.0	609
pBhyoa7455_07	BHWA1_02670	8103..9020		putative glycosyl transferase, group1	8.27e-137	305
pBhyoa7455_08	BHWA1_02669	9029..10030		glycosyl transferase, group 1-like protein	0.0	333
pBhyoa7455_09	BHWA1_02668	10037..10975		glycosyl transferase, family 2	2.96e-162	312
pBhyoa7455_10	BHWA1_02667	10972..11922		NAD-dependent epimerase/dehydratase	2.44e-166	316
pBhyoa7455_11	BHWA1_02666	11935..13173		glycosyltransferase	4.61e-141	412
pBhyoa7455_12	BHWA1_02696	13206..14336		glycosyltransferase	1.95e-147	376
pBhyoa7455_13	BHWA1_02695	14561..15748		hypothetical protein	0.0	395
pBhyoa7455_14	BHWA1_02694	15756..16334	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase	3.79e-111	192
pBhyoa7455_15	BHWA1_02693	16340..17200	*rfbD*	dTDP-4-keto-L-rhamnose reductase	4.20e-154	286
pBhyoa7455_16	BHWA1_02692	17219..18082	*rfbA*	glucose-1-phosphate thymidylyltransferase	9.85e-167	287
pBhyoa7455_17	BHWA1_02691	18096..19166	*rfbB*	dTDP-glucose 4,6-dehydratase	0.0	356
pBhyoa7455_18	BHWA1_02690	19175..20155		lipopolysaccharide biosynthesis protein-like protein"	3.17e-150	326
pBhyoa7455_19	BHWA1_02688	20296..21105		integrase	9.40e-135	269
pBhyoa7455_20	Palpr_3037 *	c21123..21947		Methlytransferase type 11 [Paludibacter propionicigenes WB4]	3e-62	274
pBhyoa7455_21	BHWA1_02687	22071..23282		DNA primase-like protein	0.0	403
pBhyoa7455_22	BHWA1_02686	23279..24373		putative replicative DNA helicase	1.57e-100	364
pBhyoa7455_23	BHWA1_02685	24534..25544		hypothetical protein	0.0	336
pBhyoa7455_24	BHWA1_02684	26013..26756	*cdsM*	plasmid partition protein	7.79e-126	247
pBhyoa7455_25*	HMPREF9369-00085	26758..26994		hypothetical protein [Fusobacterium nucleatum subsp]	0.47	87
pBhyoa7455_26	BHWA1_02683	27004..27789	*rfbF*	glucose-1-phosphate cytidylyltransferase	6.15e-154	261
pBhyoa7455_27	BHWA1_02682	27808..28911		Radical SAM domain protein	0.0	367
pBhyoa7455_28	BHWA1_02681	28932..29465	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase	2.17e-104	534
pBhyoa7455_29	BHWA1_02680	29468..30328		NAD dependent epimerase/dehydratase family superfamily protein	2.70e-151	286
pBhyoa7455_30	BHWA1_02679	C30325..31590		glycosyl transferase, group 1-like protein	0.0	421
pBhyoa7455_31	BHWA1_02678	31731..32741		Fe-S oxidoreductase containing radical SAM domain"	0.0	336
pBhyoa7455_32	BHWA1_02677	32766..33767		Fe-S oxidoreductase containing radical SAM domain	0.0	333

†This gene was detected using Clone Manager * Unique genes in the plasmid sequence

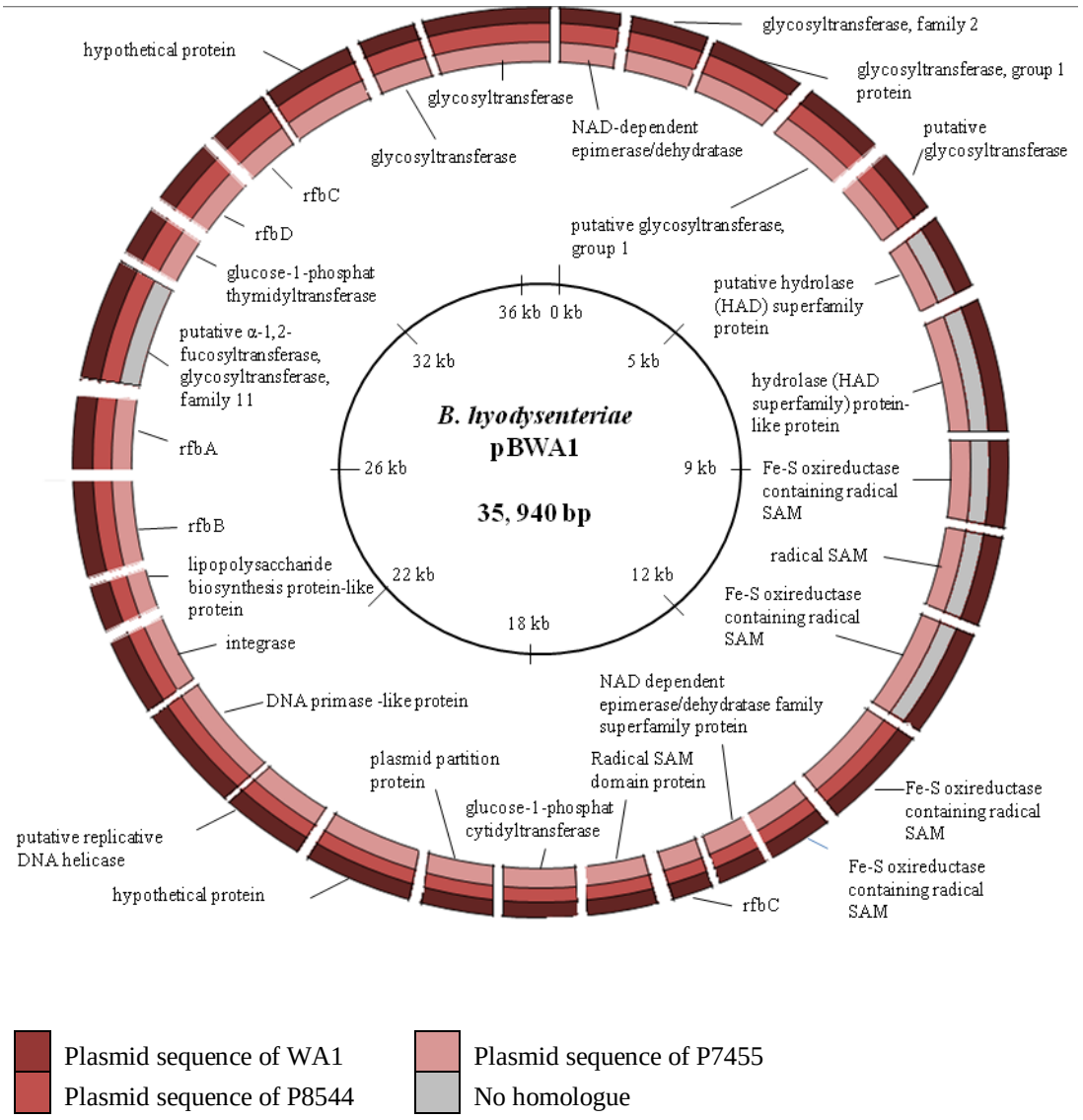


Figure 4.5: Schematic representation of the comparison of the plasmid sequence of *B. hyodysenteriae* pBWA1 to sequence data from plasmids of *B. hyodysenteriae* isolates P7455 and P8544. The outer circle represents the complete sequence of *B. hyodysenteriae* pBWA1, followed by the plasmid sequence of P8544 and P7455. The figure shows a simplified overview of genes which are present and absent in the plasmid sequences of P8544 and P7455 compared to the reference sequence of pBWA1. Grey areas indicate that no homologue in the corresponding plasmid sequence could be identified whereas coloured areas represent homologues. The plasmid sequence of P7455 lacks the gene encoding the putative α-1,2-fucosyltramnsferase;glycosyl transferase family 11 enzyme (BHWA1-02689), whereas the genes encoding the putative hydrolase (HAD) superfamily protein (BHWA1_02672), the hydrolase (HAD superfamily) protein-like protein (BHWA1_02673) and the Fe-S oxireductase containing radical SAM domain protein (BHWA1_02674, BHWA1_02676 and BHWA1_02677) were absent in the plasmid sequence of P8544.

predicted using Clone Manager 9.0 by searching for start codons in either direction, in order to find genes which were possibly missed during automated annotation using Xbase. Comparison of the plasmids showed that the sequences of P7455 and WA1 share higher similarity with each other than with P8544 (**Figure 4.5**). The plasmid sequence of P8544 is 31,469 bp in size and therefore smaller than that of WA1 and P7455. Moreover, only 29 genes have been detected in the plasmid sequence of P8544. However, due the fact that the genomes are not completed, it might be possible that these missing genes are encoded within these missing sequences.

The comparative genome analysis revealed heterogeneity among the plasmid sequences, whereby the plasmid of P8544 seems to differ more than the others by containing three novel genes. These genes encode a short (78 aa) hypothetical protein (pBhyov8544_14) sharing 46% similarity with *Fusobacterium nucleatum subsp. polymorphum* F0401 (HMPREF9369_00085), a conserved hypothetical protein (pBhyov8544_24) sharing 29% sequence identity with *Sulfurovum sp*. NBC37.1 (Sun_2139) and a gene encoding a FkbM family Methyltransferase (pBhyov8544_25) which shares 48% homology with *B. murdochii* DSM (Bmur_2571). These three genes are mainly combined in a region between 20,551 and 26,472 bp of the plasmid sequence and have been already mentioned in **Table 4.2**. The comparison has also shown that the genes encoding the putative hydrolase protein (BHWA1_02672), hydrolase protein-like protein (BHWA1_02673) and Fe-S oxireductase containing radical SAM domain (BHWA1_02674, BHWA1_02676, BHWA1_02677) present in the plasmid sequence of WA1 and P7455 were absent (**Table 4.4** and **Table 4.5**) (**Figure 4.5**).

The plasmid sequence of P7455 seems to be more homologous to that of WA1 than of P8544. In total, 32 ORFs were predicted, of which two were found to be unique (**Table 4.5**); one predicted gene encoded a Methyltransferase type 11 protein (pBhyoa7455_20), sharing 51% identity with *Pauldibacter propionicigenes* WB4 (Palpr_3037), and the second predicted gene encoding a hypothetical protein (pBhyoa7455_25), sharing 28% identity with F0401 (HMPREF9369_00085) of *Fusobacterium nucleatum* subsp. *polymorphum* , similar to that described above for P8544. Compared to WA1 and P8544, the gene encoding the putative alpha-1,2-fucosyltransferase; glycosyl transferase, family 11 protein (BHWA1_02689) was absent in P7455 (**Table 4.5**) (**Figure 4.5**). The absence of this gene was confirmed by Long Range PCR (**4.2.4**). The distribution of the gene in the virulent and avirulent panel was further assessed and will be discussed in section **4.2.2.6**.

4.2.3.1 *Novel regions among B. hyodysenteriae P7455 and P8544*

As mentioned in section **4.2.2.2**, in P8544 a total of 35 genes were identified which did not show any similarity to the genome of WA1 and P7455, whereas 27 genes were found to be unique in P7455. These ORFs were mainly clustered within intact contigs with the exception of Bhyov8544_2545 (**Table 4.2**). Particularly non-species related ORFs like Bhyov8544_2545 which span a region between two contigs may be a result of incorrect scaffolding by Mauve and PCR needed to be performed to justify the predicted contig overlap.

The novel gene sequences in each genome were further characterized by BLASTp and are summarized in **Table 4.2**. BLASTp searches revealed that 5/28 (17.85 %) of the unique protein sequences in P7455 shared high homology with *B. intermedia* and 2/28 genes (7.14 %) exhibited the highest matches to proteins of *B. murdochii*. In *B. hyodysenteriae* 8,544 4/32 (12.12%) genes were found to have homology to *B. intermedia*, 8/33 (24.24%) *B. murdochhii* and 4/33 (12.12%) *B. piloscoli*. Best matching protein hits were mainly proteins of other, related, *Brachyspira* spp. like *B. murdochii* (Bhyov8544_0632; Bhyoa7455_00626). However, the majority of the identified unique genes in both genomes 48.48% (16/33) in P8544 and 75% (21/28) in P7455 showed a best hit for protein sequences belonging to other bacterial genera, such as *Helicobacter cinaedi* and *Campylobacter jejuni*.

In P8544, 60.6% (20/33) of the novel genes encoded products of unknown function, while the same was true of 64.28% (18/28) of those in *B. hyodysent*eriae P7455. Functional annotations of genomic sequences for hypothetical proteins are of major importance in providing insights into their molecular functions and pathogenicity, thus helping in the identification of diagnostic targets. Those identified hypothetical proteins were further characterized by InterProScan, including the Gene 3D application to predict protein functional domains (**Appendix 7**). However, some of the hypothetical proteins, mainly those consisting of < 90 aa, were not successfully assigned any function. Therefore, the function of these acquired genes related to virulence remains speculative. It also questions whether the ORFs encoding these < 90 aa sequences are true genes.

4.2.4 Screening for potential target genes to distinguish between virulent and putative avirulent isolates

In order to identify genes which could serve as potential markers to differentiate between virulent and putatively avirulent isolates, the two genomes of the virulent *B. hyodysenteriae* strains WA1 and 8544 were compared against the genome of the avirulent *B. hyodysenteriae* P7455.

Genes which were present/ absent in the analysed genomes have already been presented in **Table 4.2**.In total, 7 genes which were shared among WA1 and P8544 were absent in P7455. The 7 genes, consisting of 4 genes encoding hypothetical proteins, (BHWA1_00637, BHWA1_00638, BHWA1_00723, BHWA1_1359), Appr-1-p processing enzyme family protein (BHWA1_01319), Ycfa like protein (BHWA1_02574) and putative-α-1,2-fucosyltransferase;glycosyltransferase, family 11 protein (BHWA1_02689) were amplified by PCR to ensure their absence in the putative avirulent genome (**Table 4.6)**. However, PCR revealed that 6/7 genes were present in the isolate P7455 (**Figure 4.6**). The gene encoding for the putative-α-1,2-fucosyltransferase;glycosyltransferase, family 11 present on the plasmid sequence of WA1 and P8544 was the only gene found to be truly absent in the genome of P7455. Primers were created spanning the entire coding region of the putative alpha-1,2-fucosyltransferase with oligonucleotide primers starting at the end of lipopolysaccharide biosynthesis protein-like protein (BHWA1_02690) and beginning of gene sequence encoding an integrase (BHWA1_02688) to ensure that the whole gene was amplified and the entire CDS was not present in P7455. An expected product of 2,373 bp could be amplified from WA1 and P8544, whereas a small product of only *ca.* 300 bp could be amplified from P7455 (**Figure 4.5**). Sequencing of the 300 bp product confirmed that the gene encoding the putative alpha-1,2-fucosyltransferase was absent from that location in P7455 due to amplification of the non-coding region between the ORFs encoding the lipopolysaccharide biosynthesis protein-like protein (pBhyoa7455_18) and the integrase (pBhyoa7455_19) (**Figure 4.5 D2**).

The other 6 target genes which could not be detected using bioinformatic approaches are likely to exist in the unclosed gaps of the avirulent genome P7455. Furthermore, the PCR products obtained from the genes (BHWA1_00638 and BHWA1_00723), encoding a protein of unknown function, were faint in the avirulent strain compared to products amplified by the virulent strains (**Figure 4.6**).

Table 4.6: Verification of 7 genes present in the virulent and absence of genes in virulent and avirulent *B. hyodysenteriae* isolates. The two virulent strains WA1 and P8544 were compared against the genome of the putatively avirulent *B. hyodysenteriae* P7455 using WebACT, in order to identify genes which are common in the virulent isolates and absent in the putatively avirulent strain. In total, 7 genes were found to be present in WA1 and P8544 but absent in P7455. Verification of these genes being missing in P7455 by PCR revealed that 6 out of the 7 target genes could be amplified, indicating that only the gene encoding for putative alpha-1,2-fucosyltransferase;glycosyltransferase family was truly absent in the genome of P7455.

Locustag WA1	Locustag P8544	E-value †	Protein	WebACT *			PCR		
				WA1	P8544	P7455	WA1	P8544	P7455
BHWA1_00637	Bhyo8544_1442	0	hypothetical protein	+	+	-	-	-	-
BHWA1_00638	Bhyo8544_1443	2.40E-79	hypothetical protein	+	+	-	+	+	+
BHWA1_00723	Bhyo8544_0808	7.40E-93	hypothetical protein	+	+	-	+	+	+
BHWA1_01319	Bhyo8544_1746	7.25E-173	Appr-1-p processing enzyme family protein	+	+	-	+	+	+
BHWA1_01320	Bhyo8544_1747	9.25E-119	hypothetical protein	+	+	-	+	+	+
BHWA1_02574	Bhyo8544_1369	2.07E-29	Ycfa like protein	+	+	-	+	+	+
BHWA1_02689	pBhyo8544_08	0	putative alpha-1,2-fucosyltransferase;glycosyltransferase family	+	+	-	+	+	-

†e-value corresponds to the sequence homology to *B. hyodysenteriae* WA1.

*WebACT was used as a bioinformatic comparison tool (2.12.1)

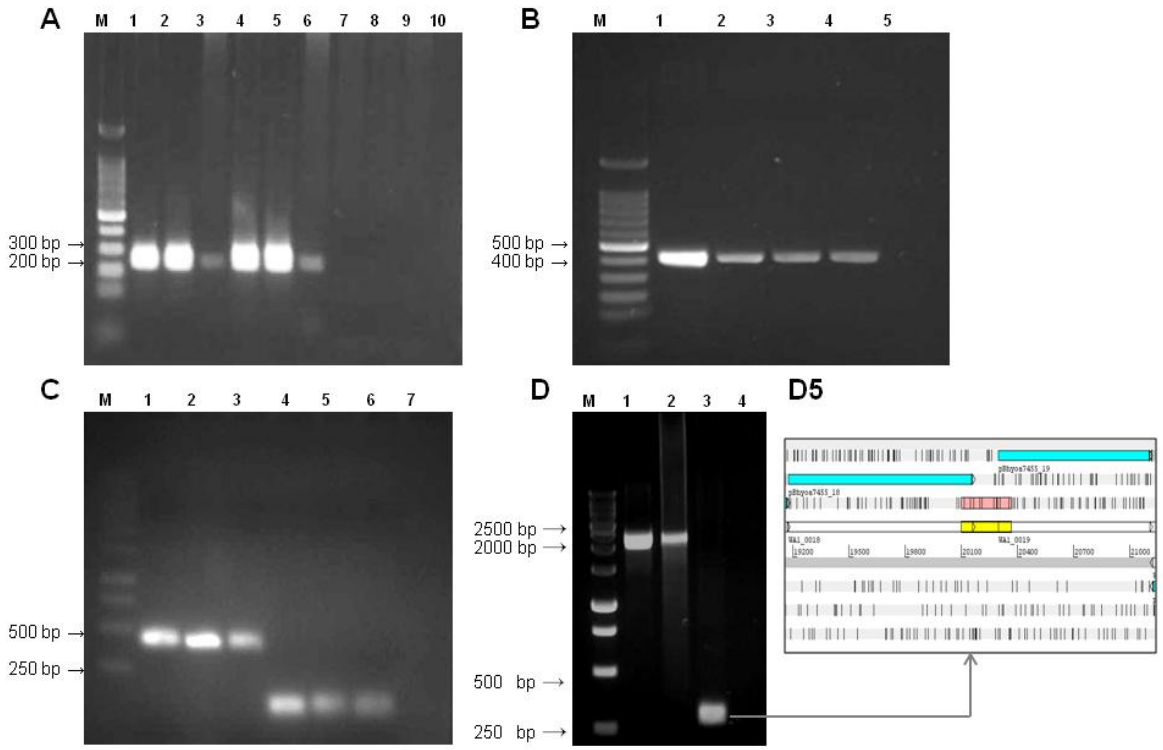


Figure 4.6: Amplification of the 7 target genes predicted to be absent in the genome of putatively avirulent *B. hyodysenteriae* P7455. Using WebACT 7, genes were predicted to be present in the genomes of pathogenic *B. hyodysenteriae* WA1 and P8544 but absent in the genome of the putatively avirulent P7455. The 7 target genes were amplified by PCR from WA1, P8544 and P7455 in order to confirm their presence/absence. Samples correspond to 100 bp DNA ladder (AM, BM) and 1kb DNA ladder (CM; DM); negative controls (A10, B5, C7,4D); PCR product of BHWA1_00638 encoding hypothetical protein in WA1 (A1), P8544 (A2) and P7455 (A3); PCR product of BHWA1_00723 encoding a hypothetical protein in WA1 (A4), P8544 (A5) and P7455 (A6), PCR product of BHWA1_00637 encoding a hypothetical protein in WA1 (A7), P8544 (A8) and P7455 (A9), PCR product of BHWA1_01320 encoding a hypothetical protein in WA1 (B1), P8544 (B2), P7455 (B3) and P8226/7 (B4); PCR products of BHWA1_01319 encoding Appr-1-p processing enzyme family protein in WA1(C1), P8544 (C2) and P7455 (C3), PCR product of BHWA1_02574 encoding YCFA like protein in WA1(C4), P8544 (C5) and P7455 (C6), PCR product of BHWA1_02689 encoding putative-α-1,2-fucosyltransferase; glycosyltransferase, family 11 in WA1 (D1), P8544 (D2) and P7455 (D3). The image (D5) presents a section of the plasmid sequence of P7455; the region highlighted in pink was amplified using primers specific for the gene encoding a putative-α-1, 2-fucosyltransferase; glycosyltransferase, family 11 protein, producing a 300 bp product, thus confirming the absence of that gene in P7455.

As the same concentration of template (100ng/µl) was used it is possible that differences in the primer-binding site exists which leads to a weaker amplicon in the isolate P7455.

It was not possible to obtain a PCR product corresponding to the gene encoding the hypothetical protein (BHWA1_00637) in any of the tested isolates (**Figure 4.6**). Different primer pairs (BHWA1_00637 F/R and BHWA1_00637 F1/R1) (**Table 2.6** in **Chapter 2**), targeting different regions of the gene sequence, as well as various target templates of *B. hyodysenteriae* were chosen, but the amplification was not successful. Therefore, the presence of the gene encoding the hypothetical protein, predicted to be 1,103 aa in size and confirmed to be present in the genomes of WA1 and P8544, could not be proven by PCR.

Additionally, PCR products of the genes encoding the App-1-p processing enzyme family protein (BHWA1_01319) and the hypothetical protein (BHWA1_00723) in P8544 and P7455 were sequenced to confirm that the correct gene was amplified. The alignment of the sequenced products showed high sequence similarity between the isolates of each gene as already indicated by PCR, confirming that the genes are present in the genome sequence of P7455 (**Figure 4.7** and
Figure 4.8).

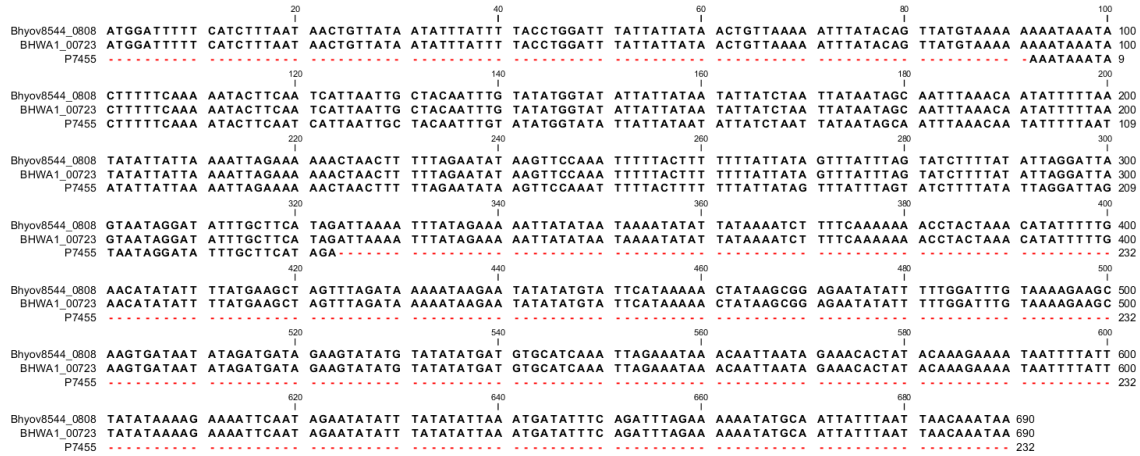


Figure 4.7: Sequence of the PCR product BHWA1_00723 in *B. hyodysenteriae* P8544 and P7455. The gene BHWA1_00723 encoding a hypothetical protein in WA1 and P8544 was not detectable in P7455 using WebACT. However, the gene was successfully amplified from P7455 by PCR; the sequenced 233 bp product shows high similarity with the genes in WA1 and P8544 confirming its presence in the avirulent *B. hyodysenteriae* isolate.

Figure 4.8: Sequence of the PCR product BHWA1_01319 in *B. hyodysenteriae* P8544 and P7455. The gene BHWA1_01319 encoding the Appr-1-processing enzyme family protein in WA1 and P8544 was not detectable in P7455 using WebACT, however, the gene was amplified by PCR. The sequenced product shows near identity to the genes of WA1 and P8544**.**

4.2.4.1 *Distribution of potential marker genes among virulent and putatively avirulent isolates*

In order to assess the distribution of the gene encoding the putative-α-1,2-fucosyltransferase; glycosyltransferase protein, as described in section **4.2.4.**, it was intended that the carriage of the gene be assessed among a panel of 22 virulent and 19 avirulent *B. hyodysenteriae* isolates (the same panel exploited in **Chapter 3**). Unfortunately, by this stage in the study, available genomic DNA had become limited. Therefore, not all strains of the previously used panel in **Chapter 3** could be tested. Nevertheless, during the time of the project two putative avirulent German field isolates assigned P010573/2 and P010573/3 were kindly provided by Dr. Judith Rohde (Tieraerztlichen Hochschule von Hannover, Germany) and were included in further testing of the putative avirulent panel. PCR was conducted using the primer pair 02689-F2 and 02689-R2 (**Table 2.7**). As shown in **Figure 4.9** and **Table 4.7**, the gene encoding the putative-α-1,2-fucosyltransferase; glycosyltransferase protein was amplified from 19/22 virulent and 16/19 putatively avirulent isolates. Although apparently not present in all isolates tested, the conservation of the gene was not significantly different between virulent and putatively avirulent isolates ($p>0.05$), and is therefore not a potential candidate for a diagnostic test.

Additionally, 27 genes were identified which were only present in the genome of the putatively avirulent P7455 (**Table 4.2**). The gene pBhyoa7455_20 encoding a 274 aa protein which showed the highest match to the Methyltransferase type 11 of *Pauldibacter propionicigenes* WB4, could only be identified in the plasmid sequence of *B. hyodysenteriae* P7455. In order to determine if this gene might be conserved among other putatively avirulent isolates of *B. hyodysenteriae* PCR screening was carried, which revealed that the gene was present in 1/22 virulent and 5/19 avirulent strains **(Table 4.7) (Figure 4.10)** and thus significantly more likely to be present in putatively avirulent isolates ($p=0.049$). However, a bigger panel needs to be analyzed to conclude if this gene is significantly more present in avirulent samples. Interestingly, the two avirulent German field isolates (P010573/2, P010573/3) and the virulent English field isolate P7645/09 showed an amplified product which was approximately 600 bp in size and thus approximately 90 bp smaller than the products amplified from P7455 and P7377/3 **(Figure 4.9)**. Sequencing of the 600 bp PCR products confirmed that 90 bp were missing **(Figure 4.11)** indicating that a deletion had taken place within the gene.

Table 4.7: Distribution of potential target genes in virulent and putative avirulent *B. hyodysenteriae* field isolates. Comparative genome analysis of the plasmids showed that the gene coding for the putative alpha-1,2-fucosyltransferase (pBWA1_02689) was only present in the plasmid sequence of pathogenic *B. hyodysenteriae* WA1 and P8544 whereby the gene coding for methyltransferase type 11 (pBhyoa7455_20) was only detected in the putative avirulent *B. hyodysenteriae* P7455. The distribution of these genes among the panel of virulent and putative avirulent *B. hyodysenteriae* field isolates was tested by PCR. The carriage of the gene Bhyoa7455_20 differed significantly between virulent and possibly avirulent isolates (*P*=0.049).

virulent strain	**Locus-ID**			**putative avirulent strain**	**Locus-ID**		
	BHWA1_01319	pBWA1_02689	Bhyoa7455_20		BHWA1_01319	pBWA1_02689	Bhyoa7455_20
WA1	+	+	-	P7455/1/08	+	-	+
QCR1	+	-	-	P7377/03/08	+	-	+
QCR2	+	+	-	P1093/6/01	+	+	-
P8544	+	+	-	P949/4/00	+	+	-
P8226/7	+	+	-	P949/5/00	+	+	-
P8404	+	-	-	P949/9/00	+	+	-
P5943/07	+	+	-	P278/97	+	n	N
P6129/2/08	+	N	n	P944/14/00 †	+	+	-
P6812/1/08	+	+	-	P271/97	+	n	N
P6858/08	+	+	-	P246/1/97	+	n	N
P7124/08	+	+	-	P265/97	+	+	-
P7210/1/08	+	+	-	P271/98	+	n	N
P7286/1/08	+	+	-	P246/2/97	+	+	-
P7309/1/08	+	+	-	P935/LI/00 †	+	n	N
P7271/1/08	+	+	-	P949/3/LI/00	+	+	-
P7346/08	+	-	-	P354/2/97	+	-	+
P7343/4/08	+	N	n	P264/97	+	n	N
P7381/2/08	+	+	-	P257/97 †	+	+	-
P7458/1/08	+	+	-	P252/A/97	+	+	-
P7458/2/08	+	-	-	P252/B/97 †	+	+	-
P5683/07	+	+	-	P944/15/00 †	+	+	-
P7645/09	+	+	+	P7649/2/09	+	+	-
P7624/1/09	+	+	-	P935/15/00	+	+	-
P7620/1/09	+	+	-	P010573/2*	n	+	+
P7563/3/08	+	+	-	P010573/3*	n	+	+
P7486/2/08	+	N	n				
Present	26/26	19/22	1/22**		23/23	16/19	5/19**

+ a PCR product was amplified -- no PCR product was amplified

n DNA of these isolates was not available at this point of study

*putatively avirulent German field isolates ** *P*- value < 0.05 using Fisher's exact two tailed method.

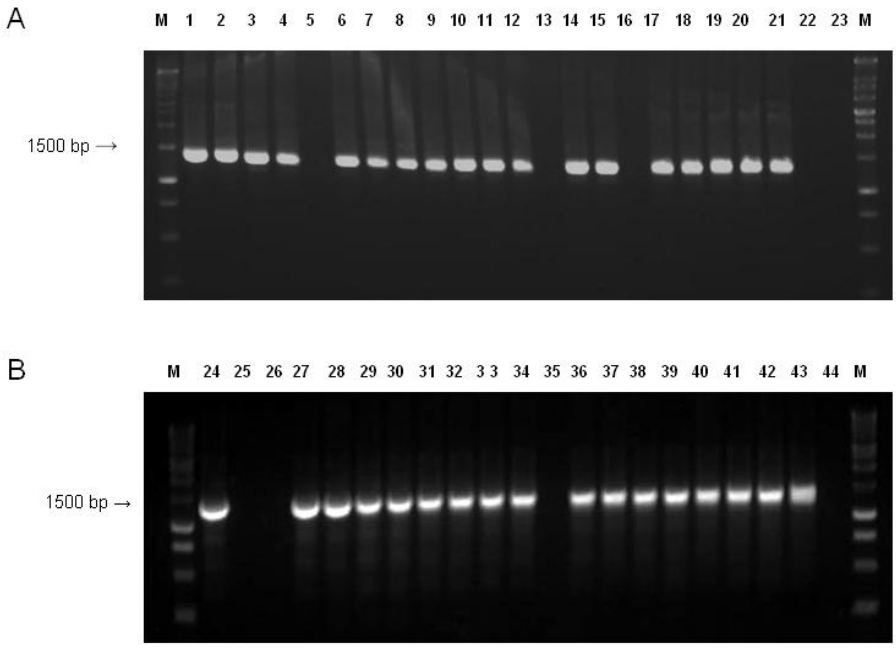


Figure 4.9: Amplification of the BHWA1_02689 gene in the virulent (A) and avirulent (B) panel of *B. hyodysenteriae* strains. A panel of 22 virulent and 19 avirulent *B. hyodysenteriae* strains were screened for the BHWA1_02689 which had been identified to be absent in the avirulent genome P7455. Samples correspond to 1kb ladder (M), P8544 (A1,B1), WA1 (A2), QCR2 (A3), P9543/07 (A4), QCR1 (A5), P6812/1/09 (A6), P6858/08 (A7), P7124/07 (A8), P7210/1/08 (A9), P7286/1/08 (A10), P7309/1/08 (A11), P7271/1/08 (A12), P7346/08 (A13), P7381/2/08 (A14), P7458/1/08 (A15), P8408 (A16), P7563/3/08 (A17), P5683/07 (A18), P7645/09 (A19), P7624/1/09 (A20), P7620/1/09 (A21), P7458/2/08 (A22), P7455 (B24), P7377/3 (B25), P1093/6/01 (B26), P949/4/00 (B27), P949/5/00 (B28), P949/9/00 (B29), P944/14/00 (B30), P265/97 (B31), P246/2/97 (B32), P949/3/LI/00 (B33), P357/2/97 (B34), P354/2/97 (B35), P257/97 (B36), P252/A/97 (B37), P252/B/97 (B38), P935/2/00 (B39), P944/15/00 (B40), P010573/2 (B41), P010573/3 (B43) and negative control (A23, B44).

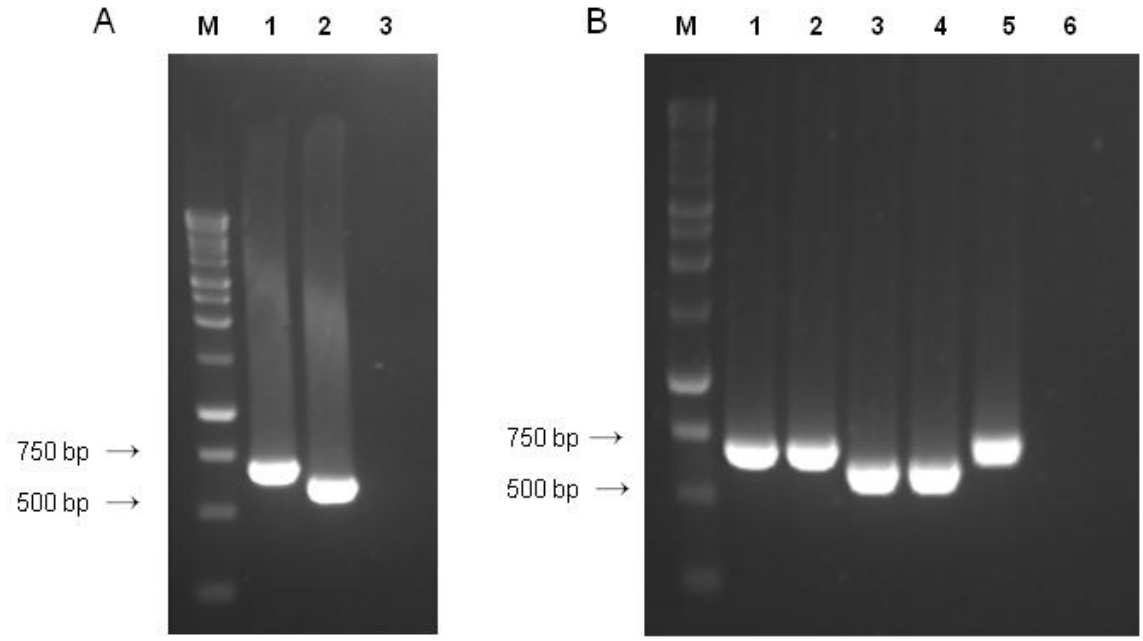


Figure 4.10: PCR screening of the novel plasmid related gene, pBhyoa7455_20, in avirulent *B. hyodysenteriae* P7455 by PCR. A panel of 22 virulent and 19 avirulent *B. hyodysenteriae* strains were screened for the gene Bhyoa7455_20 which had been identified to be unique on the plasmid sequence of the avirulent strain. The figure presents the successful amplification of the gene _0020 in 1/22 virulent (A) and 5/19 avirulent strains (B) of *B. hyodysenteriae*. Samples correspond to 1kb ladder (M), P7455 (A1, B1), negative control (A3), P7377/3 (B2), P010573/2 (B3), P010573/ (B4) (B5) and negative control (B6). The German avirulent field isolates (P010573/2, P010573/3) and the virulent English field isolate (P7645/09) produced approximately 90 bp smaller PCR product than the control (P7455).

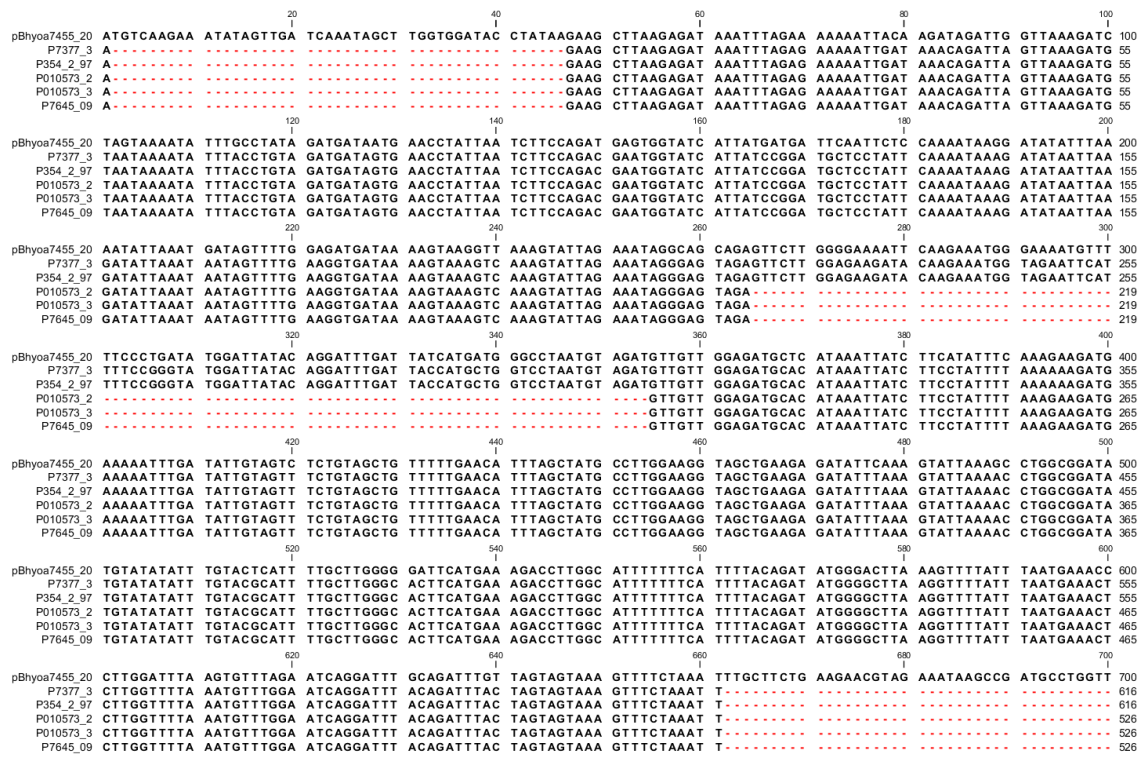


Figure 4.11: Comparison of the nucleotide sequences of the plasmid related gene pBhyoa7455_20 of the avirulent *B. hyodysenteriae* genome P7455 with other *B. hyodysenteriae* isolates. Amplified products of the gene Bhyoa7455_20 were obtained for the *B. hyodysenteriae* strains P7377/3 (avirulent), P354/2/97 (avirulent), P010573/2 (avirulent, German isolate), P010573/3 (avirulent, German isolate) and P7645/09 (virulent). Nucleic acid products were sequenced, and sequences were aligned using Clone Manager. The German avirulent isolates (P010573/2, P010573/3) and the virulent isolates (P7645/09) are missing a 90 bp region of the pBhyoa7455_20 gene.

Although the gene seems to occur in a significantly higher number of putatively avirulent strains, the gene sequence seems to vary among *B. hyodysenteriae* isolates from different geographical regions, complicating its potential use as a genetic marker. Therefore, avirulent isolates from more countries needed to be assessed to determine whether this gene has any diagnostic potential. In addition, the distribution and impact of the 90 bp deletion needs to be assessed in a wider panel.

Although the gene encoding the App-1-p processing enzyme family protein was not predicted as a potential target gene after its confirmation by PCR, it was included as representative out of the seven genes in **Table 4.6** into the screening to further assess its conservation among virulent and avirulent *B. hyodysenteriae* isolates. As summarized in **Table 4.7** all tested isolates amplified a PCR product of the expected size, demonstrating that the gene is conserved among *B. hyodysenteriae* isolates and thus not appropriate for use as a marker.

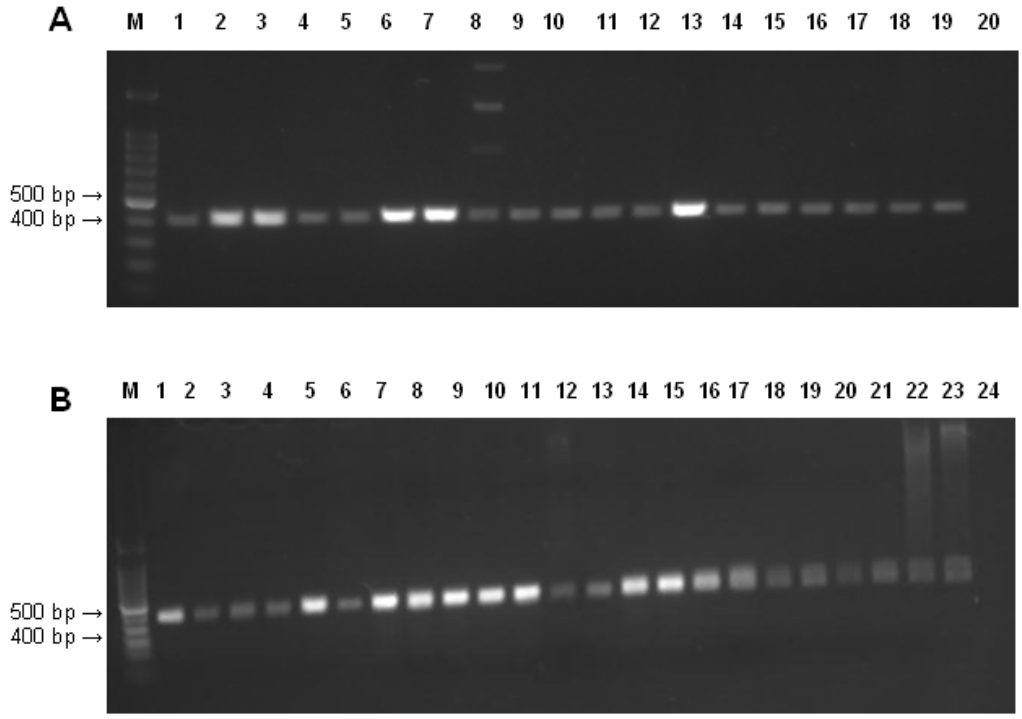


Figure 4.12: Amplification of the gene BHWA1_01319 in the virulent (A) and avirulent (B) panel of *B. hyodysenteriae* strains. The presence of the gene encoding the App-1-p processing enzyme family protein (BHWA1_01319) was screened in a panel of 19 virulent and 23 avirulent *B. hyodysenteriae* isolates. The samples correspond to 100 bp DNA ladder (M); negative controls (A20; B24) and a 417 bp PCR product amplified by following isolates: WA1 (A1), P6858/08 (A2), P7124/08 (A3), P7210/01/08 (A4), P7286/1/08 (A5), P7309/1/08 (A6), P7271/1/08 (A7), P7346/08 (A8), P7343/4/08 (A9), P7381/2/08 (A10), P7458/1/08 (A11), P7458/2/08 (A12), P5683/07 (A13), P7645/09 (A14), P7624/1/09 (A15), P7620/1/09 (A16), P7563/3/08 (A17), P7486/2/08 (A18), OCR2 (A19), P7455 (B1), P7377/03/08 (B2), P1093/6/01 (B3), P949/4/00 (B4), P949/5/00 (B5), P949/9/00 (B6), P278/97 (B7), P944/14/00 (B8), P271/97 (B9), P246/1/97 (B10), P265/97 (B11), P271/98 B12), P246/2/97 (B13), P935/LI/00 (B14), P949/3/LI/00 (B15), P354/2/97 (B16), P264/97 (B17), P257/97 (B18), P252/A/97 (B19), P252/B/97 (B20), P944/15/00 (B21), P7649/2/09 (B22) and P935/15/00 (B23).

4.3 Discussion

In **Chapter 3** a preliminary comparison of the phenotypic and genotypic features of virulent and avirulent isolates of *B. hyodysenteriae* was performed, the results of which indicated a high degree of similarity between all tested isolates. In the current chapter the draft genomes of the virulent *B. hyodysenteriae* P8544 and the avirulent *B. hyodysenteriae* P7455 were analysed using bioinformatic tools in order to find genes which are present and distinct in each of the genomes. This was done primarily to discover potential target genes to distinguish between pathogenic and non-pathogenic *B. hyodysenteriae* isolates.

4.3.1 Annotation and general properties of virulent and avirulent *B. hyodysenteriae* isolates

Due to the time-saving impact and decreasing costs of next-generation sequencing, the number of sequenced genomes has increased drastically in the last couple of years making automatic annotation mandatory for the majority of laboratories. However, the accuracy of these automatic tools has been questioned.

The draft genomes of P8544 and P7455 were 200-26,000 bp shorter and approximately 55-74 genes were additionally predicted to be present compared to the complete genome of WA1. This is likely due to the fact that the genomes were incomplete, and thus sequences were missing due to the unclosed gaps between the assembled contigs. Illumina/SOLEXA sequencing as used for the sequencing of the *B. hyodysenteriae* isolates P8544 and P7455, is known to produce short sequence reads between 30-50 bases and hence cannot resolve small sequence repeats (Bentley, 2006) which leads to contigs smaller than 150 bp as seen in the genome sequence of P8544 and P7455. In the two genomes 51 and 53 contigs were found to be less than 150 bp.

Re-ordering of contigs was performed using the Mauve software. Unfortunately the software was not able to assemble all contigs into an appropriate order, as highlighted (for example) by the VSH-1 like element which was spread over different contigs. Specifically, contigs < 150 bp were positioned to the end of the sequence which resulted in prediction of portions of the same ORF in different. For instance 15 copies of the gene *vspF* and 9 copies of a gene encoding a MCP (BHWA1_01857) were predicted in different contigs, showing variation in length over the entire genomes of P8544 and P7455. Thus, it can be

assumed that incorrect ORFs have been predicted by mapping non-related contigs leading to a higher number of predicted ORFs in the genomes of P8544 and P7455 than in the complete WA1 genome. Therefore, manual re-ordering of the contigs was performed using Artemis. Small contigs were not discarded due to possible unique sequence regions and thus mainly larger contigs have been able to assemble correctly. Annotation of the re-assembled contigs sequences did not reveal any major difference to the previous sequence and was used for further studies. However, it should be emphasised that closing the genomes of *B. hyodysenteriae* P8544 and P7455 was not a priority of the project. First of all, the draft genomes served as tool for comparative genome and proteomic analyses **(Chapter 6)** in this study, despite the known limitation of automatic annotation software and missing sequence data. Although more time spent closing the remaining gaps and manually correcting ORF predictions would have contributed to a higher quality genome sequence, it was not a primary objective of this project, as time was not available for this undertaking.

The KO analysis indicated that approximately 39.45% of the predicted proteome of *B. hyodysenteriae* P7455 and 39.83% of predicted proteins in P8544 have orthologues in the KO database. The large majority of these proteins are associated with metabolism, more specifically carbohydrate, energy and amino acid metabolism. There is also a considerable proportion of proteins concerned with the processing of genetic and environmental information, specifically in membrane transport. The number of *B. hyodysenteriae* proteins with KEGG orthologues associated with organism systems and human disease is minimal. Using the current methodology, the observed dominance of protein functional category by metabolic proteins is likely enhanced by the large focus of KEGG databases on this functional class. An alternative, widely-used, database that could have been analysed to characterize proteins by functions is 'The Cluster of Orthologous Groups' (COG) database. However, this database does not appear to be updated and still only contains proteins from 66 genomes as described elsewhere (Tatusov *et al.*, 2003); thus, this database was not used in the current study. The similar percentage distribution of functions in the genomes of P8544, P7455 and WA1 predicted by KAAS indicates that the three isolates are harboured in the same environmental niche and also mirrors their close phylogenetic relationship.

4.3.2 Virulence factors in virulent and avirulent *B. hyodysenteriae*

Known virulence factors recognized in the genome of WA1 were also identified in the genomes of P8544 and P7455. As described in **Chapter 3**, the panel of avirulent *B. hyodysenteriae* isolates were all strongly β-haemolytic. This observation is in agreement with the analysis of the genome sequence of avirulent P7455, as the 7 genes associated with haemolysin production were detected. Former studies already showed that the genes *tlyA, tlyB, tlyC* and *hylA* were highly conserved among virulent and avirulent isolates (Walker, 2001). Therefore, it can be assumed that virulent and avirulent *B. hyodysenteriae* isolates are not able to be distinguished on the basis on their haemolytic activity.

Due to the observation that presumably avirulent isolates are less frequently detected in the crypt compared to virulent isolates of *B. hyodysenteriae*, it was proposed that avirulent strains may lack components of the bacterial flagellum which would have an impact on their ability of host invasion. The PCR screening in **Chapter 3** already showed that the *flgG* gene was present in 97.8% of avirulent *B. hyodysenteriae* isolates. Moreover, the analysis of the flagella apparatus of the avirulent genome did not reveal any obvious difference as the full set of 22 core genes and the 9 additional structural genes found in WA1 and P8544 were also present in P7455. All the three genomes were lacking the genes *flgH* and *flgI* which is typical for spirochaetes as the flagellum is located in the periplasm (Bellgard *et al.*, 2009). The current study suggests that the flagella apparatus is conserved among the virulent and avirulent isolate of *B. hyodysenteriae*, however, transcription of these flagella genes (and hence expression of their encoded proteins) might differ between isolates, affecting their motility behaviour and thus colonization ability.

Additionally, the VSH-1 transfer agent which is likely to be involved in the transfer of genes and thus contributes to the adaptation of *B. hyodysenteriae*, was predicted in the isolates of P8544 and P7455. All 19 genes belonging to the bacteriophage in WA1 were identified in the genome of P7455, whereby the gene *orfC* was missing in the virulent genome P8544. Differences among the VSH-1 like agent in different *B. hyodysenteriae* isolates have been reported before (Motro *et al.*, 2009). Nevertheless, it can be assumed that virulent and avirulent isolates are able to acquire new genes via the same mechanism which enables them to expand their gene repertoire and thus enhance their adaptation to changing environmental conditions. Evidence of gene acquisition in virulent and avirulent was shown and will be discussed in section **4.3.3**.

The analysis of the distribution of the associated genes with virulence in *B. hyodysenteriae* WA1 could all be detected in the avirulent genome P7455 indicating that the presence of these genes does not allow discrimination between pathogenic and non-pathogenic *B. hyodysenteriae*. Due to similar gene carriage between virulent and avirulent *B. hyodysenteriae* isolates, but the huge differences observed regarding the severity of SD in pigs, it can be considered that deviation between two types of *B. hyodysenteriae* is associated with essential variations in the nucleotide sequence with associated alteration of protein expression, rather than in the presence/absence of particular genes. Single base pair variations, termed single-nucleotide polymorphisms (SNPs), are the most abundant form of sequence variation in mammals and bacteria and are known to have an enormous impact on the biology, capability of causing disease as well as susceptibility of diseases in various species (Boddicker *et al.*, 2002; Franchimont *et al.*, 2004; Pouttu *et al.*, 1999). SNPs have also been used as genetic markers to identify and distinguish bacteria to the sub-species level, as reported for *Brucella* (Gopaul *et al.*, 2008; Koylass *et al.*, 2010).

Single-nucleotide polymorphism can lead to a single amino acid replacement which can result in a dramatic effect on the function of a protein and hence on microbial physiology. In bacterial pathogens, several SNPs have been shown to provide a selective advantage and enhance pathogenicity potential. For instance, two phenotypically-comparable strains of *Salmonella typhimurum* demonstrated differences in their ability to adhere to eukaryotic cells. Further investigation revealed that the higher binding ability was associated with the replacement of the amino acids Gly61Ala and Phe118Ser caused by SNPs in the adhesion gene *fimH* (Boddicker *et al.*, 2002). Therefore, SNP analysis of the genomes of the virulent and avirulent *B. hyodysenteriae* isolates, particularly of the known virulence factors, would provide a deeper insight into possible sequence variations among conserved genes and may resolve differences in the assembly and confirmation of entire proteins and thus pathogenicity among both types. However, due to lack of other available avirulent genome sequences and limited DNA material, the following work in this study was focused on differential protein expression between virulent and avirulent *B. hyodysenteriae*, as described in **Chapter 6**.

4.3.3 Novel genes among virulent and avirulent *B. hyodysenteriae*

Comparison of the three genomes revealed that the field isolates acquired a series of unique genes which were not associated with *B. hyodysenteriae* WA1. However, novel

ORFs need to be appraised carefully due to possible false prediction of ORFs by Xbase (as discussed above). Genes encoding proteins consisting of < 50 aa have been identified in both new genomes of *B. hyodysenteriae* resulting in low scores and e-values, thus bringing the likelihood that these short sequences represent true ORFs into question. Further work is required to verify whether the unique ORF predicted within the genomes of P8544 and P7455 encode functional products. Significantly, a number of the unique ORF are predicted to encode proteins ranging in size from 270 aa to 1,579 aa, with acceptable scores and e-values having been obtained from BLAST searches. Interestingly, most of these genes were annotated as coding for proteins produced by other *Brachyspira* spp., in particular *B. murdochii* and *B. intermedia*. This might not be surprising as comparative genomics of *Brachyspira* has shown that the greatest number of shared genes were found between *B. intermedia*, *B. hyodysenteriae* and *B. murdochii* (Hafstrom *et al.*, 2011). In addition, a cluster of a VSH-1 like element and two additional bacteriophages termed pI1 and pI2 have been detected in *B. intermedia* (Hafstrom *et al.*, 2011). Similar clusters have been also identified in the genomes of *B. murdochii* and *B. piloscoli* indicating that all *Brachyspira* spp. are capable of exchanging genetic material.

Horizontal gene transfer between *B. intermedia* and *B. hyodysenteriae* has been suggested by the identification of a gene encoding a hypothetical inner membrane protein in the bacteriophage pI1 in *B. intermedia* (Bint_0072) sharing high sequence similarity with the gene in *B. hyodysenteriae* (BHWA1_02012) (Hafstrom *et al.*, 2011). Therefore it can be assumed that the novel genes described in **4.2.2.3** were acquired via the VSH-1 element of *B. hyodysenteriae*.

The translated products of most of the novel ORF identified in this study were of unknown function. The majority of unique genes annotated as hypothetical in this study have been also reported in other *Brachyspira* genomes (Bellgard *et al.*, 2009; Hafstrom *et al.*, 2011); however, further investigations of the translated products did not reveal any additional knowledge about the function of the proteins. Interestingly, some unique genes identified in both Scottish field isolates revealed high similarity by exhibiting low e-values and high scores of genes identified in other bacteria. Genes encoding proteins present in *Clostridium clostrioforme* were detected in P7455 while one gene present in *Clostridium difficile* was identified in the virulent genome of P8544. High numbers of significant gene similarities between *Brachyspira* spp. and *Clostridium* spp. have been reported earlier (Bellgard *et al.*, 2009). Particularly genes acquired from *Clostridium* spp. involved in metabolism have

been suggested to enhance *B. hyodysenteriae* survival (Bellgard *et al.*, 2009). However, only hypothetical proteins showing the highest match to *Clostridium* spp. have been identified in P8544 and P7455, of which the functions remain unknown. Additionally genes from other gastrointestinal anaerobic bacteria, including *Bacteriodes fragilis*, *Helicobacter bilis* and *Helicobacter cinaedi* as well as *Campylobacter jejuni* were identified. The acquisition of these genes might have an enhancing effect on *B. hyodysenteriae* persistence in the intestine and thus virulence. The helicase of *Bacteriodes fragilis*, as detected in *B. hyodysenteriae* P7455 (Bhyoa7455_1378), has been shown to have a protective effect when cells of *Bacteriodes fragilis* were subjected to metronidazole (Paul *et al.*, 2011). Therefore, the acquisition of the helicase would benefit P7455 regarding antibiotic resistance and thus its survival in the host in the face of antibiotic treatment. Moreover, *Bacteriodes* has been shown to be the most-frequently isolated bacterial species from pig faeces, suggesting that gene transfer between *B. hyodysenteriae* and species of *Bacteriodes* may occur frequently due to the high abundance of *Bacteriodes* in the large intestine.

4.3.4 The plasmid sequence of virulent and avirulent *B. hyodysenteriae*

Recently, a published manuscript suggested that *B. hyodysenteriae* strains lacking plasmid are less virulent. The current study has shown that the isolate P8544 and P7455 both contain the plasmid which was already indicated by the results reported in **Chapter 3**. To-date only two strains were found to lack the plasmid showing, that the majority of analysed *B. hyodysenteriae* isolates world-wide possess the plasmid. Thus, the findings in this chapter are in agreement with recently published data showing that the plasmid seems to be ubiquitous among *B. hyodysenteriae* isolates. The data obtained in this study suggest that low-virulence is not necessarily related to the absence of the plasmid in *B. hyodysenteriae*, since all avirulent strains assessed by PCR **(Chapter 3**) were found to possess plasmid, as determined by the amplification of the gene encoding a radical SAM protein (BHWA1_02682) which is associated with the plasmid sequence in WA1, P8544 and P7455. In the published study, non-pathogenicity was reported to result from the lack of the plasmid-encoded *rfbABCD* operon in the Australian avirulent *B. hyodysenteriae* isolates A1 and WA400 (La *et al.*, 2011). However, the *rfB* cluster, encoding the rhamnose pathway, was found to be conserved in the sequenced English virulent and avirulent field strains, suggesting that non-pathogenicity cannot be associated with the absence of this region in this study. Moreover, the current study as well as that of La *et al.* (2011) indicate

that the plasmid is ubiquitous in *B. hyodysenteriae* and the detected isolates A1 and WA400 seem to be another atypical form of *B. hyodysenteriae* and so far very uncommon. However, as more atypical subspecies of *Brachyspira* spp., particularly of *B. hyodysenteriae* and *B. intermedia*, have been reported recently, further investigation of these Australian isolates (using approaches including MLST and genome sequencing) would provide information about their relationship to other *B. hyodysenteriae* described thus-far.

Heterogeneity among the plasmid sequences could be shown, since some genes were absent and distinct in P8544, P7455 and WA1. The comparison of the plasmid sequences revealed that the avirulent isolate, P7455, shared higher similarity with the plasmid sequence of WA1 than with P8544. This is surprising as the isolates P7455 and P8544 were geographically more-closely related. However, to-date nothing is known about the extent of heterogeneity in the plasmid sequences in field isolates of *B. hyodysenteriae* in the U.K (or elsewhere) due to lack of available data. Therefore, sequencing of additional plasmid sequences of different *B. hyodysenteriae* isolates would give deeper insight into the sequence diversity. For future research prospects, generating a plasmid-free *B. hyodysenteriae* strain would serve as a useful tool for studying its contribution to pathogenicity and would increase the understanding of the plasmid's biological role. Plasmids have been successfully cured *in vitro* in various bacterial species to investigate their contribution to virulence (Gulig & Curtiss, III, 1987). However, plasmid curing has not been described in *Brachspira* spp. thus far mainly due to its fastidious nature and lack of established genetic manipulation techniques. Conventional curing methods using chemical or mutagenic agents such as ethidium bromide or treatment with UV-light (Nohmi *et al.*, 1991; Spengler *et al.*, 2006) involve screening colonies for plasmid-cured derivatives, which in the case of *B. hyodysenteriae* is hampered by the fact that colonies tend to grow under the agar surface and the presence of colonies is sometimes only detectable by the appearance of haemolytic zones. For a long time *B. hyodysenteriae* was considered non-transformable; however, the pathogen has been successfully transformed by electroporation and specific inactivation of genes has been achieved (Rosey *et al.*, 1995; ter Huurne *et al.*, 1992). Additionally, the identification and development of an appropriate selectable marker (chloramphenicol and kanamycin) with utility for allelic selection in *B. hyodysenteriae* (Rosey *et al.*, 1995; ter Huurne *et al.*, 1992) would also offer the possibility of curing the plasmid via the plasmid incompatibility method which has been successful in *B. anthracis* (Liu *et al.*, 2012b) and *Y. pestis* (Ni *et al.*, 2008).

4.3.5 Potential marker genes

Interestingly, the avirulent isolate was only lacking the alpha-1,2-fucosyltransferase which seemed to be replaced by a gene identified as a Methyltransferase type 11 protein found in *Pauldibacter propionicigenes*. The alpha-1,2-fucosyltransferase genes FUT1 and FUT2 of *E. coli* have been demonstrated to be expressed in the small intestine of the pig and to be involved in the adhesion process of the bacteria (Meijerink *et al.*, 2000). Moreover, SNPs in FUT1 resulted in the decreased ability of *E. coli* to adhere to intestinal epithelium due to changes in the structure of the adhesin (Meijerink *et al.*, 2000). It was initially thought that the absence of the putative alpha-1,2-fucosyltransferase might be associated with the non-pathogenicity of P7455. However, PCR screening showed that gene is present in the majority of avirulent *B. hyodysenteriae* isolates (84.21%) making this assumption unreasonable. That said, it is possible that the avirulent isolates carrying the gene all possess a significant SNP in the coding region which has an effect on conformational structure of the protein and thus adhesion, such as has been described in *E. coli*. Such an SNP could be targeted as a marker for virulence (or avirulence), although this hypothesis clearly requires further study.

The Methyltransferase type 11 protein, sharing the highest match with *Pauldibacter propionicigenes*, was predicted to be a potential marker for avirulent *B. hyodysenteriae*. However, the gene was subsequently found to be present in avirulent (5/19) and virulent *B. hyodysenteriae* (1/22) isolates although 3 out of the 5 avirulent isolates carried a 90 bp smaller gene, indicating that it would not be suitable for a diagnostic due to its low abundance among avirulent isolates and its sequence variation. However, the presence of this gene among avirulent strains also supports the assumption in this study that the VSH-1 like element is likely ubiquitous among non-pathogenic *B. hyodysenteriae* isolates.

Surprisingly, the comparative genome analysis of the three *B. hyodysenteriae* revealed a high degree of similarity between the three isolates of *B. hyodysenteriae*. Consequently, it is likely that differentiation between virulent and avirulent isolates of *B. hyodysenteriae* is not based on a simple presence and absence of genes and thus differentiation appears to be much more complex. Therefore, further studies reported in the following chapters were conducted to appraise differential gene and protein expression between avirulent and virulent *B. hyodysenteriae*, particularly under *in-vivo*-relevant growth environments.

Chapter 5: Iron-acquisition by *B. hyodysenteriae*

5.1 Introduction

Iron is an essential element which is required by the majority of living organism (Hentze *et al.*, 2010; Schaible & Kaufmann, 2004), as it contributes to numerous biological processes including transport and storage of oxygen, respiration, gene regulation and DNA biosynthesis (Andrews *et al.*, 2003). Although studies on the importance of iron for *Brachyspira* spp. have been limited, the annotated genome sequences of *B. hyodysenteriae* reported in **Chapter 4** indicate that iron may play an important role in *B. hyodysenteriae* life-style, as iron is needed as a co-factor for many genes. For example, the gene encoding the arylsulfatase regulator (BHWA1_00125) contains an iron-sulfur complexes suggesting that iron is important for the biological activity of this regulator protein, and this is but one example of many.

In the presence of oxygen, iron is oxidized to the ferric state, Fe^{3+}, and thus extremely insoluble. Furthermore, the accessibility of free-iron to bacteria in the mammalian host is limited by host iron-binding proteins such as lactoferrin and transferrin. In order to cause infections, bacteria need to be able to acquire essential nutrients, including iron. Bacteria generally require between 10^{-7} to 10^{-5} M of iron to support their growth (Andrews *et al.*, 2003), however, in the mammalian host, the concentration of free-iron is usually around 10^{-18} M which is far below the required level (Bullen *et al.*, 1978). Studies have shown that available iron concentration in the large intestine of rats was approximately 0.04 µmol/g and iron availability in the gastrointestinal environment is dependent upon the level of dietary iron supplementation in various mammals (Lund *et al.*, 1998). In addition to host iron-binding proteins, the host employs further mechanism to limit iron availability during inflammation of the gut. For instance, the production of the antimicrobial protein, lipochelin-2, responsible for preventing bacterial iron acquisition by capturing the siderophore enterochelin, has been shown to be increased in the inflamed intestine in response to enteric pathogens (Raffatellu *et al.*, 2009). Furthermore, mRNA levels of the hormone hepacidine were significantly increased in fish when infected with *Streptococcus iniae*, as well as in mice when inflammation was induced (Shike *et al.*, 2002). As a consequence, the gut was prevented absorbing iron from the bloodstream by inhibition the iron transport system ferropotin 1 (Ganz, 2003), thus reducing available iron in the intestine. However, bacteria not only face iron-limitation caused by the host, but also by the competition for resources with other bacterial species inhabiting the same nice. The gastrointestinal tract of pigs is densely populated with between 10^{10} to 10^{11} culturable

bacterial species (Allison *et al.*, 1979), indicating that well-adapted bacteria have developed efficient ways to scavenge nutrients to outgrow other resident bacteria (Hibbing *et al.*, 2010). Competition for iron within the bacterial community has been demonstrated between *S. typhimurium* and *E. coli* Nissle (Deriu *et al.*, 2013). Due to the fact that *B. hyodysenteriae* colonizes the large intestine, it is likely that the pathogen is exposed to an iron-restricted environment created by the host as well as the intestinal microflora. Therefore, iron-related studies were considered a relevant route by which deeper insight into *B. hyodysenteriae* pathogenicity could be elucidated.

Due to the limitation of iron in the host, microorganisms have evolved a number of diverse and highly specific mechanisms for obtaining iron from their environment, and these are often considered virulence determinants (Litwin & Calderwood, 1993). In Gram-negative bacteria, the outer-membrane allows passive diffusion of small solutes that have a molecular weight smaller than *ca.* 600 Da (Nikaido, 2003) due to the presence of β-barrel proteins termed porins. Nevertheless, porins are inadequate for efficient acquisition of low abundance iron sources, including lactoferrin or iron siderophore complexes as they exceed the Mr weight cut off for these porins. Thus, uptake of larger iron sources requires a specific outer-membrane receptor, a periplasmic binding protein (PBP) and an inner membrane ATP-binding cassette, a so -called ABC transporter. The energy required for the translocation across the OM is provided via the TonB-ExbB-ExbD protein complex (Larsen *et al.*, 1996). The TonB-dependent receptors (TBDRS) are outer-membrane proteins in Gram-negative bacteria and are mainly known for the active transport of iron siderophore complexes and Vitamin B12 into the periplasm of the cell. The periplasm-anchored TonB protein is composed of a 22-stranded-β-barrel transmembrane which enables the protein to make contact with the TonB-dependent receptor in the OM, a plug domain which binds specific metals and a periplasm exposed TonB box which has been shown to be required for TonB-dependent iron uptake. The integral cytoplasmic proteins ExbB and ExbD are believed to be involved in the conformational change of TonB and its stabilization (Held & Postle, 2002).

Another way by which bacteria acquire iron is through the production of siderophores. In response to iron starvation most pathogenic bacteria are able to secrete low molecular weight chelating agents, so called siderophores, which have a very high affinity for iron. To date, approximately 500 types of siderophores (Drechsel & Winkelmann, 1997) are known which are divided into four major classes depending on their chemical natures; so

called hydroxymates, cateholates, phenolates and carboxylates. Moreover, mixed types of siderophores have been recognised, consisting of at least two types in one molecule, such as aerobactin which is a mixed type containing citrate-hydroxymate. They all share essential features with each other, consisting of an iron-binding unit and a unit which is specific for interaction with a receptor on the bacterial membrane. The OM of Gram-negative bacteria contains several receptors that bind cognate iron-siderophores A siderophore receptor of a bacterium is usually specific for a single siderophore class (Chial *et al.*, 2003); allowing bacteria to more efficiently scavenge ferric-siderophores. All known OM siderophore receptors share a similar composition, containing a β-barrel structure consisting of 22-β-standed tube. Siderophore-uptake mechanisms have been best studied in *E. coli*, in which the three siderophore OM receptors FepA (specific for enterobactin), ferric citrate transporter (FecA) and ferrichrome OM transporter (Fhu) have been described. FepA is an *E. coli* OM receptor which binds ferric enterobactin. Once the Fe^{3+} is in the periplasm, a periplasmic-binding protein (PBP) delivers the ferric enterobactin to a specific ABC transporter, FepCDG, located on the cytoplasmic membrane.

Instead of producing their own siderophores, some bacteria like *Bordetella bronchiseptica* (Armstrong *et al.*, 2012) as well as the spirochaete *Leptospira* spp. (Louvel *et al.*, 2006) are capable of utilizing xenosiderophores (siderophores produced by other bacteria), indicating that they possess specific siderophore receptors. *Bordetella bronchiseptica* has been shown to bind enterobactin via the BfeA OM receptor (Armstrong *et al.*, 2012) while aerobactin and ferrichrome were utilized by *Leptospira biflexa* and *Leptospira interrogans*, although the uptake system still remains unknown (Louvel *et al.*, 2006).

Moreover, some bacteria are capable of obtaining iron from the host iron-binding proteins lactoferrin and transferrin which are involved in the first line of defence against invading microorganism by binding extracellular free-iron. The primary function of lactoferrin is to act as an inhibitor of microbial growth, but unlike transferrin it is not thought to be involved in eukaryotic iron transport (Mietzner & Morse, 1994). Some bacteria, including *H. pylori* (Dhaenens *et al.*, 1997) and *Bordella pertussis* (Menozzi *et al.*, 1991), are able to sequester iron directly from lactoferrin through direct binding to a specific receptor on the outer-membrane, termed lactoferrin-binding protein (Lbp). *Treponema denticola* and *Treponema palladium* are the only spirochaetes described which are able to bind lactoferrin, but no specific receptor has been identified so far (Staggs *et al.*, 1994).

The iron-binding component of haemoglobin, haem, can also be utilized as a direct source of iron by many bacteria. Pathogenic bacteria commonly secrete toxins such as haemolysins and proteases which disrupt erythrocytes and cause the release of haem bound to host carrier proteins. In a human pathogenic strain of *E. coli* EB1, haemoglobin protease (Hbp) was found to cleave haemoglobin and subsequently bind the released haem (Otto *et al.*, 1998). The direct uptake of haem in Gram-negative bacteria involves binding of haem or a haem complex to an OM receptor, followed by transport of the extracted haem group across the OM in a TonB-dependent manner. These haemophore systems have been described in enteric bacteria including *Seratia marcescens* (Letoffe *et al.*, 1994) and *Yersine enterocolitica*. *Seratia marcescens* is able to secrete haemophores such as HasA, which binds extracellular free haem or captures haem from haemoglobin. The haem-bound HasA complex is then presented to the OM receptor, HasR, which recognizes a wide range of iron-containing substrates. The transport of haem across the CM appears to require an ABC permease. Once in the CM, the haem is degraded by haem oxygenase to release the bound iron. The spirochaete *Treponema denticola*, causing periodontal disease in humans, has been found to posse's two novel OM proteins with haemin-binding ability, termed HbpA and HbpB (Xu *et al.*, 2001).

Under anaerobic or microaerophilic conditions, the ferrous iron uptake system termed Feo seems to import ferrous iron (Fe^{2+}) into the cytoplasm of cells. Ferrous iron transport systems have been described in gastro-intestinal bacteria like *E. coli* (Kammler *et al.*, 1993) and *H. pylori* (Velayudhan *et al.*, 2000). Studies suggested that Feo activity is not TonB-dependent, thus indicating that the ferrous uptake mechanism is distinct and unlike any other transporter of Fe^{3+}. In *E. coli*, the *feoABC* operon has been identified, and the product of the *feoB* gene was the major component of the Feo system and was shown to have the key role in the uptake of Fe^{2+}. Moreover, *feoA* is lacking in some bacteria. The FeoB protein is membrane-bound and has ATPase and GTPase acitivity; its function has been shown to be required for *H. pylori* in colonisation of mice and iron uptake under iron-restricted conditions (Velayudhan *et al.*, 2000).

Environmental factors including temperature, pH, osmolarity, and amino acid concentration affect bacterial gene expression, including those genes associated with virulence (Miller *et al.*, 1989). It is not surprising then that being an essential requirement for growth, the concentration of environmental iron is also "sensed" by bacteria. In *B. hyodysenteriae* the sensing of environmental iron concentration and its concomitant effect

on pathogenesis is not yet fully understood. *B. hyodysenteriae* has been shown to express at least three iron-regulated proteins when grown under iron-restricted conditions, but seems to be unable to use catechol and hydroxymate siderophores (Li *et al.*, 1995). Moreover, a periplasmic ATP-binding cassette iron import system has been detected. However, to-date there has been no other published research on the requirement of iron or its potential link to gene expression and therefore virulence. Nevertheless, it is of interest that iron has been shown to play an important role in the closely-related spirochaete *Leptospira* (Louvel *et al.*, 2005).

Upon infection with a microorganism and as part of the innate defence mechanism, the host specifically limits iron. The shift from high to low iron is an important signal for bacteria to coordinate the regulation of gene expression (Litwin & Calderwood, 1993). Subsequently, in addition to iron-acquisition genes, the expression of many genes involved in virulence are also regulated in response to environmental iron concentration. Therefore, by assessing the influence that iron availability has on *B. hyodysenteriae* gene expression, it was thought possible that novel phenotypes could be identified, which could be the focus of further studies in order to discover specific factors contributing to virulence which might be absent in the avirulent strains.

5.2 Results

5.2.1 Growth characteristics of *B. hyodysenteriae* under iron-limited and iron-replete conditions

In order to determine whether *B. hyodysenteriae* requires iron for growth, the iron-load in BHI was reduced by addition of the iron-chelator, 2',2' dipyridyl (subsequently referred to as dipyridyl). In the first instance, the conventional medium described in **Chapter 2** (BHI containing 5% FCS) was supplemented with concentrations of dipyridyl in the range of 0.2-0.01 mM (final concentration) to find the minimum inhibitory concentration (MIC). The results are summarized in **Figure 5.1.** After 48 hrs of incubation the isolates P8544 and P7455 were able to grow up to a concentration of 0.1 mM of dipyridyl, with OD values ranging from 0.7 to 0.85, whereas no growth could be observed for dipyridyl concentrations of 0.12 mM up to 0.2 mM after 48 hours. All experiments were carried out 6 times and results were highly reproducible between experiments. With increasing dipyridyl concentration a constant decrease in growth was expected. However, dipyridyl concentrations of 0.12 mM and greater resulted in complete cessation of growth, while concentrations between 0.1 mM and 0.01 mM yielded in OD values similar to the OD values observed in the controls (iron-replete). Moreover, a concentration of 0.1 mM resulted in increased growth in the virulent strain P8544, to a greater level than observed under standard culture conditions (BHI supplemented with 5% FCS).

To further assess the effect on growth using dipyridyl, growth curves of the isolates P8544 and P7455 were generated using concentrations of dipyridyl ranging from 0.14-0.08 mM for 64 hrs **(Figure 5.2).** The isolates P8544 and P7455 showed similar growth characteristics under iron-restricted conditions, consistent with previous results (**Figure 5.1**). Both isolates were able to grow up to a concentration of 0.1 mM dipyridyl, while dipyridyl concentrations of between 0.12 mM and 0.14 mM resulted in cessation of growth. Compared to the iron-replete (standard conditions) the yield of growth for each isolate was not affected up to a concentration of 0.1 mM of dipyridyl. The growth curve of the virulent *B. hyodysenteriae* P8544 reached stationary phase with an OD_{600nm} of 1.04 after 19 hrs under iron-replete conditions, OD_{600nm} of 1.06 after 36 hrs for 0.08 mM of dipyridyl and an OD_{600nm} of 1.03 after 50 hrs for 0.1 mM of

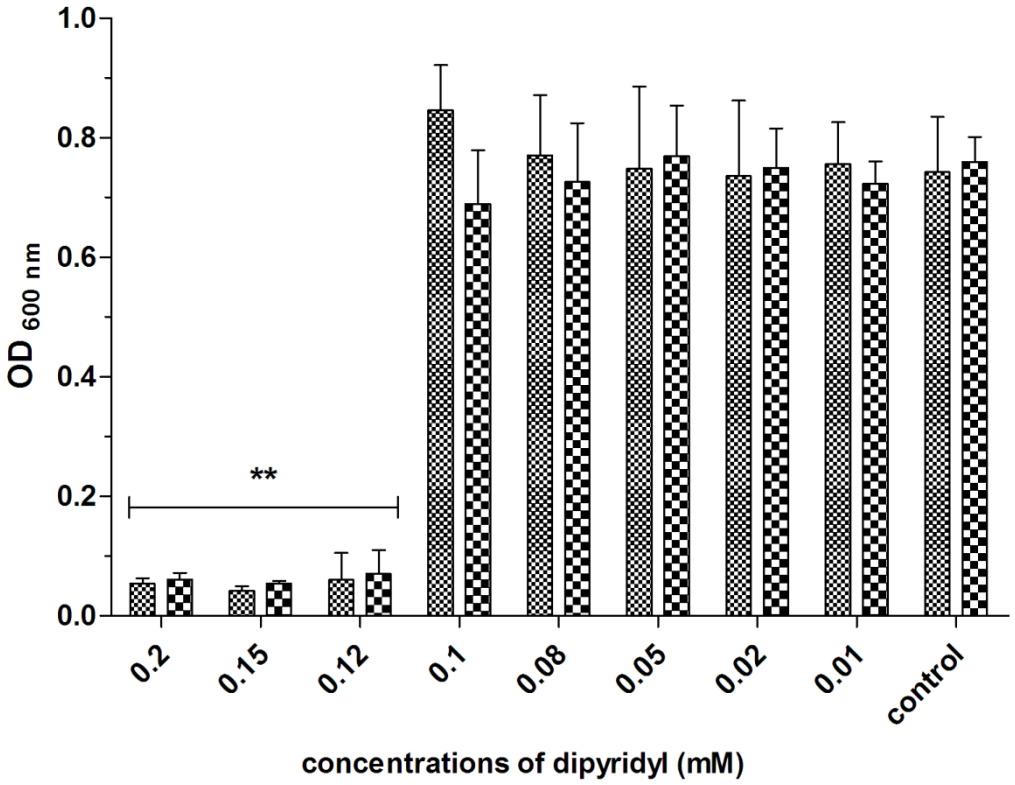


Figure 5.1: Titration of the dipyridyl in *B. hyodysenteriae* culture. The OD_{600nm} of each culture of *B. hyodysenteriae* P8544 ( ) and P7455 ( ) were measured after 48 hrs. Ten ml cultures of BHI containing 5% FCS and the stated concentration of dipyridyl were incubated at 37°C under anaerobic conditions using the Oxoid system with constant shaking at 120 rpm. The OD values of *B. hyodysenteriae* grown in iron-replete cultures (without dipyridyl) served as control. The figure presents the means and SD of six independent experiments on different days. The OD values obtained from both isolates under 0.12 mM dipyridyl were significantly lower (**; $P<0.01$) than the OD values obtained from cultures growing under 0.1 mM dipyridyl. No significant growth variations between P7455 and P8544, cultured in a medium containing dipyridyl in a range of 0.01 mM up to 0.1 mM as well as conventional conditions were detected ($P>0.05$).

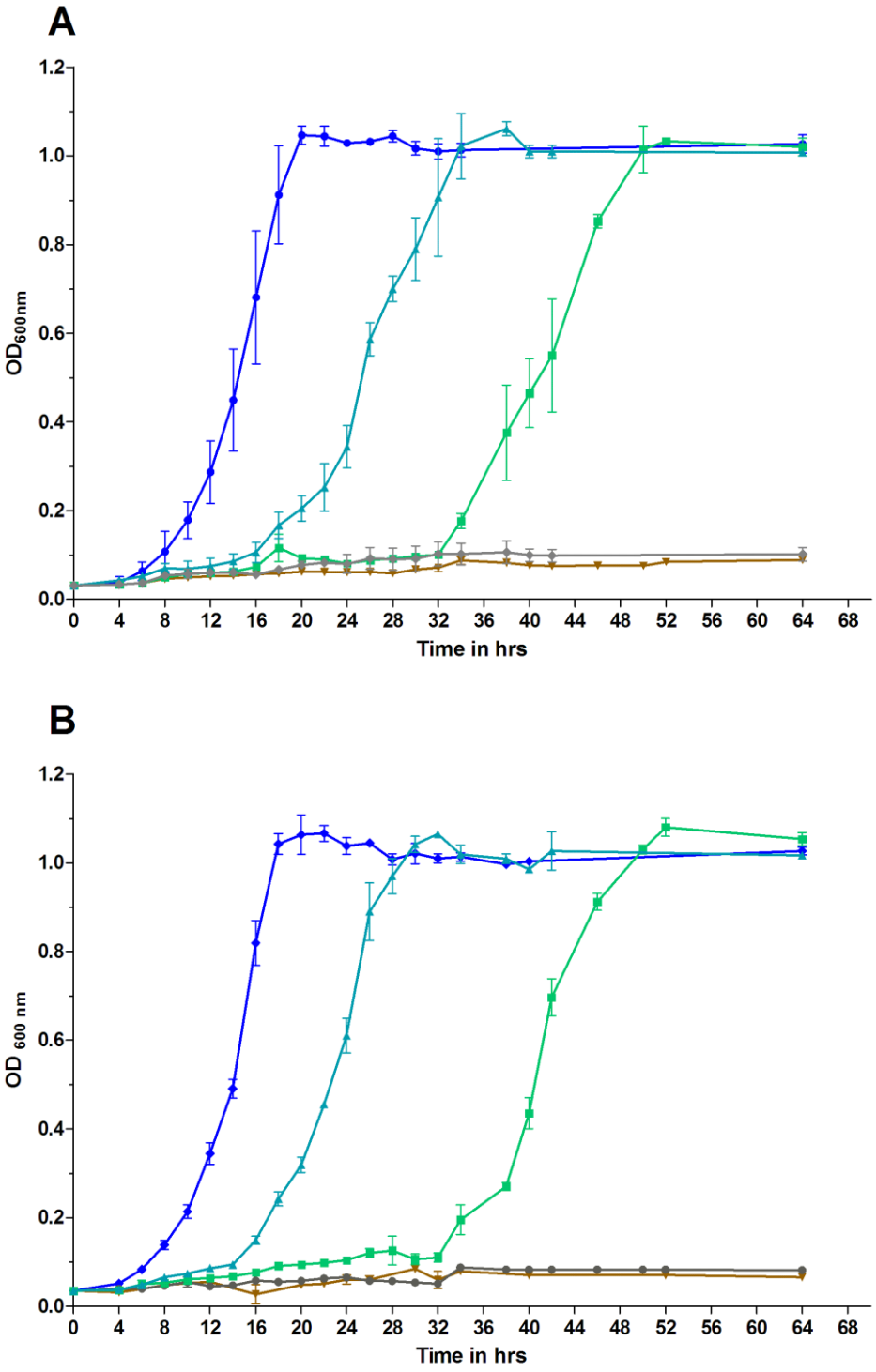


Figure 5.2: Growth of *B. hyodysenteriae* in conventional media supplemented with varying concentrations dipyridyl. *B. hyodysenteriae* 8544 (A) and 7455 (B) were cultured in BHI + 5% FCS (conventional growth medium) ( ), and conventional medium supplemented with 0.08 mM dipyridyl ( ), 0.1 mM dipyridyl ( ), 0.12 mM dipyridyl ( ), and 0.14 mM dipyridyl ( ). The OD_{600nm} was measured at for 64 hours. Cultures were incubated at 37°C with constant agitation in an anaerobic workstation. The data represent the mean of 3 separate experiments carried out on different days.

dipyridyl. With an increasing concentration of the iron chelator, the lag phase of cultures was extended. While the mid-log phase (OD_{600nm}=0.6) for *B. hyodysenteriae* P8544 under standard conditions was reached after approximately 17 hrs, the mid-log phase for BHI supplemented with 0.08 mM dipyridyl was reached after 26 hrs and 42 hrs for a concentration of 0.1 mM of dipyridyl. A similar growth profile was determined for *B. hyodysenteriae* P7455 which reached mid-log phase after 15 hrs under iron-replete conditions, after 24 hrs grown in 0.08 mM of dipyridyl and 41 hrs in a concentration of 0.1 mM dipyridyl. The greater growth of P8544 observed with 0.1 mM dipyridyl in the previous experiment might have been due to the fact that cells were still in late log-phase after 48 hrs whereas cells growing under standard conditions reached the stationary phase approximately 24 hrs earlier resulting in decreased cell size or indeed cell death and therefore slightly lower OD values in the isolate P8544. In addition, the experiments presented in **Figure 5.1** and **Figure 5.2** were carried out using two different systems. At the time the titration experiment was performed, no anaerobic cabinet was available which could have affected the growth behaviour and therefore the resulting OD values.

From this work, an iron-limited medium was defined as BHI supplemented with 5% FCS and 0.1 mM dipyridyl (final concentration) and was used for further iron-related studies; this was the highest concentration of dipyridyl which still yielded sufficient growth while being likely to exert an effect on the expression of iron-dependent genes.

In a further experiment, the isolates P8544 and P7455 were grown under iron-replete and iron-limited conditions supplemented with 10 µM and 50 µM of ferric chloride $FeCl_3$ (final concentration) to determine if the addition of iron improved the growth of *B. hyodysenteriae*. The outcome on growth was assessed (**Figure 5.3**); the addition of 10 µM and 50 µM $FeCl_3$ enhanced the growth of P8544 and P7455 under standard growth conditions (BHI containing 5% FCS) and also iron-limited conditions. The extended lag phase which was observed under iron-restricted conditions using 0.1 mM dipyridyl was drastically reduced by the addition of 10 µM and 50 µM $FeCl_3$. Apparently, with increasing $FeCl_3$ concentration under iron-restricted conditions, there was a quicker doubling time which led to the fact that *B. hyodysenteriae* was able to reach mid-log phase between 8 to 24 hrs earlier (depending on the $FeCl_3$ concentration and isolate) than under iron-limited conditions.

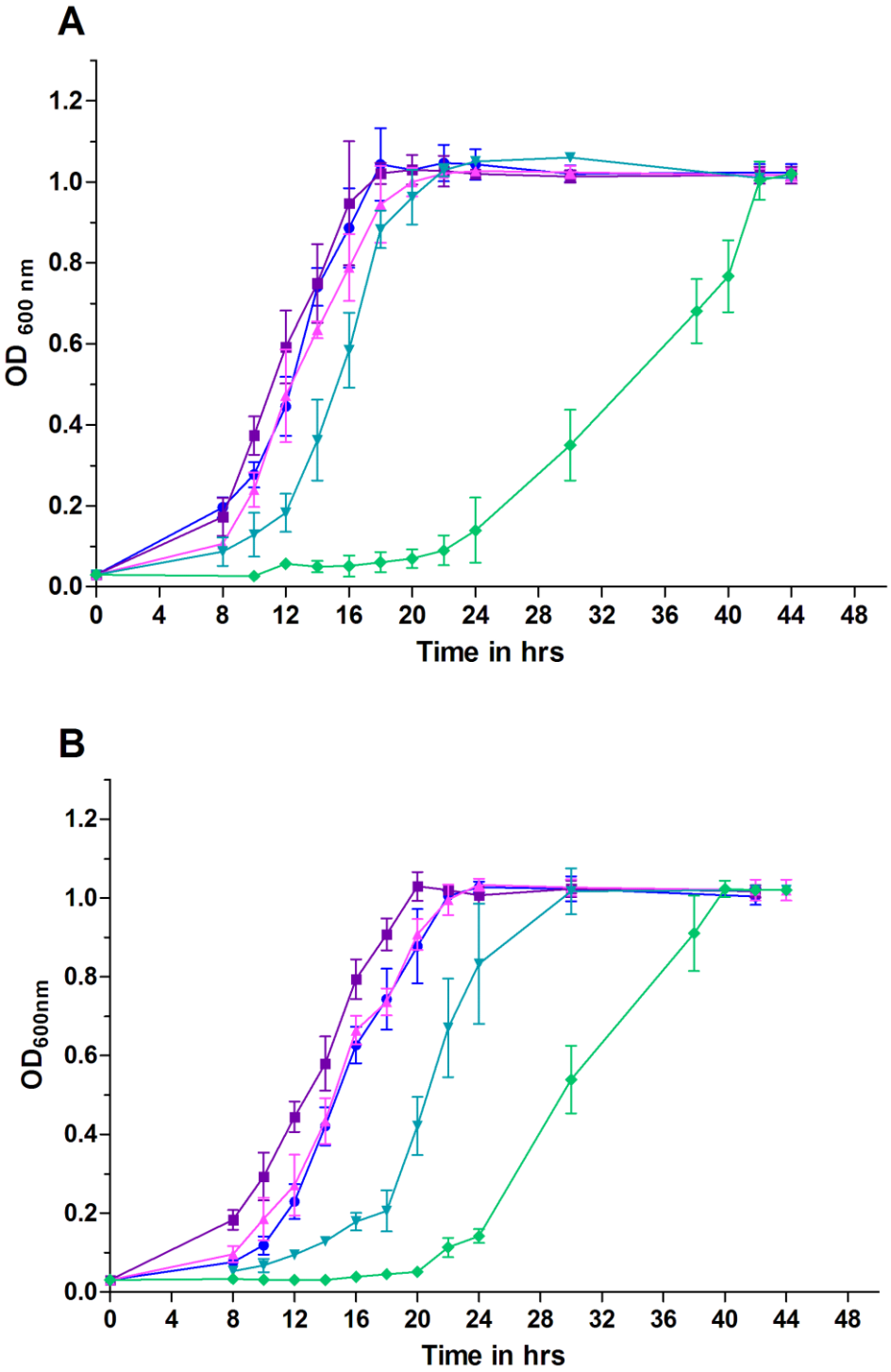


Figure 5.3: Growth of *B. hyodysenteriae* in conventional media with varying concentrations of iron chloride ($FeCl_3$). *B. hyodysenteriae* 8544 (A) and 7455 (B) were cultured in BHI + 5% FCS (conventional medium) (◆), conventional medium + 50 µM $FeCl_3$ (■), conventional medium + 0.1 mM dipyridyl + 50 µM $FeCl_3$ (▲), conventional medium + 0.1 mM dipyridyl + 10 µM $FeCl_3$ (▼), and conventional medium + 0.1 mM dipyridyl (◆). The OD_{600nm} was measured for 44 hours. Cultures were grown at 37°C with constant agitation in an anaerobic workstation. The data represents the mean of 3 separate experiments on different days.

Therefore, the data suggests that for the virulent as well as the avirulent *B. hyodysenteriae* isolate iron was required for growth. In addition, supplementation with 50 µM $FeCl_3$ enhanced the growth of both *B. hyodysenteriae* isolates slightly under standard conditions indicating that the iron content in BHI supplemented with 5% FCS is not optimal for *B. hyodysenteriae* growth.

5.2.2 *B. hyodysenteriae* acquisition of iron from host iron-binding proteins

In order to determine whether *B. hyodysenteriae* was able to acquire iron from the host-iron-binding protein lactoferrin (Lf), 0.5 mg/ml of Lf was added to aliquots of BHI, with and without a final concentration of 0.1 mM of dipyridyl. It should be noted that the Lf used in this experiment, which was purchased from Sigma-Aldrich, was obtained from bovine colostrum (bLf), containing 0.1% (wt %) of iron (personal communication with the Sigma-Aldrich Technical service). The iron-depleted form of Lf, called apolactoferrin, of bovine colostrum was not available for purchase in the UK.

The results indicated that 0.5 mg/ml of Lf enhanced the growth of *B. hyodysenteriae* P8544 and P7455 under iron-limited and iron-replete conditions *in vitro* (**Figure 5.4**). Cells grown under iron-restricted conditions reached mid-log phase (OD_{600nm} of 0.6) after approximately 36 hrs, whereby the growth of P8544 and P7455 was notably accelerated when 0.5 mg/ml Lf was added, resulting in mid-log phase being reached about 6 hrs earlier than seen in the iron-restricted medium. Thus, the data suggest that *B. hyodysenteriae* may be able to use Lf-bound-iron for growth.

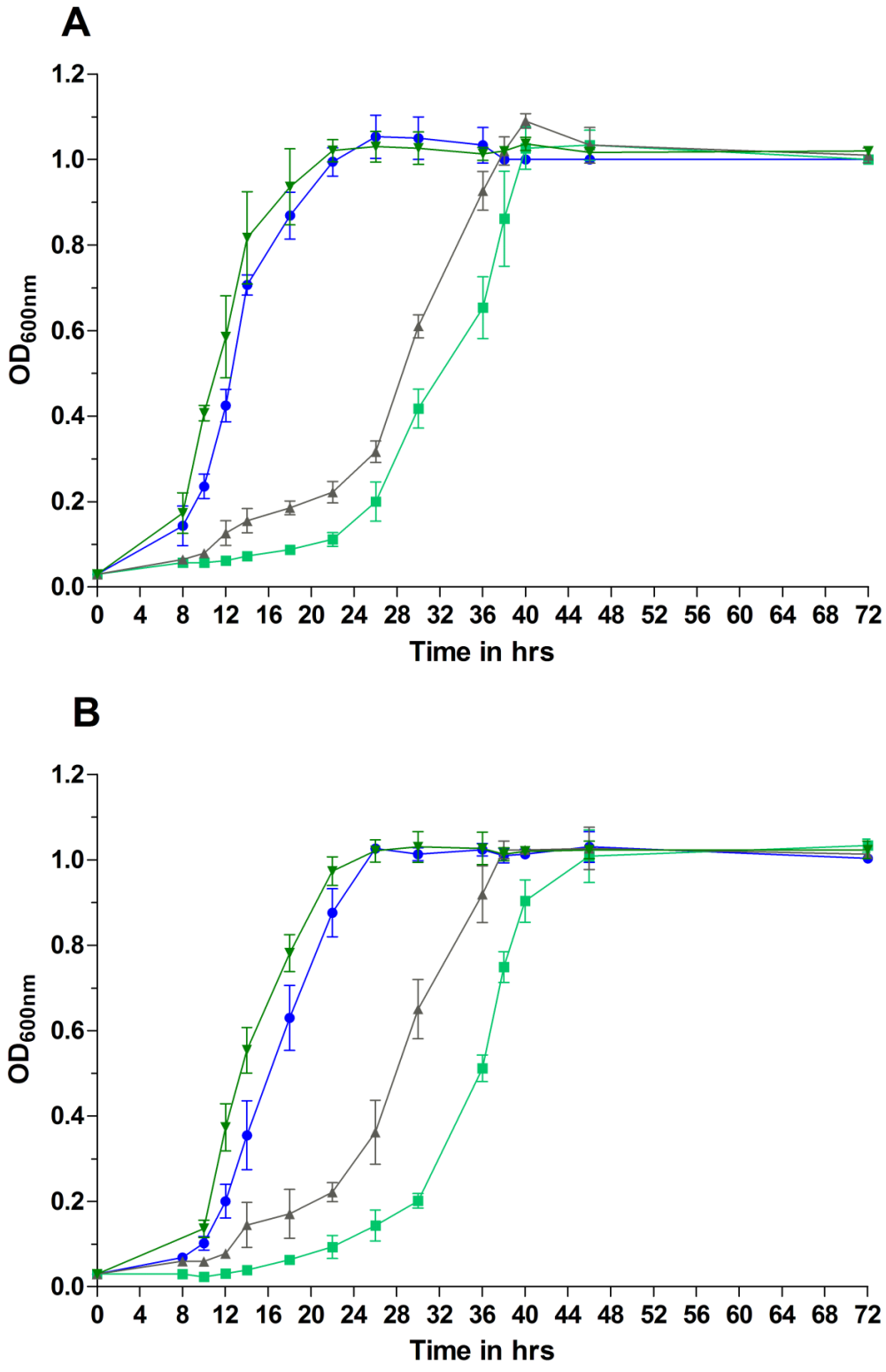


Figure 5.4: Growth curves of *B. hyodysenteriae* P8544 (A) and P7455 (B) in iron-replete and iron-restricted conditions supplemented with and without lactoferrin *B. hyodysenteriae* P8544 (A) and P7455 were cultured in conventional medium (BHI+5% FCS) ( ), conventional medium supplemented with 0.5 mg/ml lactoferrin ( ), conventional medium supplemented with 0.14 mM dipyridyl and 0.5 mg/ml lactoferrin ( ), and conventional medium supplemented with 0.14 mM dipyridyl ( ).The OD_{600nm} was measured for 72 hours; the data represents the average of three separate experiments conducted on different occasions.

Due to the possibility that *B. hyodysenteriae* can use Lf as an iron source, a preliminary experiment was carried out with *B. hyodysenteriae* P8544 growing in BHI containing 0.12 mM of dipyridyl and supplemented with 0.5 mg/ml Lf (**Figure 5.5**); this concentration of Lf being used because it induced a complete cessation of *B. hyodysenteriae* P8544 and P7455 growth in a previous experiment (**Figure 5.1** and **Figure 5.2**). While 0.5 mg/ml of Lf slightly enhanced the growth rate of *B. hyodysenteriae* P8544 under conventional conditions as seen in **Figure 5.4**, the presence of Lf did not restore growth of P8544 when grown in 0.12 mM dipyridyl.

However, the results need to be taken with care, as Lf was partially saturated with iron. Therefore it remains unclear whether the growth was stimulated due to the existence of a specific lactoferrin iron-uptake system in *B. hyodysenteriae* or was dependent on the saturation level of Lf.

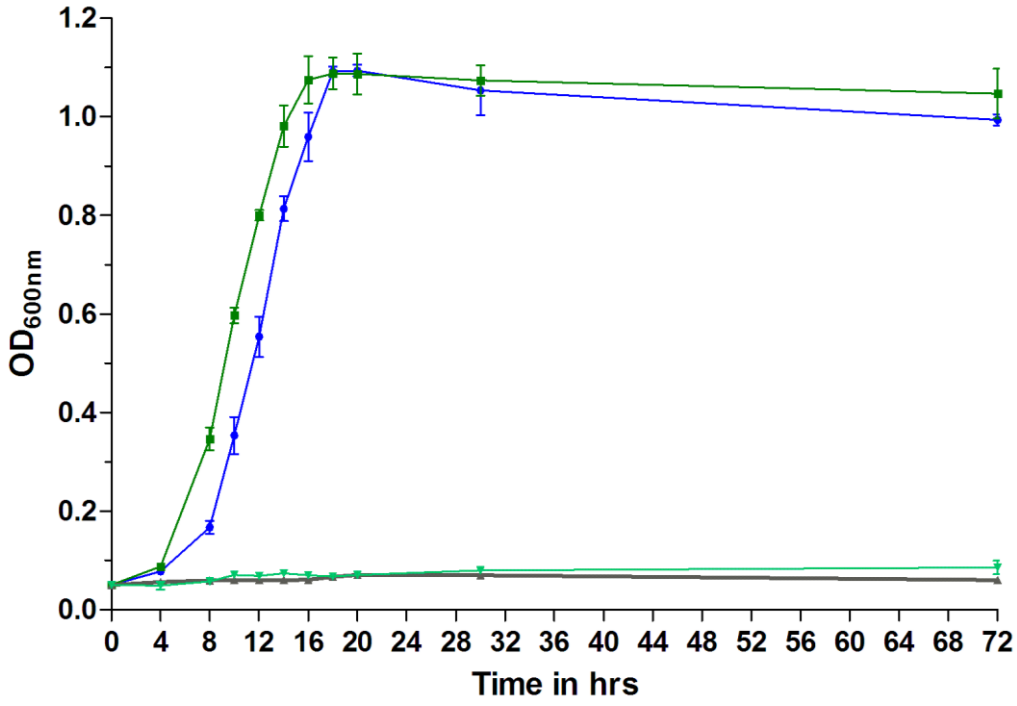


Figure 5.5: Growth curve of B. *hyodysenteriae* P8544 under conventional and iron-restricted conditions with and without 0.5 mg/ml lactoferrin. *B. hyodysenteriae* P8544 was cultured in conventional medium (BHI + 5% FCS) ( ), conventional medium + 0.5 mg/ml lactoferrin, ( ), conventional medium + 0.12 mM dipyridyl + 0.5 mg/ml lactoferrin ( ), and conventional medium + 0.12 mM dipyridyl ( ). The OD_{600nm} was recorded for 72 hours, and the data represents the average of 3 separate experiments conducted on different days.

5.2.3 Detection of lactoferrin-binding proteins

To-date, no specific uptake-system for Lf-bound iron has been described in *B. hyodysenteriae*. Due to the observed growth promotion of virulent and avirulent *B. hyodysenteriae* P8544 and P7455 following addition of 0.5 mg/ml of Lf to the conventional and iron-restricted media, a lactoferrin-binding assay was performed in order to identify possible Lf-binding proteins. Surface-exposed receptors specific for lactoferrin have been identified in many pathogenic bacteria under iron-starvation (Dhaenens *et al.*, 1997; Menozzi *et al.*, 1991). Therefore the detergent phase of OM proteins extracted from cultures grown under iron-replete and iron-restricted conditions (**Chapter 6**) of both *B. hyodysenteriae* isolates were probed with lactoferrin. Blotting was performed twice and results were identical between experiments (**Figure 5.6**).

The results indicated that Lf binds non-specifically to OM proteins expressed under iron-replete and iron-restricted conditions in the virulent and avirulent isolate of *B. hyodysenteriae*. Based on a simple observation of band patterns, the proteins reacting with Lf appeared to be very similar between isolates and conditions. However, two bands of around 51-54 kDa appeared only under iron-limited conditions, labelled as "b_1" in the avirulent isolate P7455 and "b_2" in the virulent isolate P8544 in (**Figure 5.6**). Moreover another protein band approximately 80-85 kDa in size was only present under iron-replete conditions in P8544, labelled as "a" in **Figure 5.6**. Additionally, protein patterns under both conditions were detected in the OM of P7455 ranging from 41-43 kDa (d) and 45-48 kDa in P8544 (c).

Extracted OM proteins of *B. hyodysenteriae* P7455 and P8544 expressed under iron-replete and iron-restricted conditions were analysed by LC-ESI-MS/MS. These results will be discussed in **Chapter 6**. In order to identify possible Lf-binding proteins, the list of proteins to be discussed in **Chapter 6** was analysed in an attempt to provide a possible identity for those proteins determined to be of interest by Western blot. Due to likely variations in the protein gel and corresponding marker size, LC-ESE-MS/MS identified proteins which were of the approximate size observed by Western blot were appraised, and 43 proteins that may be involved in Lf-binding were selected via process of elimination; however, only 13 of these proteins matched the set criteria to be short-listed and are possibly involved in Lf-binding (**Table 5.1**).

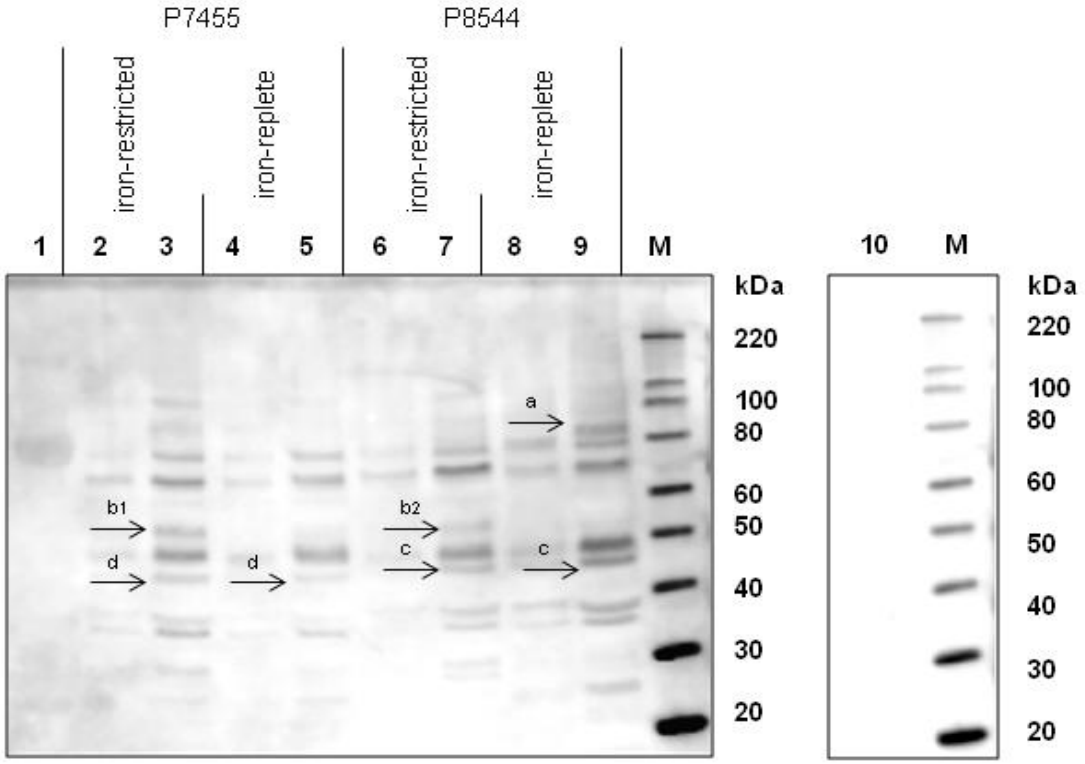


Figure 5.6: Western blot analysis of putative lactoferrin-binding proteins from the outer-membrane of *B. hyodysenteriae*. Approximately 0.6 mg/ml and an additional 1:10 dilution of each sample (2, 4, 6 and 8) of the detergent phase of OMP of *B. hyodysenteriae* P7455 and P8544, grown under iron-restricted and iron-replete conditions, were separated by SDS-PAGE and transferred onto nirocellulose membrane. The membrane was probed with 25 µg/ml colostrum-derived bovine lactoferrin. Lactoferrin-binding proteins were detected by far Western blot technique with anti-lactoferrin rabbit polyclonal antibody and subsequently detected with sheep anti rabbit IgG HRP. Moreover 0.6 mg/ml lactoferin (1) was inculded as a positive control. Lane (M) shows the Magic Marker™ XP Western Protein Standard. 10 served as a negative control with the secondary sheep anti rabbit IgG HRP antibody only. The labelled bands a, $b_{1,2,}$ c and d were identified through the analysis LC-ESI-MS/MS in chapter 6 and further shorted-listed to identify possible lactoferrin-binding proteins (Table 5.1). The blot is representative of two indepent experiments.

Table 5.1: Detection of possible lactoferrin-binding of proteins in the OM of *B. hyodysenteriae*. Proteins matching similar size ranges as observed in Western-blot analysis were filtered from the OM data set of *B. hyodysenteriae* P8544 and P7455 in Chapter 6. Proteins expressed under the conditions corresponding to the Western-blot analysis and size range were highlighted in green and are considered to be possible Lf-binding proteins.

Locustaq	Protein	kDa	Score[1]	Score	Label[2]	Growth condition	
			Iron-replete	Iron-restricted		iron-replete	iron-restricted
Bhyov8544_2636	**hypothetical protein**	**85.6**	**272.8**	**-**	**a**	**+**	**-**
Bhyov8544_0631	porA pyruvate ferredoxin reductase	82.9		287.3	a	+	+
Bhyoa7455_0064	**TPR domain-containing protein**	**52.5**	**621.5**	**-**	**b1**	**-**	**+**
Bhyoa7455_0301	response regulatory protein (atoC)	51.1	103.1	-	b1	+	-
Bhyoa7455_0825	hypothetical protein	51.2	593.1	681.9	b1	+	+
Bhyoa7455_0232	hypothetical protein	51.4	1293.4	-	b1	+	-
Bhyoa7455_1163	extracellular solute-binding protein, family 5	51.8	108.7	-	b1	+	-
Bhyoa7455_0064	TPR domain-containing protein	52.5	891.3	-	b1	+	-
Bhyoa7455_0338	TPR domain-containing protein	53.6	1072.1	916.2	b1	+	+
Bhyoa7455_1340	glutamyl-tRNA(Gln) amidotransferase, B subunit	54.5	89.6	-	b1	+	-
Bhyov8544_2195	**outer membrane protein**	**52.7**	**-**	**136.9**	**b2**	**-**	**+**
Bhyov8544_0523	**TPR domain-containing protein**	**53.6**	**-**	**1144.7**	**b2**	**-**	**+**
Bhyov8544_1067	glutamate synthase (DPH)	51.0	167.5	-	b2	+	-
Bhyov8544_1057	hypothetical protein	51.2	565.2	698.7	b2	+	+
Bhyov8544_0417	hypothetical protein	51.3	1473.0	150.5	b2	+	+
Bhyov8544_1661	lmp1 TPR domain-containing protein	52.5	1027.3	956.4	b2	+	+
Bhyov8544_1278	gatA glutamyl-tRNA(Gln) amidotransferase, A subunit	52.7	156.9	-	b2	+	-
Bhyov8544_2483	undefined product	53.4	75.4	91.2	b2	+	+
Bhyov8544_0523	TPR domain-containing protein	53.6	862.6	368.5	b2	+	+
Bhyov8544_2078	**argH argininosuccinate lyase**	**45.6**	**333.0**	**369.5**	**c**	**+**	**+**
Bhyov8544_1150	**rnfC electron transport complex protein**	**46.8**	**1266.2**	**1631.4**	**c**	**+**	**+**
Bhyov8544_0584	**eno Phosphopyruvate hydratase**	**47.0**	**793.4**	**515.4**	**c**	**+**	**+**
Bhyov8544_1649	**transporter, MFS superfamily [Brachyspira pilosicoli 95/1000_0041]**	**47.0**	**136.7**	**103.9**	**c**	**+**	**+**
Bhyov8544_0837	**pep peptidase**	**47.1**	**110.0**	**117.6**	**c**	**+**	**+**
Bhyov8544_1130	**integral membrane transport protein**	**47.7**	**196.6**	**198.7**	**c**	**+**	**+**
Bhyov8544_2348	hypothetical protein	47.8	170.0	-	c	+	-
Bhyov8544_0378	Na+-transporting methylmalonyl-CoA/oxaloacetate decarboxylase, beta subunit	48.2	157.2	-	c	+	-
Bhyov8544_0610	atpB V-type ATP synthase subunit B	48.4	184.1	-	c	+	-
Bhyov8544_0924	preprotein translocase subunit SecY	49.2	177.9	-	c	+	-
Bhyov8544_2196	mtrC membrane fusion protein	45.0	-	424.3	c	-	+
Bhyov8544_0143	potD spermidine/putrescine transport ATP-binding protein	45.3	-	915.1	c	-	+
Bhyov8544_0150	variable surface protein VspI	47.0	-	246.7	c	-	+
Bhyov8544_0378	Na+-transporting methylmalonyl-CoA/oxaloacetate decarboxylase, beta subunit	48.2	-	154.4	c	-	+
Bhyov8544_1894	aspC aspartate aminotransferase	45.8	307.7	-	c	+	-
Bhyoa7455_0115	**putative dynein heavy chain**	**42.4**	**968.9**	**795.5**	**d**	**+**	**+**
Bhyoa7455_2111	**ankyrin repeat-containing protein**	**42.6**	**294.8**	**87.5**	**d**	**+**	**+**
Bhyoa7455_1252	**Cysteine suLfinate desuLfinase/cysteine desuLfurase-like enzyme**	**42.9**	**76.0**	**219.9**	**d**	**+**	**+**
Bhyoa7455_1560	hypothetical protein	43.1	221.3	-	d	+	-
Bhyoa7455_0442	hypothetical protein	41.7	-	199.6	d	-	+
Bhyoa7455_0408	hypothetical protein	41.0	296.3	-	d	+	-
Bhyoa7455_1662	iron-containing alcohol dehydrogenase	42.0	96.4	-	d	+	-
Bhyoa7455_1642	putative lipid A disaccharide synthase; LpxB	42.5	66.6	-	d	+	-

\+ protein was expressed under these growth conditions – protein was not identified under these growth conditions

[1] highest scores detected within replictates [2] selected proteins identified via Western Blot in Figure 5.6

One possible candidate for Lf-binding protein was the hypothetical protein (Bhyov8544_2636), which was only expressed under iron-replete growth conditions in the OM of P8544 (a). The data set in **Chapter 6** also confirmed that the hypothetical protein was not expressed under iron-restricted conditions in P8544 and was neither expressed in iron-replete and iron-restricted conditions in the isolate P7455. However, the gene encoding this hypothetical protein was found to be present in the genome of avirulent P7455 (Bhyoa7455_2584). InterProScan analysis of the hypothetical protein (Bhyov8544_2636) did not reveal any specific function.

The proteins between 51-54 kDa in P8544 (b2) and P7455 (b1), present under both tested growth conditions, were predicted to contain an outer-membrane protein (Bhyov8544_2195) as well as TPR domain-containing protein (Bhyov8544_0523) in P8544 corresponding to the set criteria whereby only one protein in P7455 could be identified as a TPR domain-containing protein (Bhyoa7455_0064). The two detected TPR domain-containing proteins of P8544 and P7455 shared similar size but only 20% amino acid sequence identity with each other; thus, indicating that two different TPR domain proteins were expressed in both isolates under iron-restricted conditions. Nevertheless, the genes encoding the TPR domain-containing protein and the outer-membrane protein reacting with LF in P8544 were identified within the genome of P7455 (Bhyoa7455_0338; Bhyoa7455_2261). Furthermore, the gene encoding the TPR domain-containing protein expressed in P7455 under iron-restricted conditions was also found to be present in the genome of the virulent isolate P8544 (Bhyov8544_1661), suggesting that the different expressed TPR domain-containing protein in the two isolates are not due to the absence of the corresponding genes.

Moreover, six proteins identified as argininosuccinate lyase (argH), electron transport complex protein (rnfC), Phosphopyruvate hydratase (eno), transporter MFS superfamily, peptidase (pep), and one integral membrane transport protein were short-listed as possibly being involved in lactoferrin-binding in P8544 under iron-replete and iron-restricted conditions (c). The data in **Chapter 6** revealed that 5 out of these 6 proteins, except for the transporter, MFS superfamily, were also expressed in iron-replete and and iron restricted conditions in the avirulent isolate P7455 (Bhyoa7455_2347; Bhyoa7455_1328; Bhyoa7455_1522; Bhyoa7455_0941 and Bhyoa7455_1308). The unique gene (**Chapter 4**) shared the closest similarity with the transporter, MFS superfamily from *B. pilsocoli* 95-1000-0041 (Bhyov8544_1649) and was also identified in the genome of P7455

(Bhyoa7455_0076) but was not found to be expressed in any OM samples analysed in **Chapter 6**.

Moreover, three proteins annotated as putative dynein heavy chain, ankyrin repeat-containing protein and cysteine sulfinate desulfinase/cysteine desulfurase-like enzyme (NifS) were identified as putatively playing a role in Lf-binding in P7455 under both growth conditions (d). Interestingly, the protein putative dynein heavy chain was also present in the OM profile of *B. hyodysenteriae* P8544 in the two tested conditions, whereby cysteine sulfinate desulfinase/cysteine desulfurase-like enzyme was expressed only in an iron-limited environment in P8544 (**Chapter 6)**.

Only three proteins in total were detected under iron-restricted conditions in both *B. hyodysenteriae* isolates to possess possible Lf-binding activity. The virulent isolate P8544 expressed two proteins: TPR domain-containing protein (Bhyov8544_2195) and an outer-membrane protein (Bhyov8544_0532) while the avirulent isolate expressed only one TPR domain-containing protein (Bhyoa7455_0064). As described earlier the two expressed TPR domain-containing proteins seemed to differ. The expressed outer-membrane proteins in P8544 comprise a signal peptide and an OM efflux protein family region (IPR003423) which is widely distributed among Gram-negative bacteria and known to be involved in the export of various substrates.

5.2.4 Assessment of siderophore production and uptake

The chrome azurol S (CAS) assay (Schwyn & Neilands, 1987) demonstrates siderophore production by bacteria. A colour change in the medium corresponds to iron being liberated from the ferric iron complex of the CAS indicator dye by bacterial siderophores which have a higher affinity for the complexed iron. Siderophore production was initially examined in *B. hyodysenteriae* WA1, P8544, P7455, QCR1, P8226/7 and P7377/3. After incubation at 37°C for 2 hrs distinctive yellow ‘halos’ were seen around the colonies of all tested strains, suggesting siderophore production (**Figure 5.7**). However, yellow halos could be observed under iron-replete (data not shown) and iron-restricted conditions indicating that that possible siderophore production took place under both tested conditions. Additionally control plates which did not contain any *B. hyodysenteriae* isolates were overlaid with the CAS agar and the blue indicator dye faded completely within 4-6 hrs completely.

Since many pathogenic bacteria have been shown to use exogenously-produced siderophores (xenosiderophores) it was tested if *B. hyodysenteriae* is also able to use siderophores secreted by other microorganisms. Thus, conventional and iron-restricted media were supplemented with 20 µM ferrichrome and 20 µM enterobactin and the growth characteristics of *B. hyodysenteriae* P8544 and P7455 were monitored (**Figure 5.8**). The data indicated that *B. hyodysenteriae* P8544 and P7455 were not able to utilize the siderophores ferrichrome and enterobactin as no growth could be observed when cells were grown in BHI supplemented with 0.1 mM dipyridyl and 20 µM of siderophores. Besides the growth rate of both *B. hyodysenteriae* isolates was slightly compromised in the presence of 20 µM ferrichrome and enterobactin under iron-replete conditions, signifying that the iron level in the medium was reduced by chelation of iron by the siderophores. Thus, the data indicates that *B. hyodysenteriae* is not able to acquire iron when bound to ferrichrome or enterobactin.

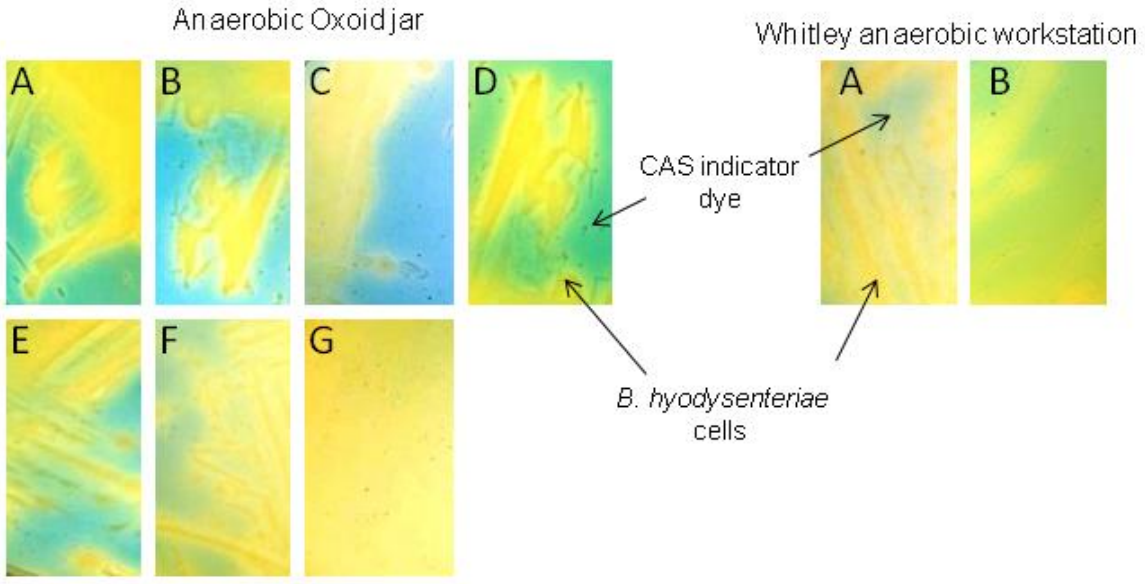


Figure 5.7: Investigation of siderophore production of *B. hyodysenteriae* using the Chrome azoroul CAS agar plate assay. The isolates P8544 (A), 8266/7 (B) 7455 (C), 7377/3 (D), WA1 (E), QCR1 (F) and a control plate (without any bacteria on it) (G) were grown on BHI agar plates supplemented with 5% FCS and 0.1 mM of dipyridyl under anaerobic conditions until growth was evident. Following the plates were overlaid with 20 ml of CAS agar and incubated for 2 days under anaerobic conditions. The yellow colour around the colonies may be indicative of siderophore production by *B. hyodysenteriae*, and requires further investigation.

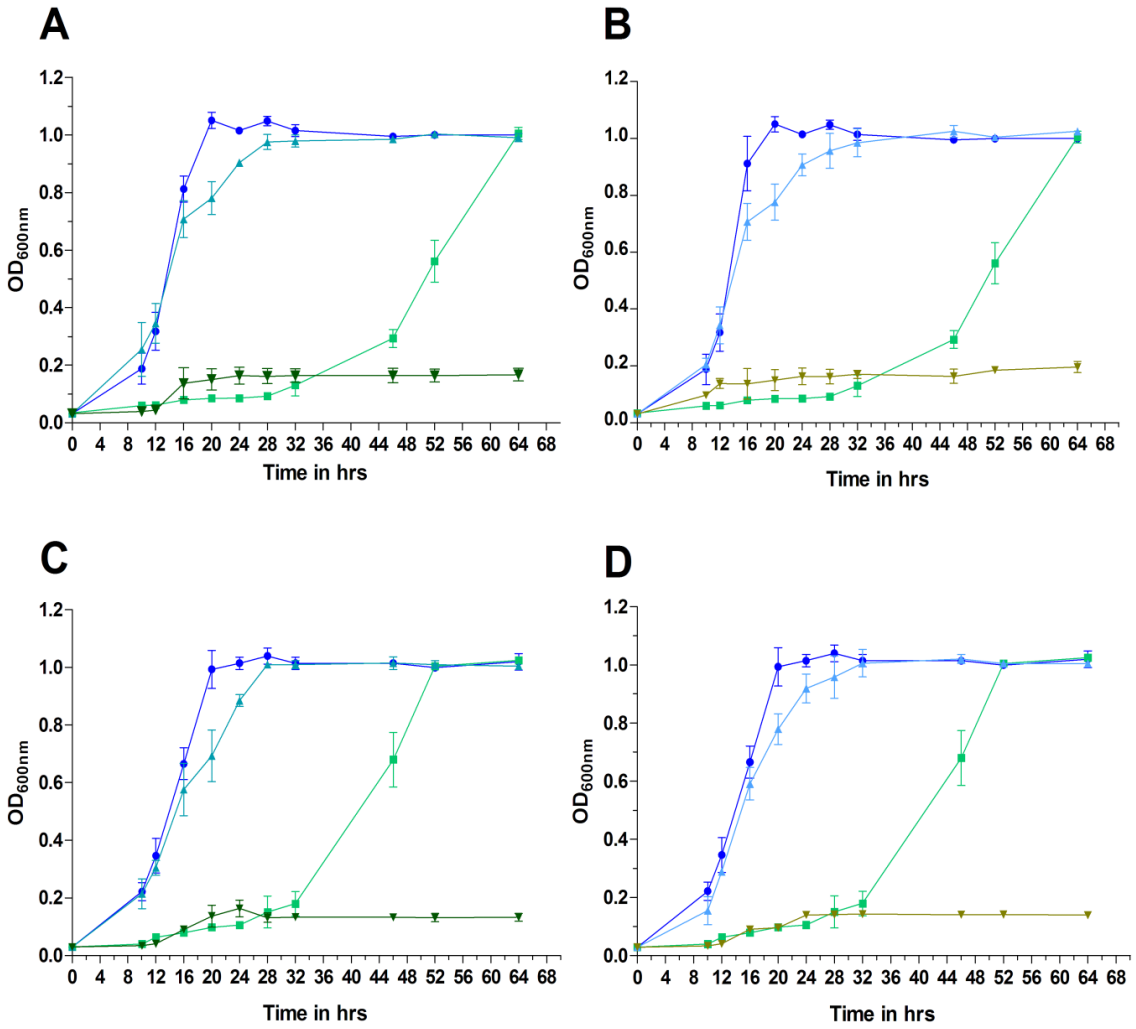


Figure 5.8: Assessment of xenosiderophore utilisation by *B. hyodysenteriae* P8544 and P7455. *B. hyodysenteriae* P8544 (A+B) and P7455 (C+D) were grown under iron-replete conditions (◆), iron-restricted conditions (■), iron-replete conditions supplemented with 20 µM Ferrichrome (▲), iron-restricted conditions supplemented with 20 µM Ferrichrome (▼), iron-replete conditions supplemented with 20 µM Enterobactin (▲) and iron-restricted conditions supplemented with 20 µM Enterobactin (▼). The OD_{600nm} was recorded for 64 hours. Data represent the mean of two separate experiments and SD.

5.2.5 Genes involved in iron-uptake in *B. hyodysenteriae*

Due to the possible ability to produce siderophores, as determined by the CAS agar plate assay (**Figure 5.7**) as well as the adaptation to iron-limited conditions **(Figure 5.2)**, the genomes of *B. hyodysentriae* WA1, P8544 and P7455 were screened for the presence of genes likely to encode proteins involved in iron-acquisition.

A total of 23 genes were suggested to play a putative role in iron-acquisition in *B. hyodysenteriae* (**Table 5.2**). The listed genes were present in all of the three analyzed genomes. The identified genes mostly encoded ABC type transporters. Even though genes encoding siderophore biosynthesis proteins could not be identified in the three genomes of *B. hyodysenteriae* a couple of listed ABC-transporters seemed to share similarity with other known iron-uptake systems coupled with ABC-transport mechanisms in different pathogenic bacteria. The gene (BHWA1_00535), encoding an iron-compound ABC transporter, ATP-binding protein shared 60 % amino acid identity (score 306; e-value 1e-101) with the ferrichrome transport ATP-binding protein, FhuC, in *B. piloscoli* WesB and 45% identity with the ferrichrome transport ATP-binding protein FhuC in *Clostridium botulinum* B str. Eklund 17B. The BIT-system described earlier has been shown to be unique to *B. hyodysenteriae*, and the presence of the complete ABC transporter cassette consisting of four copies of *bitB* and one copy of each *bitC*, *bitD* and *bitE* gene was found in P8544 and P7455 (**Chapter 4**).

The three *B. hyodysenteriae* genome sequences contained a single known ferrous uptake gene, *feoB*, whose amino acid sequence shares 27% identify with FeoB of *E. coli* which has been shown to play an important role in iron-acquisition under anaerobic conditions in several pathogenic microorganisms. However, compared to *E. coli* no *feoA* and *feoC* gene homologues could be identified in any of the *B. hyodysenteriae* genome sequences. Despite this, the FeoB protein sequences of *B. hyodysenteriae* are homologous with each other and, at 829 aa, longer than that of *E. coli* (773 aa). Interestingly, BLASTp of the FeoB sequence of all three *B. hyodysenteriae* isolates revealed that the sequence contains conserved domains of FeoA and FeoB, suggesting that a previous operon was probably merged together to create one gene termed *feoB*.

Table 5.2: Genes associated with iron-acquisition in *B. hyodysenteriae* WA1, P8544 and P7455.

locus tag for WA1	Gene	Product	locus tag 7455	locus tag 8544
BHWA1_00040		**Metal binding protein**, function in the ABC transport of ferric siderophores and metal ions such as Mn2+, Fe3+, Cu2+ and/or Zn2+.	Bhyoa7455_1934	Bhyov8544_1172
BHWA1_00042		**ABC-type Mn/Zn transport system,** involved in the uptake of siderophores, haem, vitamin B12, or the divalent cations Mg2+ and Zn2	Bhyoa7455_1932	Bhyov8544_1170
BHWA1_00175		**lipoprotein releasing system, ATP-binding protein**	Bhyoa7455_1010	Bhyov8544_1490
BHWA1_00176	*†	**ABC transporter, transmembrane region** is a mitochondrial ATP-binding cassette protein involved in iron homeostasis and one of four ABC transporters expressed in the mitochondrial inner membrane	Bhyoa7455_1009	Bhyov8544_1489
BHWA1_00194		**hypothetical ABC transporter ATP-binding protein**	Bhyoa7455_2490	Bhyov8544_2575
BHWA1_00200		**ABC-type transport system, ATP-binding component,** ABC (ATP-binding cassette) transport system involved in resistant to organic solvents	Bhyoa7455_2484	Bhyov8544_2569
BHWA1_00535		**iron compound ABC transporter, ATP-binding protein,** ATP-binding protein, ABC-type cobalamin/Fe3+-siderophores transport systems, ATPase components [Inorganic ion transport and metabolism / Coenzyme metabolism	Bhyoa7455_0041	Bhyov8544_1603
BHWA1_00536		**ferric ion ABC transporter**, transporters involved in the uptake of siderophores, haem, vitamin B12, or the divalent cations Mg2+ and Zn2+	Bhyoa7455_0040	Bhyov8544_1602
BHWA1_00537		**iron compound ABC transporter, periplasmic iron compound-binding protein t**ransport of ferric siderophores and metal ions such as Mn2+, Fe3+, Cu2+ and/or Zn2+	Bhyoa7455_0039	Bhyov8544_1601
BHWA1_00859	*feoB**†	**ferrous iron transport protein B,** may make an important contribution to the iron supply of the cell under anaerobic conditions	Bhyoa7455_2574	Bhyov8544_2645
BHWA1_00866	*bitB**	**periplasmic-iron-binding protein BitB**	Bhyoa7455_2668	Bhyov8544_2672
BHWA1_00867	*bitA*†	**periplasmic-iron-binding protein BitA**	Bhyoa7455_2671	Bhyov8544_2671
BHWA1_00868	*bitB*†	**periplasmic-iron-binding protein BitB**	Bhyoa7455_2670	Bhyov8544_2669
BHWA1_00869	*bitC**	**periplasmic-iron-binding protein BitC**	Bhyoa7455_2675	Bhyov8544_2193
BHWA1_00870	*bitD*	**periplasmic-iron-binding protein BitD,** putative ABC transporter	Bhyoa7455_1764	Bhyov8544_2192
BHWA1_00871	*bitE*	**periplasmic-iron-binding protein BitE**	Bhyoa7455_1765	Bhyov8544_2191
BHWA1_00888		**ABC-3, ABC 3 transport family protein,** uptake of siderophores, haem, vitamin B12, or the divalent cations Mg2+ and Zn2+	Bhyoa7455_1782	Bhyov8544_2174
BHWA1_00891	*†	**periplasmic solute binding protein,** function in the ABC transport of ferric siderophores and metal ions such as Mn2+, Fe3+, Cu2+ and/or Zn2+	Bhyoa7455_1785	Bhyov8544_2171
BHWA1_00932		**putative iron depended repressor**	Bhyoa7455_1752	Bhyov8544_1909
BHWA1_01632	*modA*	**molybdenum ABC transporter, molybdate-binding protein**	Bhyoa7455_1623	Bhyov8544_1103
BHWA1_02299	*Fur*	**ferric uptake regulator,** typically iron-dependent, DNA-binding repressors and activators	Bhyoa7455_0610	Bhyov8544_0071
BHWA1_02586	*TonB*	**TonB-dependent-receptor ,** outer-membrane channel shares β-barrel structure	Bhyoa7455_1387	Bhyov8544_1358
BHWA1_02570	*bitB**†	**periplasmic-iron-binding protein BitB**	Bhyoa7455_1375	Bhyov8544_1373
BHWA1_02571	*bitB**†	**periplasmic-iron-binding protein BitB**	Bhyoa7455_1376	Bhyov8544_1372

Highlighted genes in blue were used for further qRT-PCR studies to assess their role in the iron-uptake.

*proteins detected by *B. hyodysenteria* P8544 in **Chapter 6**

†proteins detected in *B. hyodysenteriae* P7455 in **Chapter 6**

In addition, the analysis of the genomes also revealed the presence of one copy of genes encoding a TonB-ExbB-ExbD complex in the virulent and avirulent strains of *B. hyodysenteriae* which has been described in many Gram-negative bacteria to energize the uptake of large iron complexes, including Lf and ferric siderophores, across the outer-membrane into the cytoplasm.

The ferric iron uptake (Fur) protein, acting as transcriptional regulator controlling the expression of genes encoding proteins involved in iron storage and iron uptake, was present in all three genomes of *B. hyodysenteriae* isolates. The amino acid sequence of this protein was 100% identical in all three isolates, suggesting that iron metabolism in *B. hyodysenteriae* might be similarly regulated to that in other Gram-negative bacteria in which Fur has been studied.

Eight target genes (highlighted in blue) were chosen out of this panel to assess them further by qRT-PCR in order to identify genes which are transcribed when subjected to low iron conditions and thus play a role in pathogenicity.

5.2.6 Analysis of expression of *B. hyodysenteriae* putative iron-acquisition genes

5.2.6.1 *Selection of housekeeping genes*

In the first instance, the expression of the three housekeeping genes (HKG) 16S-RNA, *recA* and *gyrB* (encoding gyrase B) was studied in order to identify a HKG with minimal variability under the chosen experimental conditions. Total RNA of *B. hyodysenteriae* P8544 and *B. hyodysenteriae* P7455 was isolated under standard and iron-restricted growth conditions at an OD_{600nm} of 0.6. To give an overview of the relative abundance of the three candidate genes the cycle thresholds (Ct) values were determined for each HKG across the experimental sample sets of RNA isolated under iron-replete and iron-restricted growth conditions of both *B. hyodysenteriae* isolates. Vertical scatter plots were produced which all exhibited a wide range of mean Ct-values for all tested candidate reference genes under the chosen conditions and isolates ranging from 12.51 to 17.84 (
Figure 5.9). The Most abundant transcripts were 16 S rRNA, with an average Ct-value of 12.51, followed by *recA* with an average Ct-value of 16.47.

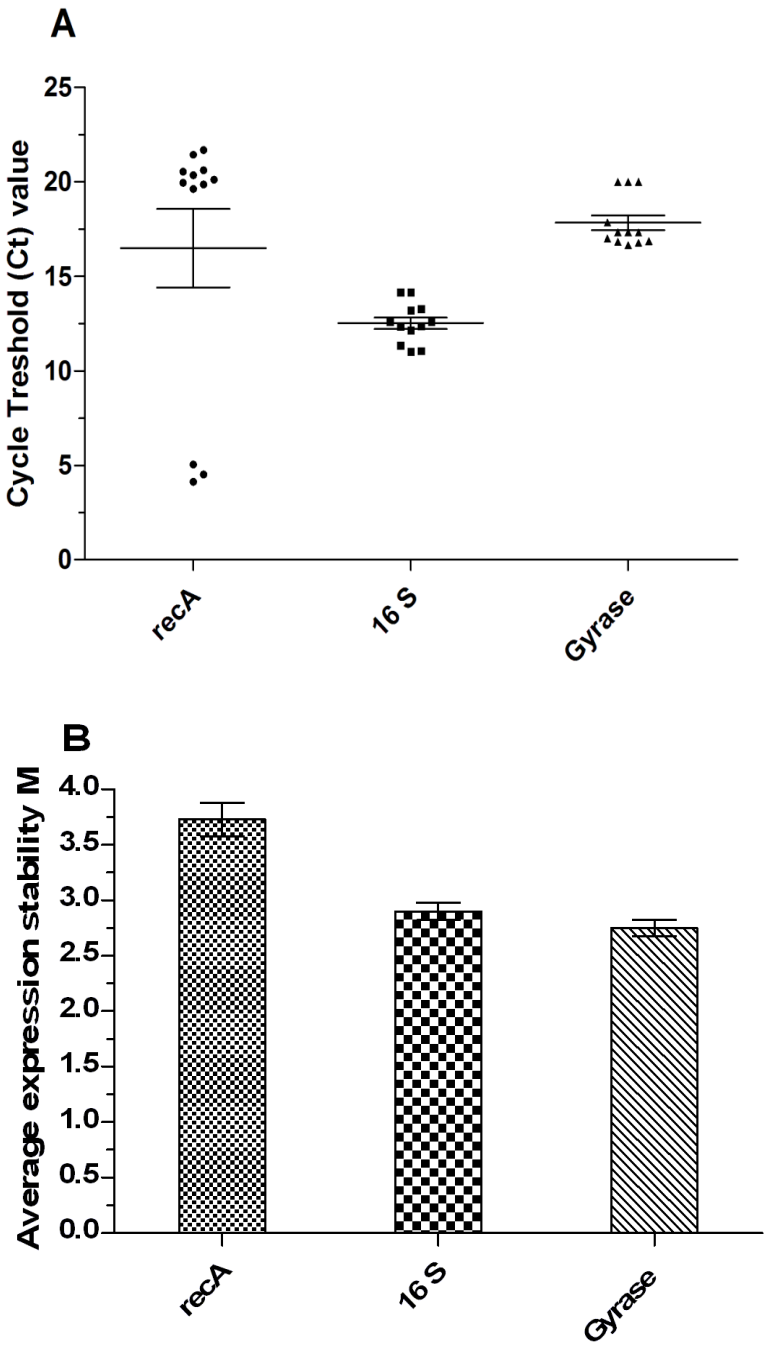


Figure 5.9: Evaluation of housekeeping genes. (A) Vertical scatter plot of the mean expression levels, presented as cycle threshold numbers (Ct values) of the candidate reference genes in iron-restricted and iron-limited RNA samples of *B. hyodysenteriae* P8544 and P7455 in triplicates. (B) Average expression stability values M of the candidate reference genes calculated by the geNorm^{Plus} software. The least stable gene is on the left with the highest predicted M-value, and the most stable gene is on the right with the lowest M-value predicted in the set of chosen housekeeping genes.

However, *recA* showed the most variation between iron-replete and iron-restricted samples with Ct-values ranging from 4.5 to 20.45. *B. hyodysenteriae* P7455 RNA from iron-restricted culture revealed noticeably lower Ct- values compared to RNA from iron-replete cultures, causing a huge variation across the sample set. Moreover, the lower Ct-values indicated a higher abundance of *recA* when cells of P7455 were subjected to a lower-iron environment. The least abundant transcript was *gyrB*, with an average Ct-value of 17.84.

In addition, expression stability (M) was determined for every chosen reference candidate gene using the software geNorm^{plus}. Genes with the lowest M value were considered the most stably expressed genes while a higher M values was an indicator of less stably expressed genes. According to the geNorm^{plus} software, *gyrB* was predicted as the most stable HKG in this experiment with an average M value of 2.75 and was thus used as the reference gene for further *q*RT-PCR studies.

5.2.6.2 *Efficiency for three housekeeping genes*

The efficiency of each primer pair for *recA*, *gyrB*, and 16S RNA (**Table 2.8**) was determined by pooling three RNA samples of each strain to accumulate all possibilities of the PCR kinetic. Therefore, a serial dilution up to 1:1000 of pooled RNA (40 ng), in triplicates, was performed which served as a template for qRT-PCR. The PCR efficiency was calculated according to the equation $E= 10^{-(1/slope)}$. All PCR assays displayed efficiency between 86 and 103 % and R^2 values ranged from 0.9715-0.9997 (**Table 5.3**).

5.2.6.3 *Quantitative real-time PCR of genes associated with iron-uptake*

Quantitative RT-PCR was performed in order to find out if the 8 chosen target genes putatively associated with iron-uptake in *B. hyodysenteriae* were differentially expressed under iron-limited conditions and thus may play a role in iron acquisition. The data presents the expression profiles of the target genes under iron-restricted conditions with regard to the expression profiles under iron-replete conditions (control) (**Figure 5.10**). Cells of *B. hyodysentyeriae* P8544 and P7455 were harvested from the same logarithmic growth phase (OD_{600nm}=0.6) in both media to ensure that sufficient RNA was generated and isolates were in comparable stages of growth.

Table 5.3: Determination of the efficiency of the target genes and housekeeping gene for qRT-PCR

Strain	Locus tag	R^2	Slope	E
B. hyodysenteriae P8544	Gyrase B	0.9956	-3.43	1.95
	BitC	0.9974	-3.94	1.93
	FeoB	0.9836	-3.61	1.9
	BHWA1_00040	0.9929	-3.38	1.97
	BHWA1_00042	0.9993	-3.70	1.86
	BHWA1_00535	0.9856	-3.61	1.9
	BHWA1_00536	0.9812	-3.49	1.9
	BHWA1_00888	0.9981	-3.39	1.94
	BHWA1_00891	0.9893	-3.87	1.81
B. hyodysenteriae P7455	Gyrase B	0.9936	-3.33	1.99
	BitC	0.9715	-3.48	1.93
	FeoB	0.9995	-3.23	2.03
	BHWA1_00040	0.9947	-3.49	1.93
	BHWA1_00042	0.9879	-3.4	1.96
	BHWA1_00535	0.9883	-3.38	1.97
	BHWA1_00536	0.9989	-3.38	1.97
	BHWA1_00888	0.9997	-3.34	1.99
	BHWA1_00891	0.9993	-3.43	1.95

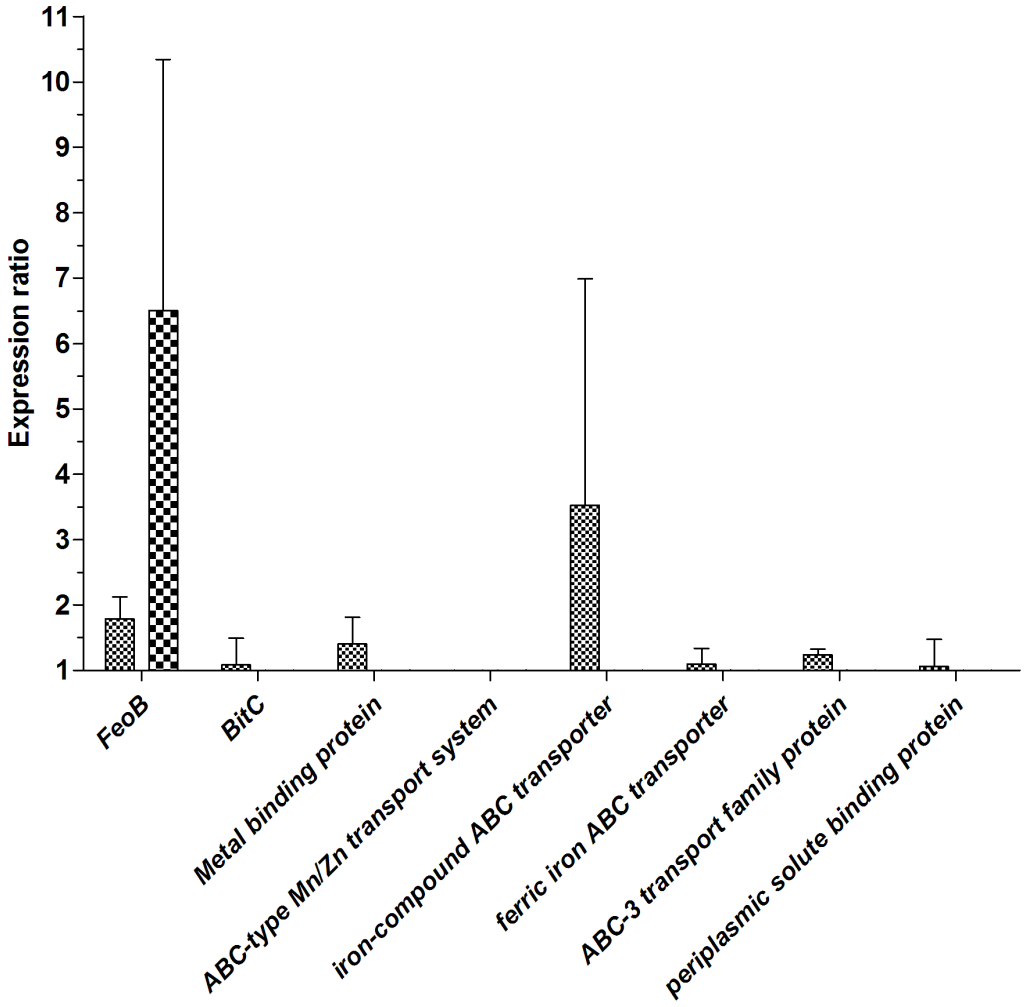


Figure 5.10: qRT-PCR analysis of genes related to iron-uptake under standard and iron-restricted growth conditions of *B. hyodysenteriae* P8544 ( ) and *B. hyodysenteriae* P7455 ( ). RNA was isolated under conventional and iron-restricted conditions when cells reached OD $_{600nm}$ value of 0.6. Data were normalized to that of *gyrB*. The figure presents the means and SD of three independent experiments and only illustrates genes which showed a fold change > 1 and thus exhibit increased expression under iron-restricted conditions compared to iron-replete conditions. The non-parametric Mann-Whitney U-test showed that the median transcription fold of the tested genes of the avirulent *B. hyodysenteriae* isolate P7455 and the virulent *B. hyodysenteriae* isolate P8544 were not significantly different (P> 0.05).

The **Figure 5.10** illustrates only genes which showed an increased expression (>1). A fold change < 1 indicated decreased expression of the targets compared to the control whereas a fold change > 1 illustrated increased expression. The results show that all tested target genes, with the exception of the ABC-type Mn/Zn ABC transporter, were differentially expressed under iron-restricted conditions in the virulent *B. hyodysenteriae* isolate P8544. Genes encoding the FeoB protein and iron-compound ABC transporter were the genes most up-regulated by P8544 in this study (1.8 fold to 3.5 fold, respectively). The genes encoding the Metal-binding protein and ABC-3 transport family protein were slightly up-regulated (1.4 fold and 1.2 fold respectively) than in iron-replete conditions followed by *bitC* (1.14 fold) and the periplasmic solute binding protein (1.06 fold).

In comparison, the *feoB* gene was the only differentially transcribed gene under iron-limited conditions in the avirulent *B. hyodysenteriae* isolate P7455 whereas all the other target genes were less transcribed than under iron-replete conditions. The *feoB* gene in P7455 was, with an expression ratio of 6.5 fold, 3.5 times more transcribed than in P8544 although the non-parametric Mann-Whitney U-test showed that the exhibited median expression folds of all obtained Ct-values of each tested genes were not significantly different (P>0.05) between the virulent and avirulent isolate.

Nevertheless, the observed up-regulation suggested that *feoB* plays a role in iron-acquisition under anaerobic conditions in the virulent and avirulent isolate. Interestingly, the variable expression profile of the two isolates is the first study indicating a difference between the virulent and avirulent isolate of *B. hyodysenteriae* at the level of gene transcription, even though further work is required to increase the statistical validity of the observations.

5.3 Discussion

To-date nothing is known about the requirement for iron and its means of uptake by *Brachyspira* spp. As stated previously, iron is required by most bacteria for survival; however, the genome of the closely-related species, *B. burgdorferi* and *T. Palladium*, have been reported not to contain any genes associated with iron-acquisition. Furthermore, *B. burgdorferi* has been shown to be able to grow under highly iron-restricted conditions, indicating that this organism adapted to its host niche by eliminating genes which require iron as a co-factor; instead, the alternative transition metal, manganese, has been reported as being essential (Ouyang *et al.*, 2009; Troxell *et al.*, 2012). In contrast, the genomes of *B. hyodysenteriae* have shown that these bacteria contain many iron acquisition protein-encoding genes, as well as genes which need iron as a co-factor, such as catalase and cytochromes, suggesting that iron is an essential nutrient for this pathogen. Therefore, iron uptake and acquisition in *B. hyodysenteriae* has been the focus of the work described in this chapter.

5.3.1 Response of *B. hyodysenteriae* to iron-limitation

The fact that iron plays a major role in the life-style of *B. hyodysenteriae* could be observed in the results obtained from culturing strains in medium containing different concentrations of dipyridyl, revealing that this organism requires iron. The assessment of different concentrations of dipyridyl added to the medium determined that a concentration of 0.1 mM of dipyridyl was sufficient to restrict but not abrogate growth and it is likely that this degree of restriction exerts an effect on expression of iron-dependent genes. Li *et al.* (1995) cultured type strains of *B. hyodysenteriae* under iron-limited conditions using BHI supplemented with 5% FCS and 0.15 mM of dipyridyl for 4 days. Interestingly, in this study a concentration of 0.12 mM of dipyridyl and higher resulted in no growth after 64 h of incubation at 37°C. This indicates that different strains seem to tolerate lower concentrations than others. However, these strain variations could not be observed in the two tested field isolates P8544 and P7455. Technical differences including the usage of glass-ware or plastic as well as variations in the BHI batches could have contributed to further differences between the two studies. The results obtained from the current study were unexpected, as dipyridyl has been used in many iron related studies of different bacteria (Barua *et al.*, 1990; Chart *et al.*, 1986; Lin *et al.*, 2012). Generally, a decrease in the bacterial growth rate is expected with an increase in the concentration of the iron-

chelator, with an associated reduction in the maximal levels of growth compared to iron-replete conditions. For example, the growth rate of *E. coli* was retarded by a concentration of 200 µM of EDDA and dipyridyl (Chart *et al.*, 1986). Moreover, iron-related studies of *Y. enterocolitica* resulted in similar reduction of growth when deferiprone was added (Lesic *et al.*, 2002)**.** None of the published studies were able to detect similar yields in growth compared to iron-replete conditions when iron-chelators were added. Although dipyridyl is known to be a strong chelator of iron, it is also acknowledged that it binds other transition metal ions. In this respect, although it was considered most likely that iron-chelation was affecting growth, there was a slight possibility that the removal of other metals such as manganese or zinc might have an effect on *B. hyodysenteriae* growth kinetics and might be the reason that *B. hyodysenteriae* did not grow at a concentration of 0.12 mM of dipyridyl and higher.

Despite an extended lag phase of approximately 32-34 hrs, the eventual rate of exponential growth in iron restricted culture was near identical to that under iron-replete conditions, and the final optical density of cultures was equivalent. This indicates that virulent and avirulent *B. hyodysenteriae* isolates were adapting to the iron-limited conditions, and were overcoming this limitation, such that the maximum level of growth in restricted conditions was not reduced as compared to iron-rich conditions. Although it is unclear what the nature of this adaptation is, it could possibly be due to the adjustment of the cells to utilise an alternative metal ion to iron. However, the likely candidates for alternative metal ions (primarily manganese or zinc) would also be chelated by dipyridyl and hence reduced in availability. That said, no efforts were made to quantify the amounts of any ion within the dipyridyl-treated growth medium, so it is possible that some transition metals were present in greater abundance than others. Another possible explanation for the adaptation to iron-restricted growth was through the scavenging of dipyridyl-chelated iron. In this respect, the ability of *B. hyodysenteriae* to produce a molecule of greater affinity for iron than dipyridyl is questionable, although this possibility prompted an investigation into the production of siderophore by *B. hyodysenteriae*.

Preliminarily work conducted during this project suggested that virulent and avirulent *B. hyodysenteriae* may both be able to produce a functional siderophore molecule since the CAS assay appeared to demonstrate that the bacteria were obtaining iron from the external medium, resulting in a characteristic colour change. The iron sequestration appeared to happen almost immediately after overlaying the plate with CAS agar. Nevertheless, the

colour change around colonies did not seem to be influenced by the absence or presence of 0.1 mM of dipyridyl within agar plates. Interestingly, the supplementation of 50 µM ferric iron to the conventional medium (BHI containing 5% FCS) resulted in a slightly reduced doubling time of virulent and avirulent *B. hyodysenteriae* isolates compared to the conventional growth medium without any additional iron source. Thus, the data suggests that the iron content in the conventional media used in this study, although sufficient for the maintenance of *B. hyodysenteriae*, is not perfectly optimized since additional iron sources seem to further enhance the growth rate of these bacteria. These findings might be an explanation for why CAS agar showed a colour change around colonies under iron-replete as well as iron-restricted conditions. The iron content in the conventional medium remains unknown. However, microorganisms have different threshold values at which they stop or start siderophore production (Mahmoud, 2001). Hydroxymate siderophore production in *Pseudomonas aeruginosa* has been shown to increase by increasing the iron concentration from 20 µM up to 40 µM (Mahmoud, 2001) indicating that siderophore production under even iron-richer conditions (as was the case in the present study) might not be unusual. The colour change around *B. hyodysenteriae* colonies in the CAS assay was stable until plates were discarded (after 4 days or more). Unfortunately, however, the CAS agar plate assay is not able to quantify siderophore production, and therefore no conclusion about the degree of siderophore production between strains and conditions could be made using this approach.

Unfortunately, control plates containing no bacteria were included in the CAS agar plate assay, and were observed to lose their blue colour after *ca.* 2 h of incubation. The reason for this is unclear, but might be due to the anaerobic atmosphere under which plates were incubated, causing the reduction of Fe^{3+} to the soluble form Fe^{2+}, and thus potentially influencing the colour change. However, what is not clear is why an equivalent loss of colour did not occur on inoculated plates, except for in the areas surrounding colonies, which is exactly where secreted siderophore would be expected to be located. In addition, following application of the CAS overlay, the colour change on inoculated plates was immediate, as compared to the colour transition taking several hours on uninoculated plates. To-date there have been no reports of the CAS plate agar assay being used under anaerobic conditions (Garcia *et al.*, 2012; Milagres *et al.*, 1999; Pakchung *et al.*, 2008). Therefore, no similar observations regarding the control in the CAS assay have been made. It should be mentioned, however, that the CAS plate agar assay was performed in Oxoid anaerobic jars, meaning that oxygen was present at the beginning of the period of

incubation, before an anaerobic atmosphere was reached. To see whether the presence of oxygen may have interfered with the production of orange halos around *B. hyodysenteriae* colonies, the same experiment was carried out later on during the project when the anaerobic workstation became available. However, the same results were observed under strict anaerobe conditions, resulting in orange halos around colonies of *B. hyodysenteriae* P8544 and P7455 under iron-replete and iron-restricted conditions. Thus, the data indicates that if a siderophore is produced it does not seem to be triggered by the presence of oxygen. Previous investigations suggested that *B. hyodysenteriae* did not produce either of the two most common siderophores, these being the catcheol and hydroxymate types. Moreover, the literature assumes that spirochaetes do not produce siderophores. The main reason for this assumption is that spirochaetes predominantly inhabit the anaerobic milieu, such as the intestine, which means that iron is more easily accessible under the redox state than the oxidised state.

Further assessment and quantification of possible siderophore production using the CAS microtitre plate assay was unsuccessful due to false-negative/positive results produced by the complex media BHI (data not shown). A published report was subsequently found which revealed that the CAS liquid assay is only suitable for measuring siderophore production when bacteria are cultured in chemically-defined media and is not suitable for bacteria growing in complex media like BHI (Shin *et al.*, 2001). Attempts have been made to culture *B. hyodysenteriae* in chemically defined media (CDM) supplemented with and without 5% serum (data not shown). Unsurprisingly, *B. hyodysenteriae* cells did not grow due to their fastidious nature. Moreover, the cells formed spherical bodies, indicating that CDM is not sufficient for growth. Consequently, although the CAS agar plate results offer the tantalising possibility of the existence of a siderophore in *B. hyodysenteriae*, clearly further work is required.

Analysis of the three genomes of *B. hyodysenteriae* did not identify any genes that encode proteins related to siderophore production or secretion. Genomes of other spirochaetes like *Leptopspira* have been also found to lack genes involved in siderophore production or secretion. Nevertheless, *Leptospira* spp. has been previously shown to utilize exogenous ferrichrome, aerobactin and desferrioxamine *in vitro* (Louvel *et al.*, 2006). Due to the diverse pig gut microflora (Lamendella *et al.*, 2011), it is likely that other intestinal bacteria are able to produce siderophores. To test whether *B. hyodysenteriae* is also able to use xenosiderophores produced by other microorganisms as an iron source, 20 µM of the

hydroxymate-type siderophore ferrichrome (iron-free) and 20 µM of enterobactin were added to iron-replete and iron-restricted conditions. The experiment showed that ferrichrome and enterobactin were not utilized by the virulent and avirulent *B. hyodysenteriae in vitro*. The results are in agreement with the comparative analysis of the genomes (**Chapter 4**) as no specific receptor for ferrichrome or enterobactin could be identified. The lack of specific receptors for these siderophores is reflected by the observation that *B. hyodysenteriae* cells were able to grow in media containing ferrichrome or enterobactin, although slightly more slowly as compared to the iron-replete medium, as the siderophores likely chelated free iron and thus reduced the iron content of the medium. If the microorganism possessed specific cell-surface siderophore receptors, addition of an excess of siderophore would either enhance or show no effect on growth, as demonstrated in *C. albicans* (Lesuisse *et al.*, 2002). However, it might be possible that *B. hyodysenteriae* contains a yet undefined siderophore receptor to bind a siderophore type which has not been tested in any *B. hyodysenteriae* strain.

5.3.2 Expression of iron-acquisition genes

In various environments, iron is present in two forms depending on the pH and the oxygen level. Under aerobic conditions, Fe^{3+} is very insoluble at neutral pH and requires active transport into the cell. In contrast, Fe^{2+},which exists under anaerobic conditions such as in the intestine is relatively soluble and obtaining ferrous iron is a much easier task for bacteria, and siderophore mediated transport is presumably not required. As the CAS plate assay showed siderophore production in *B. hyodysenteriae* the genomes were screened for genes which might be involved in iron-uptake. Even though the existence of secretion and/or utilization of siderophores could not be proven in *B. hyodysenteriae*, 23 genes potentially involved in iron and other metal transport as well as regulation were detected in all three genomes of *B. hyodysenteriae*.

Most of these genes were annotated as ABC transporters, with some of them sharing similarity with other ABC-transporters shown to play an important role in iron-acquisition in many bacteria. The gene encoding an iron-compound ABC transporter, ATP-binding protein (BHWA1_00535) shared 60 % identity with the ferrichrome transport ATP-binding protein, FhuC, in *B. piloscoli* WesB. However, the virulent and avirulent isolates of *B. hyodysenteriae* were not able to uptake ferrichrome *in-vitro*. To-date, utilization of exogenous siderophore has not been investigated in *B. piloscoli*. Thus, it remains unclear if

other *Brachyspira* spp. are able to sequester iron from siderophores and whether the ATP-binding protein, FhuC, in *B. piloscoli* WesB is actually involved in the uptake of iron-ferrichrome.

In order to test whether the identified genes are actually transcribed when subjected to iron-restricted conditions and thus play a potential role in pathogenicity, quantitative real-time PCR was performed for 8 of the most likely candidates to be involved in iron acquisition. In the first instance it was essential to evaluate a stably expressed housekeeping gene for data normalization of mRNA levels across samples of varying quality, to allow reliable results to be produced (Thellin *et al.*, 1999). Housekeeping genes are generally genes which are required for the maintenance of the cell and are thus consistently expressed under varying physiological conditions. However, it became evident that the reference genes commonly used for eukaryotes and prokaryotes, including beta-actin, GAPDH and 16S, vary considerably in their levels of expression under different conditions, leading to erroneous results (Glare *et al.*, 2002; Lin & Redies, 2012; Vandecasteele *et al.*, 2001). Thus, it is necessary to determine an appropriate reference gene for every individual experiment. In this study three candidate reference genes were chosen and their expression stability was determined using the software geNorm^{Plus}. According to the calculation by geNorm^{Plus} Gyrase B was predicted to be the most stably expressed HKG among experimental samples, followed by the 16 S RNA.

Variations in transcript level in virulent and avirulent *B. hyodysenteriae* could be confirmed by qRT-PCR. Interestingly, qRT-PCR data showed that seven genes, with the exception of the gene encoding for the ABC-type Mn/Zn transport system, were up-regulated in P8544 under iron-restricted conditions, whereby only the gene *feoB* in P7455 was found to be up-regulated. To-date no characterization of the seven differentially expressed genes detected in P8544 has been conducted in *B. hyodysenteriae* by others, with the exception of *bitC*. However, as the seven genes encode proteins which share similar ABC-transporter domain features with proteins studied in other bacteria a functional role can be predicted.

Generally, ABC transporters have been shown to contribute to virulence in various Gram-positive and Gram-negative bacteria as well as to iron-acquisition (Basavanna *et al.*, 2009; Dugourd *et al.*, 1999; Rodriguez & Smith, 2006). The fact that more ABC transporters in the virulent isolate were up-regulated under iron-restricted conditions compared to the

avirulent isolate might be a possible reason for the reduced pathogenicity of *B. hyodysenteriae* P7455. A metal-ABC transporter (mtsABC) has been described in *Streptococcus iniae*, which also contains a TroA-like domain found within the periplasmic solute-binding protein of *B .hyodysenteriae* (BHWA1_00891); the *S. iniae* gene was shown to be expressed *in vivo* and the resulting protein had haem-binding activity (Zou *et al.*, 2010). Although all tested ABC transporters were present in the genome of the avirulent *B. hyodysenteriae* strain, they were, with the exception of *feoB*, not up-regulated under iron-limited conditions. The down regulation of these transporters may contribute to lack of scavenging for important metals, such as iron and zinc, which are essential for survival. In *Treponema pallidum* TroA is a periplasmic metal binding protein (MBP) and has been demonstrated to bind zinc (Deka *et al.*, 1999). Zinc is another crucial element required by many microorganisms to maintain key biological functions such as gene expression (Berg & Shi, 1996). Competition for zinc between bacteria and their host, as well as between different bacterial species in the gut (Gielda & DiRita, 2012), has been suggested due to the presence of a high-affinity zinc transporter, known as the ZnuABC transport system in *E. coli* (Patzer & Hantke, 1998) and *C. jejuni*. By studying the intra-species variation in the transcriptional response of *B. hyodysenteriae* to iron-limitation, a set of ABC transporters was identified as being important to low-iron adaptation in the virulent strain but seemed to lack expression in the avirulent isolate, probably influencing its persistence and pathogenicity potential in the host. However, further work is required to confirm this preliminary observation.

The most up-regulated gene under iron-restricted conditions in both *B. hyodysenteriae* isolates was *feoB*. The Feo system has been recognized as playing a critical role in ferrous-acquisition under anaerobic conditions by *E. coli* (Kammler *et al.*, 1993) and *Salmonella typimurium* (Tsolis *et al.*, 1996). Moreover, this iron-acquisition system has been found to play a key role in bacterial virulence and gastrointestinal tract colonization (Naikare *et al.*, 2006). In all three sequenced *B. hyodysenteriae* genomes, the *feoB* gene, encoding the ferrous iron transport protein B (BHWA1_00859) was identified, and is likely to play a role in ferrous iron uptake under anaerobic conditions. To-date, this gene has not been further characterized in *B. hyodysenteriae*. However, *feoB* mutants of *Campylobacter jejuni* were significantly affected in their ability to colonize the chicken caecum (Naikare *et al.*, 2006). In the present study, the *feoB* gene was shown to be differentially transcribed in *B. hyodysenteriae* P8544 and P7455 when cells were exposed to iron-limited conditions. The gene was 2 fold more transcribed in P8544 and 6 fold more transcribed in P7455 when

grown in iron-limited media, suggesting that the Feo-transport mechanism is indeed involved in iron-acquisition in *B. hyodysenteriae*. This may not be surprising, since ferrous iron is predominant under the reducing conditions and low pH which are associated with the intestinal tract. In *H. pylori*, the uptake of Fe^{2+} was increased three-fold when cells were shifted from an aerobic to an anaerobic environment, suggesting that uptake of Fe^{2+} occurs under both conditions but is induced anaerobically (Velayudhan *et al.*, 2000). Interestingly, the *feoB* gene was 3.5-fold more transcribed in the avirulent *B. hyodysenteriae* isolate than in the virulent isolate, which might be due to the down-regulation of the other tested ABC-transporters lacking the import of other important metals. Most of the tested ABC-transporters are known to be involved in the uptake of various metals. Therefore, down-regulation of the seven ABC-transporters in P7455 may not only result in insufficient uptake of iron but also of other metals like zinc and magnesium under iron-restricted conditions, as suggested earlier. For instance, in *E. coli* (Hantke, 1997) and *C. jejuni* (Raphael & Joens, 2003) Fe^{2+} uptake was significantly enhanced in low Mg^{2+}-containing medium, suggesting that a deficiency of essential metals in the avirulent isolate (caused by down-regulation of ABC-transporter gene expression) results in a higher level of transcription of the *feoB* gene to compensate for this shortage and to maintain cell function.

Taken as a whole, the result of the qRT-PCR work described in this chapter offers the suggestion that differential expression of specific genes may be responsible for the difference in disease-causing ability between virulent and avirulent *B. hyodysenteria* strains; to study this further, proteomic analyses were conducted, which are described in **Chapter 6**.

5.3.3 Detection of lactoferrin-binding proteins

Lactoferrin is a multifunctional protein which binds free iron in hostexocrine fluids, such as milk and mucosal secretions. To test the ability of *B. hyodysenteriae* to use lactoferrin as an iron source, cells were grown in iron-replete and iron-limited medium supplemented with 0.5 mg/ml of partially iron-saturated bovine lactoferrin. The addition of 0.5 mg/ml lactoferrin led to acceleration in doubling time in P8544 and P7455 when grown under iron-limited and iron-replete conditions. Nevertheless, the level of iron-saturation of lactoferrin can affect its ability to chelate additional iron from the medium as well as increase the amount of lactoferrin-bound iron available for use. Unfortunately,

apolactoferrin (iron-free) was not available to compare the effect of iron-free and iron-saturated Lf on the growth rate of *B. hyodysenteriae*. However, unlike in human breast milk where Lf is found as apolactoferrin, the native state of bLf is partly saturated with an iron content of 15-20%, whereas the iron-depleted form is defined as having an iron content of 5% or less (Steijns & van Hooijdonk, 2000). Therefore, using the partially iron-saturated form of bLf in this study is more likely to mirror a comparable level of iron saturation *in vivo*. The supplementation of 0.5 mg/ml partially saturated Lf did not show any obvious antimicrobial effect of *B. hyodysenteriae*, as observed for some other bacteria resulting in growth inhibition or possible membrane degeneration (Ellison, III *et al.*, 1988; Yekta *et al.*, 2011). Inhibition of growth by Lf has been shown to be concentration and iron-saturation dependent. Various studies reported an increase in growth by *Helicobacter pylori* with either partially or fully iron-saturated Lf (Husson *et al.*, 1993; Velayudhan *et al.*, 2000), while Lf also enhanced the growth of anaerobic *Bifidobacterium* spp., and increasing the concentration of iron-saturated Lf did not influence the stimulation of growth (Petschow *et al.*, 1999). Compared to that, apolactoferrin has been demonstrated to be bactericidal to a wide range of bacteria, including *H. pylori*, *E. coli* and *Vibrio cholorae*, due its iron chelating ability (Arnold *et al.*, 1980; Wang *et al.*, 2001). Thus, different concentrations and different iron-saturated forms of Lf need to be tested to further investigate any antimicrobial effect of Lf on *B. hyodysentriae*. However, the main focus for testing Lf in this study was to investigate whether *B. hyodysenteriae* was able to sequester iron from Lf when exposed to iron-restricted conditions. Nevertheless, 0.5 mg/ml Lf did not restore the growth of *B. hyodysenteriae* P8544 when exposed to an iron-restricted environment containing 0.12 mM dipyridyl, possibly due to the chelation of other non-ferrous transition metals.

Even though the work described in this chapter indicates that *B. hyodysenteriae* is able to use Lf-bound iron, the mechanism of the iron-acquisition and its importance to pathogenesis are still unknown. The study provided presumptive identifications of possible lactoferrin-binding activity, whereby nine proteins were identified in the virulent and 4 proteins were found in the avirulent isolate. However, the specifity of binding of *B. hyodysenteriae* proteins to lactoferrin should be interpreted with care due to the known interaction of lactoferrin with acidic and charged macromolecules (Damiens *et al.*, 1998; Roux-de *et al.*, 1998). Moreover, out of the predicted panel of possible lactoferrin-binding proteins only two proteins in the virulent and one protein in the avirulent isolates were induced under iron-limited conditions and thus are targets of further interest. Both isolates

expressed a TPR-domain-containing protein under iron-limited conditions which reacted with bLf. The TPR-domain is known to be involved in protein-protein-interaction and is found in many organisms. In a few cases, the specificity of interaction is well-described; for example, the TPR-domain of the protein-serine phosphotase (PP5) binds to the heat shock protein 90 (hsp90) which is essential for signalling *in vivo* (Chen *et al.*, 1996). Nevertheless, TPR-domain-containing proteins have not been reported to be involved in Lf-binding or general iron-uptake in bacteria. Therefore, it remains to be determined if the Lf-binding activity found in the TPR-domain-containing protein in *B. hyodysenteriae* P8544 and P7455 resulted from a non-specific interaction between the protein and Lf or due to a specific receptor interaction. This could be further investigated by the protein-protein interaction between the TPR-domain-containing protein and Lf via the Two-Hybrid System performed in yeast or bacteria.

Interestingly, the data obtained from the Lf-binding assay as well as from the transcriptional profiling of the ABC-transporters are in agreement by identifying consistently more genes/proteins as being up-regulated under iron-limited conditions in the virulent isolate as compared to the avirulent isolate. Therefore, investigation of iron-limitation and its impact on the transcriptome (and hence proteome) of virulent and avirulent *B. hyodysenteria* via microarray or RNA-Seq, and quantitative proteomic methodologies would contribute to a deeper insight into the likely different responses in iron-homeostasis of both isolates.

Previous work discussed in **Chapter 3** and **Chapter 4** did not detect any obvious difference between virulent and avirulent *B. hyodysenteriae*. Consequently, the work discussed in this chapter was conducted to investigate the response of iron-limitation on *B. hyodysenteriae,* particularly regarding the response of virulent versus avirulent. Although the isolates responded phenotypically similarly when exposed to an iron-restricted environment, differences in gene transcription could be detected, suggesting that avirulent and virulent isolates are more likely to be discriminated at the level of gene expression rather than gene carriage. Therefore, in order to continue the investigation of differences between virulent and avirulent strains, a study into differential protein expression under iron-restricted conditions was conducted, and is the focus of the next chapter.

Chapter 6: Proteomic analysis of *B. hyodysenteriae*

6.1 Introduction

Prior to the findings in **Chapter 3** to **Chapter 5**, it became apparent that differences among virulent and avirulent isolates are not related to presence/absence of the single genes targeted. Preliminary studies presented in **Chapter 5** revealed that the virulent isolate P8544 and avirulent isolate P7455 differed at the transcription level when cells were exposed to an iron-restricted environment, suggesting that discrimination between strains might be possible on mRNA or protein level. The genome is usually considered as static in an individual living organism, whereas both the pattern and quantitative regulation of expressed proteins is dynamic and differs over time in direct response to changes in the extracellular environment (Hegde *et al.*, 2008). Compared to genomics the application of proteomics technologies has enabled the detailed study of these fluctuations and provided fresh insight into not just protein expression *per se*, but the post-translational modification of individual proteins and protein-protein interactions. In turn, these new findings have facilitated a greater understanding of cellular processes including mechanisms of bacterial pathogenesis (Poetsch & Wolters, 2008; Thongboonkerd *et al.*, 2009). Therefore, proteomics analysis has provided a unique tool for the identification of diagnostic biomarkers, evaluation of disease progress, and drug development (Boguski & McIntosh, 2003; Hanash, 2003) and will thus useful in the investigations of discrimination factors between virulent and avirulent *B. hyodysenteriae* strains.

The proteome can be defined as the entire set of expressed proteins in a given type of cell or an organism at a particular time and under a certain environmental condition (Wilkins *et al.*, 1996).

The increasing availability of complete and fully annotated bacterial chromosomal sequences has facilitated the global comparative analyses of pathogens at the level of both the genome and the proteome.

The dawning of the post-genomic era triggered the development and improvement of many technologies used in proteomics research including 2D-gel electrophoresis (2DGE). 2DGE is regarded as a fickle and technically challenging laboratory technique which has enjoyed major improvements in sample preparation and first dimension separation chemistries, user-friendly purpose-built laboratory equipment and powerful software algorithms for the comparative analysis of multiple 2D-electropherograms. Reproducibility and overall

sensitivity has increased markedly and these collective improvements have progressed the identification of novel diagnostic biomarkers and vaccine candidates (Di *et al.*, 2013; Hsu *et al.*, 2008).

2DGE is one of the most widely technologies used for the identification of potential biomarkers and vaccine candidates. The method exploits two different physiochemical properties of soluble proteins allowing the individual proteins comprising a complex biological mixture to be separated and resolved based on their isoelectric point (pI) in the first dimension, and their mass in the second dimension. 2DGE has been applied in spirochaete research in order to study mainly the membrane proteome regarding its potential of vaccine candidates in *Borrelia* (Gesslbauer *et al.*, 2012), *Leptospira* (Humphryes *et al.*, 2012) and *Treponema* (Altaie & Cox, 1991). Additionally, 2D-gel electrophoresis has been used in the investigation of the OM of *B. hyodysenteriae* identifying the OM-associated proteins Bhlp29.7a and Bhlp16 on the 2D map (Cullen *et al.*, 2003b). However, no wider 2D-gel studies on different cellular compartments of different *B. hyodysenteriae* isolates or investigations of the *B. hyodysenteriae* proteome in response to stress situations have been published to date. Clearly therefore, further proteomic approaches, particularly those investigating protein expression under conditions mimicking those *in vivo* would contribute to the knowledge of *B. hyodysenteriae* virulence factors and mechanism as well as the identification of further possible vaccine targets.

Bacterial proteins of primary interest for vaccine development include those that confer virulence and which are surface-exposed. Outer-membrane proteins come into direct contact with the host cell surface and play a key role in pathogenesis by acting as adhesins. A good example of this is the opacity-associated adhesin (Opa) of *N. meningitides* which facilitates infection by adhering to host receptor molecules on the nasopharyngeal cell surface (Sadarangani *et al.*, 2012). One of the main functions of the OM is to serve as a barrier to protect the bacteria from the environment. Therefore, the composition of the OMP in Gram-negative bacteria is the key to understanding the ability of the pathogen to cause infection.

The cellular ultra structure of Gram-negative bacteria includes inner- and outer-membranes which are separated by the periplasim containing the peptidoglycan layer. The outer-membrane consists of phospholipids and lipopolysaccharides (LPS). Proteins present in the OM include lipoproteins which are anchored to the OM with an N-terminal lipid tail and

integral proteins which span the OM. Spirochaetes form an independent branch of the bacterial phylogenetic tree distinct from Proteobacteria, hence it is no surprise that the architecture of their OM differs from other Gram-negative bacteria (Plaza *et al.*, 1997).

In spirochaetes the peptidoglycan cell wall is more closely related to the cytoplasmic membrane than to the outer-membrane resulting in a much more unstable and labile outer-membrane compared to other Gram-negative bacteria (Holt, 1978). The instability was noted when *Brachyspira* spp. was observed to be forming outer-membrane blebs when cells were exposed to 0.15 mM dipyridyl (Li *et al.*, 1995).

Among spirochaetes outer-membrane composition is best studied in *Leptospira* which shares many OM features with other spirochaetes. However, studies have also shown that the composition differs among spirochaete species; unlike *Borrelia* and *Treponema* spp., *Leptopsira* spp. possess LPS and a high diversity of amphipathic β-sheet transmembrane OMPs like the porin OmpL1 (Haake *et al.*, 1993) which results in different biological functions such as adhesion. Therefore different features in the LPS compositions have an impact on the pathogenicity potential in each bacteria and are thus important research target to understand how the bacteria causes infection.

Additionally, differences in the nature of the OM of spirochaetes have rendered the selective removal of proteins belonging to this cellular fraction technically challenging. Sarkosyl and Tween 20 have been used successfully for the OM enrichment in many Gram-negative bacteria However; these detergents have been shown to cause cell lysis in *B. hyodysenteriae* resulting in the release of cytoplasmic proteins and are thus unsuitable for the extraction of OMPs in spirochaetes (Gabe *et al.*, 1995; Joens *et al.*, 1993). Studies used electron microscopy to demonstrate that the nonionic detergent Triton-X-114 removed the OM without damaging the lipid bilayer structure of the cytoplasmic membrane in *T. pallidum* (Cunningham *et al.*, 1988).

Treatment of *T. pallidum* cells with Triton-X-114 caused blebs of the OM which were released into the detergent phase and were identified as OM associated proteins. Since then Triton-X-114 phase partitioning has been widely used for the enrichment of spirochaete OMP and has been successful in the identification of many outer surface proteins including the Lip21 in *Leptospira* (Cullen *et al.*, 2003a) and the BbA66 in *Borrelia* (Brooks *et al.*, 2006). These studies have highlighted the pathogenic potential of these proteins.

However, as with almost every method Triton-X-114 has its pitfalls and limitations. Triton-X-114 forms a homogenous solution of micelles within an optimum temperature range limit at the low end by the freezing point of the solvent and at the high end by the cloud point. Temperatures above the cloud point form a turpid suspension as a result of increased micellar size or micellar aggregation caused by a decrease in polar head group hydration (Bordier, 1981). The aggregated micelles can be pelleted by centrifugation yielding in a denser detergent-rich phase and a lighter-detergent phase (aqueous phase). The cloud point alters depending on other supplements in the solution, e.g. glycerol was shown to lower significantly the cloud point of Triton-X-114 in water (Werck-Reichhart *et al.*, 1991). The Triton family is also known to strongly absorb UV light which could interfere with optical assays and tends to be problematic for ESI (Loo *et al.*, 1994). Additionally their low CMC and high molecular weight makes removing the solvent complicated by dialysis for further downstream applicants. Several investigators reported problems associated with aldehyde and peroxide impurities in Triton (Chang & Bock, 1980). Despite these potential limitations, Triton is widely accepted as suitable for OM preparation from spirochaetes.

Compared to *Treponema* spp., *Leptospira* spp. and *Borrelia* spp., little is known about the OMP composition of *Brachyspira* spp. Limited knowledge of OMP expression in *B. hyodysenteriae*, has hampered the identification of potential vaccine candidates. A deeper interrogation of the OM composition would enhance the understanding and facilitate the detection of vaccine candidates. However, phase partitioning using Triton-X-114 has been applied in *Brachyspira* spp. and detected several OMP in *B. hyodysenteriae* including the Vsp and the Bhlp family. Additionally, the outer-membrane lipoprotein BmpC was identified in *B. piloscoli* (Trott *et al.*, 2004).

Studies have shown that lipoproteins are the predominant class of proteins in the total membrane protein complement of spirochaetes (Haake & Matsunaga, 2010). Examples of high abundant lipoproteins are Lip21 in *Leptospira* (Cullen *et al.*, 2003a) and OspA of *Borrelia* (Jiang *et al.*, 1994). Several identified lipoproteins are known to be important factors in the interactions between the pathogenic spirochaete and the host. Therefore one of the main focuses in *Leptospira* research is the prediction and identification of lipoproteins in the study of physiology and pathogenesis. As mentioned previously, the OM composition differs between spirochaetes and other known Gram-negative bacteria. Additionally, amino acid sequences of their lipoproteins and signal peptides have been

reported to be distinct from other bacteria. Consequently, this affects localization prediction as well as further analysis of these sequences using established databases like PSORTb (Yu *et al.*, 2010) and LipoP resulting in false-positive or low accuracy predictions (Setubal *et al.*, 2006). However, a novel prediction tool, specifically modulated to spirochaetes, termed SpLiP has been developed in order to facilitate the prediction of lipobox sequences and hence lipoproteins with greater precision (Setubal *et al.*, 2006).

The virulent and avirulent *B. hyodysenteriae* isolates were shown to be very similar with respect to gene carriage and phenotypical characteristics (**Chapter 3** and **Chapter 4**). However, transcription analysis of various genes under iron-deplete conditions (**see Chapter 5**) provided the first indication that the two isolates differ at the mRNA level. The aim of this chapter was to perform a proteomic analysis of different cell compartments of the two isolates under iron-replete and iron-restricted growth conditions with a view to identifying differences in protein expression that reflect the differences observed at the mRNA level. It was hoped that any qualitative differences observed could lead towards the identification of a possible marker to distinguish between avirulent and virulent *B. hyodysenteriae*.

6.2 Results

6.2.1 Outer-membrane profile of *B. hyodysenteriae* under iron-replete and iron-restricted conditions

As shown in **Chapter 5**, 0.1 mM of dipyridyl was the highest concentration tested in this study which still yielded growth of *B. hyodysenteriae*. Therefore, BHI supplemented with 5% FCS (v/v) and 0.1 mM of dipyridyl was defined as iron-restricted medium for *B. hyodysenteriae*. In order to assess the influence of iron-restriction in the proteome of *B. hyodysenteriae*, cells of the isolates P8544 and P7455 were grown under iron-replete conditions and in the presence of 0.1 mM dipyridyl. Cells of three biological replicates of each isolate were grown up to an OD_{600nm} value of 0.6. Afterwards, each sample was extracted using 1% Triton-X-114 which has been shown to yield a sufficient enrichment of OMP in other spirochaetes. Subsequently, the detergent fractions, containing mainly hydrophobic proteins, were separated on a 12.5% SDS-PAGE (**2.7.2**). The electropherograms were scrutinized in order to identify proteins which were isolate-specific and exclusively expressed in an iron limited environment. An initial aim of this was to assess whether possible biomarker(s) capable of distinguishing between virulent and avirulent *B. hyodysenteriae* isolates could be identified (**Figure 6.1**).

The protein profiles of P8544 and P7455 obtained from iron-replete and -depleted growth conditions appeared to be very similar with the two most abundant protein bands appearing around 40 and 60 kDa (**Figure 6.1**). Expressed proteins were analyzed by LC/ESI-MS/MS and summarized for each isolate and condition. Proteins which exclusively met the following criteria were included in the analysis of the outer-membrane profiling: Present at least in two out of three biological replicates and contained at least two peptides containing unbroken “b” or “y” ion series of at least four contiguous amino acid residues with a sequence coverage ≥ 5% (**Chapter 2**).

The data has shown that 233 proteins were detected under iron-replete conditions in *B. hyodysenteriae* P8544 and 271 proteins in P7455 whereas 226 proteins could be identified under iron-restricted conditions in P8544 and 200 in P7455 (**Table 6.1** and **Appendix 6**). Furthermore, 25.75% (60/233) of proteins were annotated as hypothetical proteins in the OM profile under iron-replete conditions while 28.88% (65/225) of proteins were found under iron-restricted conditions in the virulent isolate P8544.

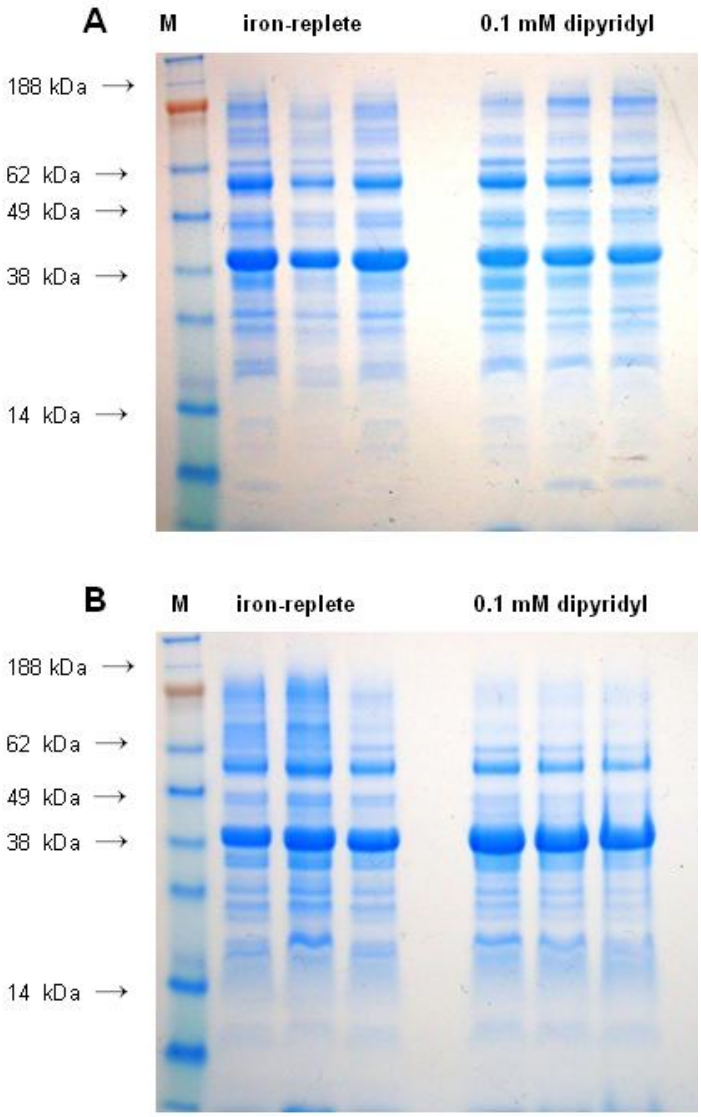


Figure 6.1: SDS-PAGE analysis of outer-membrane proteins of *B. hyodysenteriae* P8544 (A) and P7455 (B) using Triton-X-114. Cells were grown in biological triplicates under iron-replete and iron-restricted conditions. After cells reached an OD_{600nm} of 0.6, the outer-membrane proteins of each strain grown under each condition were extracted using 1% v/v Triton-X-114. The detergent phase fractions were separated using 4 µg of protein per lane and stained with SimplyBlue™ SafeStain (Invitrogen) for 1 hr. A Seeblue standard (Invitrogen) molecular weight marker (M) was used. Following this, proteins were excised manually by cutting whole gel lanes into 25 slices and further processed as described in section 2.10.2.

Table 6.1: Main features of the predicted proteins in the OM enriched fraction in *B. hyodysenteriae* P7455 and P8544.

	***B. hyodysenteriae* P8544**		***B. hyodysenteriae* P7455**	
	Iron-replete	**Iron-restricted**	**Iron-replete**	**Iron-restricted**
Number of total proteins	233	226	271	200
predicted hypothetical proteins	60	65	74	72
proteins assigned as lipoprotein by SpLiP	55	66	74	61
hypothetical proteins	25	26	32	28
Proteins assigned beta-barrel domain by BOMP[1]	26	30	32	24
Proteins detected in both conditions	174		159	
unique proteins expressed in iron-replete conditions	58[2]	/	110[2]	/
number of same proteins present in the other isolate	11		11	
unique proteins expressed in iron-restricted conditions	/	51[3]	/	41[3]
number of same proteins present in the other isolate		11		11

[1] beta-barrel integral outer-membrane protein predictor (BOMP) tool

[2] proteins are listed in Table 6.5

[3] proteins are listed in Table 6.4

The novel prediction software SpLiP which is particularly tailored for spirochaetes was used to identify lipoproteins in the OM enrichment fraction of *B. hyodysenteriae* P8544 and P7455. Additionally, the programs SpLiP 4.0 and LipoP 1.0 were used to analyze the entire translated CDS of the genomes of *B. hyodysenteriae* P8544 and P7455 to predict signal peptides and lipoproteins (**Appendix 5)**. However, as SpLiP provided a higher accuracy of predicted lipoproteins in all spirochaetes tested by Setubal (2006), only results obtained using this software were included for further analysis and discussion in this chapter.

The software SpLiP predicted that 25.75% (60/233) of proteins detected in the iron-rich fraction of P8544 were lipoproteins while 24.43% (65/226) of proteins expressed under iron-limited conditions were identified as lipoproteins. A similar number of lipoproteins were predicted in P7455 under iron-repletion 27.30% (74/271) and 36% (72/200) in iron-restricted conditions. To date the only three lipoproteins in *B. hyodysenteriae* to be reported in publications are Bhlp29.7, MglB and BitB (Dugourd *et al.*, 1999; Trott *et al.*, 2004; Zhang *et al.*, 2000). In the current study these proteins were expressed in both isolates under both growth conditions and were also assigned a lipobox by SpLiP validating the reliability of the software. Furthermore, the software also predicted several proteins which have not been reported as lipoproteins in *B. hyodysenteriae*. Other predicted lipoproteins included the oligopeptides (Opp) termed OppA (Bhyov8544_0298; Bhyov8544_1670; Bhyov8544_2610; Bhyov8544_2611; Bhyoa7455_0055; Bhyoa7455_0382; Bhyoa7455_2239; Bhyoa7455_2553; Bhyoa7455_2554), the alkaline phosphatase II (PhoB) (Bhyov8544_1024; Bhyoa7455_0792) and the outer-membrane protein A (OmpA) (Bhyov8544_2009; Bhyov8544_2010; Bhyoa7455_1693) which were all found to be expressed in both isolates of *B. hyodysenteriae* tested. Orthologues of these proteins have been identified and reported in *B. burgdorferi* (Kornacki & Oliver, 1998) and other bacteria (Kriakov *et al.*, 2003) as lipoproteins. However, half of the predicted lipoproteins in each obtained OM fraction of each isolate were assigned as hypothetical proteins (**Table 6.1**) indicating that the function of these putative lipoproteins remains unknown.

Additional proteins such as ribosomal proteins and other cytoplasmic proteins which are not associated with the bacterial membrane including flagella proteins have been detected in both isolates and conditions. Therefore, the software beta-barrel integral outer-membrane proteins predictor (BOMP) was used to identify proteins containing a defined

membrane inserted β-barrel region for localization prediction. The program predicted 24-32 (**Table 6.1**) of the identified proteins in each isolated fraction to contain a beta-barrel structure suggesting that 11-14% of the detected proteins in *B. hyodysenteriae* P8544 and P7455 in iron-replete and iron-restricted conditions are outer-membrane associated.

6.3 KEGG analysis of the OM of *B. hyodysenteriae*

The predicted protein sets of each isolate obtained from each growth condition were assigned to KO orthologues in order to identify changes in the OM profile of virulent and avirulent isolates under different environmental conditions (**Figure 6.2** and **Figure 6.3**).

In the virulent isolate P8544 a total of 41.63% (97/233) of proteins under iron-replete and a total of 29.46% (66/225) of proteins identified under iron-restricted conditions were assigned to KO ortholgues. Similar percentages of proteins were assigned to KO orthologues in the avirulent isolate P7455 with 43.91% (119/271) and 30.5% (63/200) of proteins identified under iron-replete and iron- limited conditions respectively (**Table 6.1**).

The largest single category of expressed proteins (11.1-11.8%) in P8544 and P7455 under iron-replete conditions were assigned to membrane transport followed by 8.5 % of proteins in P8544 categorized into carbohydrate metabolism and 7.0 % of proteins in P7455 classified into signal transduction (**Figure 6.2**).

Most differences between the isolates under iron-replete conditions were observed by the assignation of predicted proteins in P8544 to three further categories termed "Metabolism of other amino acids", "metabolism of terpenoids and polyketides" and "execretory system". These genes were annotated as *atoB* coding for the acetyl-CoA C-acetyltransferase (Bhyov8544_1861), *ackA* encoding for the acetate kinase (Bhyov8544_1894) and *pckA* encoding for phosphoenolpyruvate carboxykinase (PEPCK) (Bhyov8544_2426) (**Figure 6.2**). Additionally the three categories glycan biosynthesis and metabolism, biosynthesis of other secondary metabolites and cell growth and death were absent in the OM profile of P8544 but were assigned to proteins identified in the OM of avirulent P7455. These categories contained the gene *lpxB* encoding the lipid-A-disaccharide synthase (Bhyoa7455_1642), a gene coding for the Rieske (2Fe-2S) domain protein (Bhyoa74557455_2168) as well as a gene encoding anaphase-promoting complex subunit 3 (APC3) (Bhyoa7455_0338) (**Figure 6.2**).

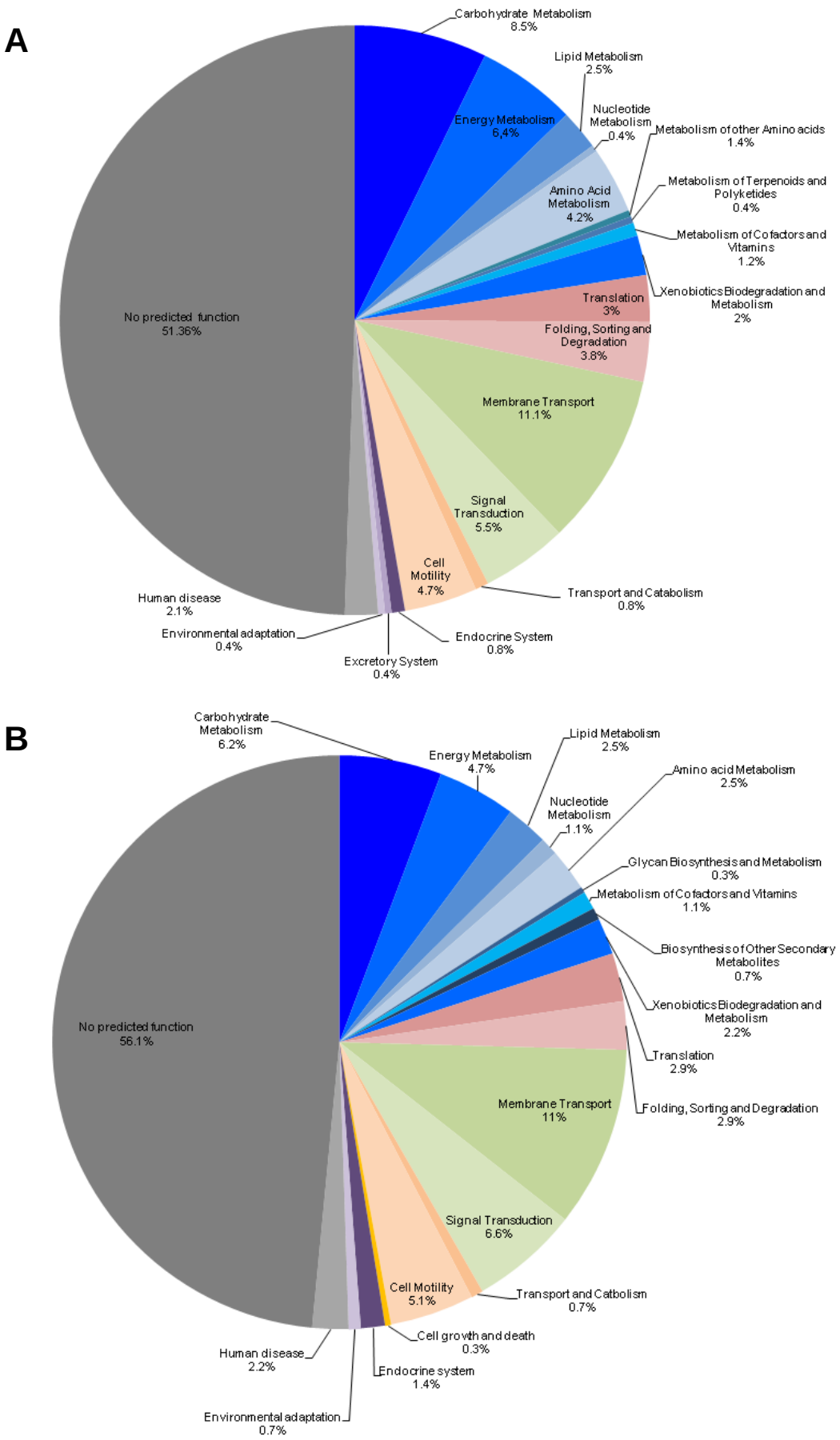


Figure 6.2: KEGG analysis of the OM of *B. hyodysenteriae* grown under iron-replete conditions. (A) represents the OM profile of *B. hyodysenteriae* P8544 and (B) *B. hyodysenteriae* P7455.

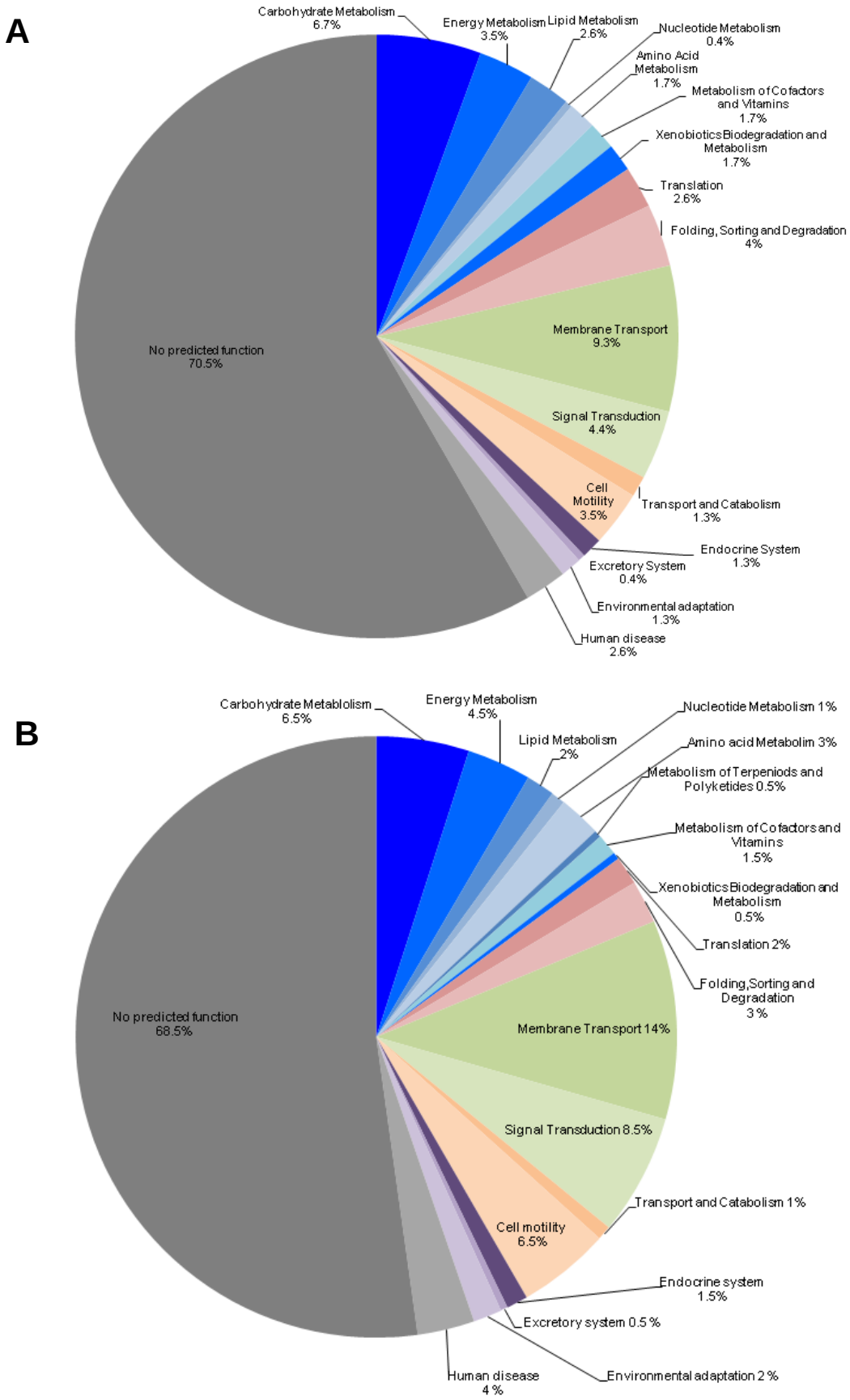


Figure 6.3: KEGG analysis of the OM of *B. hyodysenteriae* grown under iron-restricted conditions. (A) represents the OM profile of *B. hyodysenteriae* P8544 and (B) *B. hyodysenteriae* P7455.

Table 6.2: Number of protein assigned to each KO orthologue under iron-replete (STD) and iron-restricted (IR) conditions.

KO orthologues	Number of proteins assigned			
	P8544		P7455	
	STD	IR	STD	IR
Carbohydrate Metabolism	20	15	17	13
Energy Metabolism	15	8	13	9
Lipid Metabolism	6	6	7	4
Nucleotide Metabolism	1	1	3	2
Amino Acid Metabolism	10	4	7	6
Metabolism of other Amino acids	1	/	/	/
Metabolism of Terpenoids and Polyketides	1	/	1	1
Metabolism of Cofactors and Vitamins	2	4	3	3
Glycan Biosynthesis and Metabolism	/	/	1	/
Biosynthesis of Other Secondary Metabolites	/	/	2	/
Xenobiotics Biodegradation and Metabolism	6	4	6	4
Translation	7	6	8	4
Folding, Sorting and Degradation	9	9	8	6
Membrane Transport	26	21	32	25
Signal Transduction	13	10	19	17
Transport and Catabolism	2	3	2	2
Cell Motility	11	8	14	11
Endocrine System	2	3	1	3
Excretory System	1	1	4	1
Environmental adaptation	1	3	2	4
Human disease	5	6	6	8
No predicted function	119	158	143	139
Assigned function	114	66	128	63
Proteins in total	233	224	271	200

1-3 proteins 4-5 proteins ≥6- proteins

Under iron-restricted growth conditions the OM profile of P8544 and P7455 changed most obviously in a decrease of percentages of proteins and thus number of proteins assigned to KEGG orthologues (**Figure 6.3**) (**Table 6.3**). For instance, in P8544, 15 proteins expressed under iron-replete conditions were assigned to the class energy metabolism whereas only eight proteins expressed under iron-restricted conditions were assigned to the same category. Additionally, seven proteins which were assigned to membrane transport of the iron-rich protein set of P7455 were not found to be expressed under iron-restricted conditions and thus not classified as membrane transport-related proteins. However, the decrease in percentages of proteins which were detected under iron-limitation and assigned to the KO orthologues membrane transport and signal transduction was not obvious in the avirulent isolate P7455 (**Figure 6.2)** possibly due to the variation of total identified proteins detected under both growth conditions compared to the isolate P8544 (**Table 6.1**).The other orthologues changed in a smaller increase or decrease of 0.1-1.8% of classified proteins.

Orthologues of membrane transport in P7455 and energy metabolism in P8544 showed the highest discrepancy regarding number of proteins (>6) assigned to these categories when comparing the iron-restricted protein set to iron-replete protein set. Therefore, further analysis was carried out in order to assign proteins which were found to be expressed in one of the condition and thus more likely to be condition specific (**Table 6.3**). The results showed that 19 proteins assigned to membrane transport in *B. hyodysenteriae* P8544 were expressed under both tested conditions (data not shown). Only the gene *secG* coding for the preprotein translocase subunit SecG was found in this category under iron-limited conditions while seven proteins were exclusively found to be expressed under iron-replete conditions.

Interestingly, 24 proteins assigned to membrane transport were expressed under both conditions in *B. hyodysenteriae* P7455 (data not shown) whereby eight proteins were detected uniquely under iron-replete conditions. Only the Phosphoenolpyruvate-protein-phosphotransferase (Bhyoa7455_1881) was found to be expressed under iron-restriction and assigned to the same class.

Table 6.3: Proteins classified into the KO orthologues by KEGG which were detected in either iron-replete or iron-restricted conditions.

Locus tag	Gene	Protein	Peptides[1]			SC[2]	Growth condition
Membrane Transport							
Bhyov8544_1240	*secG*	preprotein translocase subunit SecG	2	2	/	14.3	IR
Bhyoa7455_0047	*modA*	molybdate transport system substrate-binding protein	4	4	/	28.9	STD
Bhyoa7455_0369	*mglC*	methyl-galactoside transport system permease protein	3	2	2	5.0	STD
Bhyoa7455_1536	/	amino acid-binding protein	4	4	/	17.3	STD
Bhyoa7455_1009	/	ABC transporter, transmembrane region	3	3	/	10.6	STD
Bhyoa7455_1535	/	PTS system, IIa component	3	3	/	14.8	STD
Bhyoa7455_1085	/	PTS system, fructose subfamily, IIC subunit	3	3	/	18.2	STD
Bhyoa7455_2467	*secF*	preprotein translocase subunit SecF	4	3	3	13.7	STD
Bhyoa7455_1632	*secY*	preprotein translocase subunit SecY	3	3	/	15.7	STD
Bhyov8544_1609	*modA*	molybdate transport system substrate-binding protein	3	2	/	17.1	STD
Bhyov8544_1391	*mglA*	methyl-galactoside transport system ATP-binding protein	21	3	/	44.7	STD
Bhyov8544_0358	*pstS*	phosphate transport system substrate-binding protein	8	8	7	44.8	STD
Bhyov8544_1986	/	extracellular solute-binding protein, family 5	2	2	/	10.8	STD
Bhyov8544_0292	/	binding-protein-dependent transport system, membrane transport	3	3	/	10.7	STD
Bhyov8544_2464	*ptsG*	fused N-acetyl glucosamine specific PTS enzyme: IIC, IIB , and IIA components	12	9	9	22.9	STD
Bhyov8544_0924	*secY*	preprotein translocase subunit SecY	6	4	/	16.4	STD
Bhyoa7455_1881	*ptsI*	Phosphoenolpyruvate-protein phosphotransferase	8	3	/	18.1	IR
Energy Metabolism							
Bhyov8544_0609	*ntpA*	V-type H+-transporting ATPase subunit A	4	3	/	11.4	STD
Bhyov8544_0610	*ntpB*	V-type H+-transporting ATPase subunit B	4	3	/	12.5	STD
Bhyov8544_1048	*tpiA*	triosephosphate isomerise	3	3	/	17.8	STD
Bhyov8544_1862	*crt*	3-hydroxybutyryl-CoA dehydratase	6	5	4	35.7	STD
Bhyov8544_1861	*atoB*	acetyl-CoA C-acetyltransferase	8	6	/	30.1	STD
Bhyov8544_1485	*ackA*	acetate kinase	8	4	/	25.6	STD
Bhyov8544_1067	*gltD;*	glutamate synthase (NADPH/NADH) small chain	4	2	/	12.2	STD
Bhyoa7455_1548	*ntpA*	V-type H+-transporting ATPase subunit B	2	2	2	7.7	STD
Bhyoa7455_0816	*tpiA*	triosephosphate isomerase (TIM)	2	2	/	19.8	STD
Bhyoa7455_1822	*porA*	pyruvate ferredoxin oxidoreductase	23	22	9	44.0	STD
Bhyoa 7455_1543	*etfB*	electron transfer flavoprotein beta subunit	8	8	4	56.3	STD
Environmental Adaptation							
Bhyov8544_2069	*flaB3*	periplasmic flagella filament protein FlaB3	16	9	9	54.0	IR
Bhyov8544_2088	*flaB*	Flagella filament core protein FlaB2	10	9	4	38.6	IR
Bhyoa7455_1135	*fliC*	Flagella protein	14	12	8	51.5	IR
Bhyoa7455_2357	*flaB*	Flagella filament core protein FlaB2	11	8	8	48.3	IR
Metabolism of other Amino Acids							
Bhyov8544_1485	*ackA*	acetate kinase	8	4	/	25.6	STD
Glycan Biosynthesis and Metabolism							
Bhyoa7455_1642	*lpxB*	putative lipid A disaccharide synthase; LpxB	3	3	/	11.7	STD
Biosynthesis of other Secondary Metabolites							
Bhyoa7455_2168		Rieske (2Fe-2S) domain protein	15	14	14	10.2	STD
Bhyoa7455_1737		aspartate aminotransferase	5	4	/	19.1	STD

IR iron-restricted growth conditions STD standard growth conditions referred to iron-replete conditions

Highlighted proteins in blue were predicted to be present in *B. hyodysenteriae* P8544 and P7455 detected under the same growth condition and classified into the same KO orthologue.

[1]Refers to number of validated peptides per protein in each of the 3 replicates.

[2] sequence coverage. The highest SC observed from all replicates of each presented protein is shown.

An obvious reduction in the number of assigned proteins (≥ 4) identified under iron-restriction was observed in the carbohydrate metabolism group in both isolates and further differences were seen in Amino Acid Metabolism in P8544 and Translation in P7455. However, compared to conventional growth conditions, some categories including Environmental Adaptation and Human Disease showed an increase of assigned protein percentages under iron-restricted conditions. In both isolates proteins of the flagella assembly apparatus were only found to be expressed under iron-limited conditions and assigned to environmental adaptation. These included the periplasmic flagella filament core protein FlaB3 (Bhyov8544_2069), and the flagella filament core protein FlaB2 (Bhyov8544_2088; Bhyoa7455_2357) (**Table 6.3**).

Four proteins of *B. hyodysenteriae* P8544 and P7455 were found to be expressed under the same condition and classified in the same orthologue group. These include the molybdate transport system substrate-binding protein (ModA), the preprotein translocase subunit (SecY), the V-type H+-transporting ATPase subunit A (NtpA) and the flagella filament core protein (FlaB2) (**Table 6.3**). The low number of shared proteins expressed under the same conditions indicates that the proteins in the OM enrichment fractions differ between the virulent and avirulent isolate. Additionally a higher percentage of proteins with no assigned function (68.5-70.5%) were observed when cells were subjected to an iron-limited environment in both isolates.

Thus, the KEGG analysis of the OMP profile of virulent and avirulent *B. hyodysenteriae* isolates has shown that the almost identical protein patterns observed in **Figure 6.1** contain different proteins within growth conditions and isolates. Thus, investigation of predicted proteins in the OM of virulent and avirulent *B. hyodysenteriae* isolates indicates that the OM profile altered between studied conditions and isolates.

6.3.1 Proteins expressed under iron-limitation in virulent and avirulent *B. hyodysenteriae*

The KEGG analysis of the OM of the *B. hyodysenteriae* isolates has already suggested that the OMP profile was influenced by the availability of iron in the growth media. However, as KEGG analysis is only taking proteins which are assigned to the matched orthologues into account, a more detailed analysis of the OM data was conducted to assign proteins

which are specifically expressed under iron-restricted conditions and unique to either virulent or avirulent *B. hyodysenteriae*.

Comparison between the predicted proteins expressed under iron-restricted and iron-replete growth conditions has shown that 41 proteins were found under iron-limitation and not under iron-replete conditions in avirulent P7455, whereby 52 proteins were identified in virulent P8544. Of these expressed proteins, 34.61% (18/52) in P8544 and 26.82% (11/41) in P7455 were annotated as hypothetical proteins. Some of these proteins have already been mentioned in **6.3**. Moreover, in the set of proteins detected to be expressed under iron-restriction in P8544, the programs BOMP and SpLiP predicted 11.76% (6/51) to contain a β-barrel domain and 25.49% (13/51) were identified as lipoproteins. In P7455 12.19% (5/41) were predicted to be lipoproteins and proteins associated with the OM (**Table 6.4**).

Additionally, the data (**Table 6.4**) showed that both isolates shared 11 proteins which were found to be expressed under iron-restriction and identified in 3 out of 3 biological replicates of each isolate. The majority of these 11 proteins have been already associated with virulence in *B. hyodysenteriae* and other bacteria. For instance, the VspI protein was identified in both isolates under iron-limited conditions and was previously described to be involved in antigenic variation in *B. hyodysenteriae* (Witchell *et al.*, 2006). Additionally, the flagella filament outer layer protein FlaA, as well as the flagella filament core protein FlaB2 were exclusively identified under these conditions.

Two proteins involved in iron-transport and storage were identified. The gene encoding FeoB has already been shown to be significantly more transcribed (1.8-3.5 fold) under iron-restricted conditions indicating its importance in the acquisition of iron-acquisition of virulent and avirulent *B. hyodysenteriae* in **Chapter 5**. The data in this chapter confirmed the results obtained by the *q*RT-PCR in **Chapter 5** showing that FeoB was highly abundant under iron-limitation in both isolates.

The non-haem iron-containing ferritin known to be involved in iron storage was detected in P8544 and P7455.

Table 6.4: Proteins expressed under only iron-restricted conditions in *B. hyodysenteriae* P7455 and P8544. Proteins shown were expressed under iron-restricted growth conditions (0.1 mM dipyridyl) in *B. hyodysenteriae* P8544 and P7455, identified by LC-ESI-MS-MS and matching the criteria: Present at least in two out of three biological replicates and contained at least two peptides containing unbroken "b" or "y" ion series of at least four contiguous amino acid residues with a sequence coverage ≥ 5%.

Locus tag[1]	Gene	Protein	MW [2] [kDa]	pI [3]	Mowse Score [4]	Peptides [5]			SC [6]
Bhyov8544_0095	*rplJ*	50S ribosomal protein L10	19.2	5.9	197.7	3	3	2	16.4
Bhyov8544_0107	*flaA*	**putative flagella filament outer layer protein FlaA**	27.6	4.6	354.9	7	5	/	37.3
Bhyov8544_0108	*flaA*	flagella filament outer layer protein FlaA	24.7	4.6	238.4	6	5	5	20.4
Bhyov8544_0150	*vspI*	variable surface protein	47.0	4.9	246.7	5	3	/	20.9
Bhyov8544_0209	/	hypothetical protein	24.2	4.7	89.3	3	2	2	19.1
Bhyov8544_0317	/	PSP1	37.6	5.0	53.9	3	3	2	5.2
Bhyov8544_0354	*arp*	ankyrin repeat-containing protein	71.0	4.6	629.9	12	6	5	23.4
Bhyov8544_0525	/	hypothetical protein	40.0	5.6	123.3	3	3	2	13.7
Bhyov8544_0531	*dnaJ*	chaperone protein dnaJ	40.9	9.6	101.1	3	3	/	11.4
Bhyov8544_0570	/	TPR domain-containing protein	20.2	4.7	217.3	5	4	4	31.5
Bhyov8544_0701	/	hypothetical protein	41.9	5.3	107.9	5	3	/	14.0
Bhyov8544_0832	*rsgA*	non-haem iron-containing ferritin	20.8	4.9	142.8	5	4	/	28.9
Bhyov8544_0953	/	hypothetical protein	14.8	4.8	157.0	4	4	/	42.5
Bhyov8544_1046	*flaA*	flagella filament outer layer protein FlaA	36.0	4.7	637.8	11	7	5	40.6
Bhyov8544_1090	/	hypothetical protein	37.2	4.9	113.3	4	3	3	11.3
Bhyov8544_1099	/	hypothetical protein	15.3	4.3	369.3	8	5	3	51.2
Bhyov8544_1201	/	hypothetical protein	22.0	9.1	294.1	5	4	3	25.9
Bhyov8544_1218	/	hypothetical protein	19.7	5.7	76.8	2	2	/	5.6
Bhyov8544_1240	*secG*	putative preprotein translocase subunit SecG	12.3	9.4	109.7	2	2	/	14.3
Bhyov8544_1303	/	hypothetical protein	16.5	9.6	127.9	3	3	2	28.5
Bhyov8544_1329	/	thiol-disulfide interchange protein DsbD-like protein	31.6	5.4	487.0	11	8	5	45.8
Bhyov8544_1330	/	thiol-disulfide interchange protein DsbD-like protein	31.2	4.6	164.0	4	3	2	19.6
Bhyov8544_1347	/	hypothetical protein	44.7	7.5	498.1	10	6	3	36.1
Bhyov8544_1352	/	TPR domain-containing protein	27.2	9.3	197.2	4	3	2	21.1
Bhyov8544_1438	/	Flavodoxin	18.8	8.8	61.4	2	2		12.2
Bhyov8544_1477	*ahpC*	alkyl hydrogen peroxide reductase	20.9	5.3	158.7	4	4	2	31.0
Bhyov8544_1510	/	hypothetical protein	26.2	4.9	255.8	5	4	2	23.9
Bhyov8544_1528	/	non-haem iron-containing ferritin	20.0	5.0	380.6	9	8	8	55.0
Bhyov8544_1587	*faa1*	Long-chain acyl-CoA synthetases (AMP-forming)	71.2	7.7	210.3	5	4	4	14.1
Bhyov8544_1645	/	putative transcriptional regulator	40.5	9.5	125.8	4	3	/	12.7
Bhyov8544_1715	/	Predicted membrane protein	23.2	10.6	124.0	2	2	/	11.6
Bhyov8544_1823	*arp*	**ankyrin repeat-containing protein**	87.9	4.6	258.2	5	4	/	9.8
Bhyov8544_1900	/	**hypothetical protein**	65.2	4.2	175.4	4	4	2	7.2
Bhyov8544_1933	/	hypothetical protein	41.2	5.0	424.9	9	7	2	25.0
Bhyov8544_2069	*flaB3*	periplasmic flagella filament protein FlaB3	30.1	5.2	869.7	16	7	7	54.0
Bhyov8544_2088	*Flab2*	Flagella filament core protein flaB2	28.2	5.4	487.2	10	9	4	38.6
Bhyov8544_2097	/	**hypothetical protein**	143.4	6.7	691.5	15	11	/	12.7

Bhyov8544_2111	*nifS*	NifS, Cysteine sulfinate desulfinase/cysteine desulfurase-like enzyme	42.9	6.1	441.5	10	5	4	37.1
Bhyov8544_2151	/	hypothetical protein	15.1	4.5	181.1	3	3	/	27.6
Bhyov8544_2195	/	outer membrane protein	52.7	4.7	136.9	3	3	/	7.3
Bhyov8544_2196	*mtrC*	membrane fusion protein	45.0	7.2	424.3	9	6	3	26.5
Bhyov8544_2284	/	Rieske (2Fe-2S) domain protein	38.0	9.7	280.4	11	10	8	8.0
Bhyov8544_2385	/	hypothetical protein	53.9	5.1	368.5	8	4	4	24.8
Bhyov8544_2410	*msrAB*	bifunctional methionine sulfoxide reductase	23.3	6.6	165.7	5	4	3	51.0
Bhyov8544_2465	*tar*	methyl-accepting chemotaxis protein	63.7	4.7	132.2	3	2	2	6.3
Bhyov8544_2503	/	hypothetical protein	18.8	7.2	363.4	6	6		33.5
Bhyov8544_2504	/	hypothetical protein	18.5	7.2	351.5	6	6	3	32.7
Bhyov8544_2611	*oppA*	ABC-type oligopeptide transport system, periplasmic component	61.2	5.4	149.5	3	3		8.4
Bhyov8544_2645	*feoB*	ferrous iron transport protein B *	92.3	6.1	1652.0	30	28	25	32.9
Bhyov8544_2667	/	**putative inversin protein alternative isoform**	64.3	4.2	420.4	8	7	7	21.0
Bhyov8544_2693	/	**basic membrane lipoprotein**	23.2	7.5	74.2	2	2	/	12.0
Bhyoa7455_0028	*mcpB*	methyl-accepting chemotaxis protein McpB	84.5	6.0	216.9	5	5	4	9.4
Bhyoa7455_0035	*argB*	acetylglutamate kinase	31.1	5.1	86.2	5	4	/	14.9
Bhyoa7455_0038	/	biopolymer transport protein ExbB	22.5	5.0	163.3	4	2	/	23.8
Bhyoa7455_0129	/	PSP1	37.6	5.0	104.0	5	3	2	7.0
Bhyoa7455_0185	/	hypothetical protein	68.5	6.4	68.8	4	3	/	10.4
Bhyoa7455_0442	/	**hypothetical protein**	41.7	4.6	199.6	3	3	/	12.9
Bhyoa7455_0469	/	hypothetical protein	24.2	4.7	368.4	6	5	5	43.9
Bhyoa7455_0531	*vspI*	variable surface protein VspI	47.0	4.9	1019.2	15	14	10	47.0
Bhyoa7455_0549	/	**hypothetical protein**	29.3	8.6	131.7	3	3	2	13.6
Bhyoa7455_0573	*flaA*	flagella filament outer layer protein FlaA	24.7	4.6	880.9	16	10	6	65.7
Bhyoa7455_0586	*rplJ*	50S ribosomal protein L10	19.2	5.9	232.5	5	3	3	39.0
Bhyoa7455_0604	*lysM*	putative peptidoglycan-binding LysM:Peptidase M23B family	67.7	9.4	138.5	4	4	/	10.2
Bhyoa7455_0685	/	putative flavoprotein	45.8	5.5	164.6	4	4	/	18.3
Bhyoa7455_0814	*flaA*	flagella filament outer layer protein FlaA	36.0	4.7	796.8	13	13	8	45.9
Bhyoa7455_0997	*ahpC*	alkyl hydrogen peroxide reductase †	20.9	5.3	366.7	9	8	6	49.7
Bhyoa7455_1048	/	non-haem iron-containing ferritin	20.0	5.0	679.6	13	11	8	69.6
Bhyoa7455_1102	*glgC*	glucose-1-phosphate adenylyltransferase	48.2	8.5	270.9	7	6	/	26.4
Bhyoa7455_1135	*fliC*	Flagellin	28.8	5.5	792.8	14	12	8	51.5
Bhyoa7455_1153	*cheY*	chemotaxis response regulator CheY	16.0	9.3	51.8	2	2	/	22.7
Bhyoa7455_1158	/	hypothetical protein	22.0	9.5	157.8	4	4	4	26.0
Bhyoa7455_1183	/	**hypothetical protein**	33.5	4.9	150.2	3	3	/	16.1
Bhyoa7455_1322	/	**hypothetical protein**	44.1	5.9	80.4	3	3	/	7.4
Bhyoa7455_1362	*fld*	Flavodoxin	15.3	4.1	355.1	5	3	/	74.1
Bhyoa7455_1625	*rplJ*	50S ribosomal protein L5	21.1	10.1	138.2	4	2	/	21.4
Bhyoa7455_1663	*mcpB*	methyl-accepting chemotaxis protein McpB	81.0	5.2	165.9	4	3	/	8.4
Bhyoa7455_1735	*adk*	adenylate kinase	20.7	5.1	100.9	3	3	/	23.2
Bhyoa7455_1864	/	hypothetical protein	28.0	10.2	90.9	4	3	/	14.0
Bhyoa7455_1881	*ptsI*	Phosphoenolpyruvate-protein phosphotransferase	64.3	4.8	381.8	8	3	/	18.1
Bhyoa7455_1922	*mcpA*	methyl-accepting chemotaxis protein McpA	45.1	5.5	286.9	6	4	/	18.1
Bhyoa7455_2148	/	-	16.8	9.9	219.7	4	3	2	32.1

Bhyoa7455_2357	*flaB2*	Flagella filament core protein flaB2 †	28.2	5.4	863.2	11	8	8	48.3
Bhyoa7455_2413	/	**hypothetical protein**	38.6	4.4	164.0	3	3	2	15.3
Bhyoa7455_2440	/	hypothetical protein	18.8	7.2	243.4	4	3	/	38.9
Bhyoa7455_2468	/	zinc finger SIM domain protein	68.3	5.1	99.1	5	2	/	9.6
Bhyoa7455_2512	*cheX*	chemotaxis protein CheX	15.2	4.3	159.6	3	3	3	36.7
Bhyoa7455_2518	/	hypothetical protein	39.5	6.3	51.2	3	3	/	12.5
Bhyoa7455_2574	*feoB*	ferrous iron transport protein B *	92.3	6.0	689.5	14	13	11	21.0
Bhyoa7455_2670	*bitB*	periplasmic-iron-binding protein BitB	17.1	5.0	155.2	3	3	2	31.8
Bhyoa7455_2679	*mcpA*	methyl-accepting chemotaxis protein McpA	18.8	4.3	226.6	4	4	2	30.6
Bhyoa7455_2723	*bitB*	periplasmic-iron-binding protein BitB	8.0	4.9	100.3	3	3	/	44.6
Bhyoa7455_2791	*bitB*	periplasmic-iron-binding protein BitB	4.8	4.7	93.6	2	2	/	60.9

1 locus tag refers to gene prefixes given by the manual annotation software Xbase using *B. hyodysenteriae* WA1 as a query.

[2] Molecular weight.

[3] Isoelectric point.

[4] Molecular Weight Search

[5]Refers to number of validated peptides per protein.

[6] sequence coverage. The highest SC observed from all replicates of each presented protein is shown.

Proteins highlighted in blue were predicted to be present in *B. hyodysenteriae* P8544 and P7455

*these genes were shown to be differentially transcribed under iron-limited conditions by *q*RT-PCR in **chapter 5.**

†these proteins were detected via 2D-gel electrophoresis to be differentially expressed under iron-restricted conditions compared to iron-replete conditions.

Underlined proteins were predicted to be lipoprotein by SpLiP.

Bold proteins were predicted to be integral β-barrel OMP by BOMP.

Moreover, alkyl hydrogen peroxide reductase (AhpC) was identified in both isolates under iron-limitation and has not been further described in *B. hyodysenteriae* within the extent of pathogenicity, but is a known stress defense protein in *C. jejuni* (Baillon *et al.*, 1999).

Therefore, the data suggests that these 11 proteins expressed by *B. hyodysenteriae* in response to an iron-restricted environment are likely to contribute to the adaptation and survival process of this pathogen in an iron-limited environment. Under conventional growth conditions, 58 proteins in P8544 and 110 proteins in P7455 were only found under this condition with 11 proteins in common between the virulent and avirulent *B. hyodysenteriae* isolate (**Table 6.5**). In total, 17 hypothetical proteins in P8544 and 30 hypothetical proteins in P7455 could only be detected under iron-replete conditions. Additionally 5.17%-5.45% was predicted to contain a β-barrel motif while 11.76% (6/51) and 39.02% (16/41) were predicted as lipoproteins in P8544 and P7455. The proteomic approach used was non-quantitative hence relative or absolute quantitation of protein abundance between growth conditions and isolates is not possible from these data. It is possible that these 11 proteins exclusively found under iron-restricted conditions may also be expressed under iron-replete conditions but were not detected due to limitations of the method used. Thus, only cautious conclusions about proteins which are solely detected in one of the growth conditions can be made.

Table 6.5: Proteins identified only under iron-replete conditions in *B. hyodysenteriae* P8544 and P7455.

locustaq	Protein	MW [kDa]	pI	Scores	Peptides			SC
Bhyov8544_0045	arp putative ankyrin repeat-containing protein	33.7	3.8	120.7	3	2	/	13.5
Bhyov8544_0060	hypothetical protein	37.5	5.9	112.4	3	2	/	13.4
Bhyov8544_0063	invasin; Opacity protein-like surface antigens	25.0	9.4	103.5	2	2	/	10.2
Bhyov8544_0067	ndhD hypothetical protein	18.9	4.8	297.0	5	4	4	51.7
Bhyov8544_0134	hypothetical protein	43.0	4.6	170.6	3	3	2	11.2
Bhyov8544_0218	*hypothetical protein*	30.1	9.7	213.3	4	3	/	17.0
Bhyov8544_0228	hypothetical protein	11.7	5.2	224.3	5	4	/	41.7
Bhyov8544_0229	hypothetical protein	11.7	7.5	118.1	3	2	/	41.7
Bhyov8544_0292	binding-protein-dependent transport system, membrane component	30.6	10.3	91.3	3	3	/	10.7
Bhyov8544_0321	mcpB methyl-accepting chemotaxis protein McpB	75.8	4.9	317.6	5	3	3	10.6
Bhyov8544_0358	ptsS phosphate ABC transporter, phosphate-binding protein	27.5	4.3	519.1	8	8	7	44.5
Bhyov8544_0493	hypothetical protein	25.7	5.4	170.0	3	2	/	25.8
Bhyov8544_0537	dnaK molecular chaperone DnaK	67.7	4.8	317.7	6	3	/	15.0
Bhyov8544_0569	carboxyl-terminal protease	55.0	5.3	246.1	4	2	/	11.5
Bhyov8544_0609	atpA V-type ATP synthase subunit A	65.1	5.0	198.1	4	3	/	11.4
Bhyov8544_0610	atpB V-type ATP synthase subunit B	48.4	4.9	184.1	4	3	/	12.5
Bhyov8544_0628	hypothetical protein	39.6	5.6	269.6	5	3	/	22.7
Bhyov8544_0779	TPR domain-containing protein	26.2	5.5	150.4	3	3	3	16.5
Bhyov8544_0866	apbE putative thiamine biosynthesis lipoprotein apbE	37.2	4.9	147.5	3	2	/	13.0
Bhyov8544_0897	**hypothetical protein**	33.6	9.0	191.4	4	3	/	15.9
Bhyov8544_0898	hypothetical protein	17.5	4.4	228.9	5	4	3	47.3
Bhyov8544_0924	secY preprotein translocase subunit SecY	49.2	10.2	177.9	6	4	/	16.4
Bhyov8544_0952	Bhyov8544_0952 hypothetical protein	18.8	4.5	160.6	2	2	2	15.5
Bhyov8544_1003	hypothetical protein	58.9	8.9	347.1	10	10	/	22.0
Bhyov8544_1048	tpiA triosephosphate isomerase	27.6	5.7	230.4	3	3	/	17.8
Bhyov8544_1067	glutamate synthase (DPH)	51.0	5.6	167.5	4	2	/	12.2
Bhyov8544_1144	**hypothetical protein**	44.1	5.9	127.5	4	3	/	10.8
Bhyov8544_1278	gatA glutamyl-tRNA(Gln) amidotransferase, A subunit	52.7	5.8	156.9	3	3	2	11.4
Bhyov8544_1372	bitB periplasmic-iron-binding protein BitB	39.0	4.6	254.0	5	3	2	29.1
Bhyov8544_1387	aroB 3-dehydroquinate synthetase	39.9	6.3	58.3	3	3	/	6.4
Bhyov8544_1391	mglA galactose/methyl galaxtoside transporter ATP-binding protein	56.1	9.1	1121.0	21	3	/	44.7
Bhyov8544_1447	anti-sigma factor antagonist	12.1	4.6	167.8	3	2	2	35.5
Bhyov8544_1473	fliF flagella MS-ring protein	64.6	6.0	111.4	3	2	/	10.2
Bhyov8544_1485	ackA acetate kinase	42.8	6.7	419.6	8	4	/	25.6
Bhyov8544_1529	mcpB methyl-accepting chemotaxis protein McpB	68.3	4.7	146.1	4	3	/	9.3
Bhyov8544_1609	modA ModA, ABC-type molybdate transport system, periplasmic component	28.5	4.6	202.2	3	2	/	17.1
Bhyov8544_1662	tsf elongation factor Ts	31.2	5.2	234.3	6	5	2	28.2
Bhyov8544_1748	iars isoleucyl-tRNA synthetase	106.0	5.8	172.3	5	4	4	7.6
Bhyov8544_1760	cheY chemotaxis protein CheY	15.6	9.2	223.2	4	3	3	32.4
Bhyov8544_1837	**acetyltransferase, GT family**	20.9	5.2	111.5	4	3	/	5.7
Bhyov8544_1851	hypothetical protein	27.3	4.8	101.6	3	3	/	14.0
Bhyov8544_1861	acetyl-CoA acetyltransferase	40.9	8.8	448.2	8	6	/	30.1
Bhyov8544_1862	crt 3-hydroxybutyryl-CoA dehydratase	27.7	5.4	292.9	6	5	4	35.7
Bhyov8544_1863	crt 3-hydroxybutyryl-CoA dehydratase	29.9	8.8	391.8	7	5	4	36.4
Bhyov8544_1894	aspC aspartate aminotransferase	45.8	5.8	307.7	7	2	/	31.9

Bhyov8544_1937	amino acid transport system permease protein	26.3	9.4	220.8	4	4	/	18.1
Bhyov8544_1986	extracellular solute-binding protein, family 5	40.0	4.7	97.5	2	2	/	10.8
Bhyov8544_1995	aspS aspartyl-tRNA synthetase	66.9	5.2	187.3	4	3	/	13.0
Bhyov8544_2103	prolipoprotein diacylglyceryl transferase	35.8	9.5	129.6	2	2	/	10.6
Bhyov8544_2169	hypothetical protein	15.5	7.1	138.0	2	2	/	17.6
Bhyov8544_2234	pgi glucose-6-phosphate isomerase	49.5	6.4	162.8	4	3	/	14.9
Bhyov8544_2279	rubrerythrin fusion protein	20.4	5.4	317.7	6	6	5	51.6
Bhyov8544_2341	hypothetical protein	11.0	9.1	122.0	3	2	/	19.1
Bhyov8544_2348	hypothetical protein	47.8	4.3	170.0	3	3	/	11.4
Bhyov8544_2468	hypothetical protein	55.9	9.2	314.5	5	5	3	15.3
Bhyov8544_2485	MoxR-like ATPase	38.5	5.1	369.0	8	7	/	33.4
Bhyov8544_2501	hypothetical protein	77.0	4.9	620.0	10	5	/	19.1
Bhyov8544_2636	hypothetical protein	85.6	5.5	272.8	6	4	/	13.6
Bhyoa7455_0047	ModA, ABC-type molybdate transport system, periplasmic component	28.5	4.7	226.2	4	4	/	28.9
Bhyoa7455_0063	elongation factor Ts	31.2	5.2	320.7	8	7	/	33.1
Bhyoa7455_0088	chemotaxis response regulator CheY	13.5	7.6	240.6	5	5	/	51.6
Bhyoa7455_0090	putative methyl-accepting chemotaxis protein	74.0	4.4	110.3	4	4	3	7.2
Bhyoa7455_0100	ABC-type uncharacterized transport system	61.4	9.4	292.8	6	4	/	21.4
Bhyoa7455_0133	methyl-accepting chemotaxis protein McpB	75.7	4.9	282.9	5	5	3	12.9
Bhyoa7455_0170	ankyrin repeat-containing protein	71.0	4.6	242.2	6	6	/	12.6
Bhyoa7455_0171	solute binding protein-like protein	97.5	4.8	828.1	13	13	8	20.7
Bhyoa7455_0194	Na+-transporting methylmalonyl-CoA/oxaloacetate decarboxylase, beta subunit	48.2	9.9	202.1	3	3	2	10.0
Bhyoa7455_0207	Peptidase	31.7	6.0	169.4	4	4	/	20.0
Bhyoa7455_0232	**hypothetical protein**	51.4	4.8	1293.4	22	20	19	55.3
Bhyoa7455_0301	response regulatory protein (atoC)	51.1	5.5	103.1	5	3		16.9
Bhyoa7455_0335	hypothetical protein	18.8	5.1	128.4	2	2		21.7
Bhyoa7455_0347	putative chaperone protein DnaJ	11.2	10.0	145.6	2	2	2	27.1
Bhyoa7455_0369	beta-methylgalactoside transporter inner membrane component	36.0	0.0	135.0	3	2	2	5.0
Bhyoa7455_0408	**hypothetical protein**	41.0	5.1	296.3	7	5	3	36.8
Bhyoa7455_0460	hypothetical protein	30.1	9.7	212.1	5	52		21.2
Bhyoa7455_0477	hypothetical protein	68.1	5.7	105.1	4	3	3	6.3
Bhyoa7455_0486	phosphotransferase system mannitol/fructose-specific IIA domain (Ntr-type)	31.2	5.4	509.5	8	8	5	39.7
Bhyoa7455_0552	**hypothetical protein**	38.4	8.8	102.9	3	3	/	13.5
Bhyoa7455_0585	50S ribosomal protein L7/L12	13.3	4.8	244.5	4	4	/	60.9
Bhyoa7455_0590	pututative preprotein translocase subunit SecE	12.1	10.4	120.3	4	4	3	14.2
Bhyoa7455_0615	hypothetical protein	18.9	4.8	113.9	2	2	2	18.4
Bhyoa7455_0619	invasin; Opacity protein-like surface antigens	25.0	9.3	113.5	3	2	/	20.5
Bhyoa7455_0623	hypothetical protein	24.7	5.3	153.5	3	3	/	33.2
Bhyoa7455_0629	PilZ domain containing protein	28.9	5.1	143.2	3	3	/	17.1
Bhyoa7455_0678	ThiJ/PfpI domain-containing protein	20.0	4.8	107.8	3	3		44.0
Bhyoa7455_0730	chemotaxis protein CheY	15.6	9.2	105.6	3	2	2	21.6
Bhyoa7455_0741	isoleucyl-tRNA synthetase	106.0	5.8	152.4	4	3	/	6.4
Bhyoa7455_0756	methyl-accepting chemotaxis-like domains (chemotaxis sensory transducer)	62.4	4.5	135.8	3	3	/	11.8
Bhyoa7455_0765	GltP, Na+/H+-dicarboxylate symporter	40.2	9.2	189.6	3	3	/	10.3
Bhyoa7455_0772	hypothetical protein	58.9	8.9	169.0	3	3	/	14.7
Bhyoa7455_0816	tpiA triosephosphate isomerise	27.6	5.7	161.2	2	2	/	19.8

Bhyoa7455_0865	hypothetical protein	27.8	5.1	107.9	2	2	/	9.6
Bhyoa7455_0888	hypothetical protein HMPREF9467_03922 [Clostridium clostridioforme 2_1_49FAA]	31.9	7.6	122.0	4	3	/	13.3
Bhyoa7455_0934	**hypothetical protein**	47.8	5.9	75.8	3	3	/	7.9
Bhyoa7455_0944	hypothetical protein	35.0	4.9	359.5	6	3	/	28.6
Bhyoa7455_0958	hypothetical protein	18.0	4.9	143.4	4	4	/	38.7
Bhyoa7455_0964	purine-binding chemotaxis protein	17.9	4.3	200.0	3	3	/	31.4
Bhyoa7455_1009	ABC transporter, transmembrane region	73.2	9.3	163.2	3	3	/	10.6
Bhyoa7455_1042	putative Na+/phosphate symporter	61.9	6.3	305.6	7	6	3	20.0
Bhyoa7455_1085	PTS system, fructose subfamily, IIC subunit	17.3	7.7	54.3	3	3	/	18.2
Bhyoa7455_1163	extracellular solute-binding protein, family 5	51.8	4.9	108.7	3	3	/	10.0
Bhyoa7455_1167	putative methyl-accepting chemotaxis protein B	75.7	4.6	226.3	6	4	3	10.2
Bhyoa7455_1188	**ankyrin repeat-containing protein**	62.1	4.5	63.5	2	2	/	6.6
Bhyoa7455_1247	putative cytidylate kinase	23.4	6.5	101.4	3	2	2	19.3
Bhyoa7455_1329	putative electron transport complex	39.4	9.9	83.6	3	3	3	9.6
Bhyoa7455_1340	glutamyl-tRNA(Gln) amidotransferase, B subunit	54.5	6.0	89.6	4	4	4	13.5
Bhyoa7455_1360	galactose/glucose-binding protein	38.7	9.4	107.5	5	4	3	10.7
Bhyoa7455_1361	3-dehydroquinate synthetase	39.9	6.1	84.8	4	4		11.7
Bhyoa7455_1376	periplasmic-iron-binding protein BitB	39.0	4.6	261.6	4	4	2	27.4
Bhyoa7455_1415	thiol-disulfide interchange protein DsbD-like protein	31.2	4.6	182.0	4	4	/	18.1
Bhyoa7455_1461	Predicted membrane protein	23.2	10.6	89.8	2	2	2	11.6
Bhyoa7455_1483	transcriptional regulator	32.3	5.8	68.3	4	4	/	14.7
Bhyoa7455_1505	dinB family protein	32.5	10.0	48.1	3	3	/	10.8
Bhyoa7455_1510	TPR domain-containing protein	20.2	4.9	140.5	3	2	/	26.0
Bhyoa7455_1535	PTS system, IIa component	16.3	4.2	53.7	3	3	/	14.8
Bhyoa7455_1536	amino acid-binding protein	28.6	4.9	237.0	4	4	/	17.3
Bhyoa7455_1543	electron transfer flavoprotein beta subunit	28.6	5.8	439.8	8	8	4	56.3
Bhyoa7455_1548	V-type ATP synthase subunit B	48.4	4.9	102.2	2	2	2	7.7
Bhyoa7455_1560	hypothetical protein	43.1	5.1	221.3	5	5	3	19.4
Bhyoa7455_1566	hypothetical protein	39.6	5.6	57.6	2	2	2	10.3
Bhyoa7455_1593	TPR domain-containing protein	77.5	5.2	218.1	4	3	/	10.9
Bhyoa7455_1603	OmpA family protein	25.2	9.6	106.8	2	2	/	9.5
Bhyoa7455_1604	hypothetical protein	26.2	9.3	120.4	3	3	/	20.3
Bhyoa7455_1605	hypothetical protein	33.6	9.0	127.6	3	3	/	12.0
Bhyoa7455_1606	hypothetical protein	17.5	4.4	136.0	4	4	/	42.4
Bhyoa7455_1610	elongation factor G	75.3	5.1	315.2	8	7	6	20.9
Bhyoa7455_1626	30S ribosomal protein S8	14.9	9.7	262.7	5	5	/	45.9
Bhyoa7455_1627	50S ribosomal protein L6	21.0	10.0	189.6	4	4	/	34.8
Bhyoa7455_1628	50S ribosomal protein L18	13.2	10.6	91.8	2	2	/	16.9
Bhyoa7455_1632	preprotein translocase subunit SecY	49.2	10.2	85.4	3	3	/	15.7
Bhyoa7455_1642	putative lipid A disaccharide synthase; LpxB	42.5	9.4	66.6	3	3	/	11.7
Bhyoa7455_1662	iron-containing alcohol dehydrogenase	42.0	5.5	96.4	4	4	4	19.4
Bhyoa7455_1710	Ferredoxin	29.6	9.4	465.4	10	9	4	45.7
Bhyoa7455_1717	MiaB-like tRNA modifying enzyme	48.1	9.3	113.6	4	4	/	5.3
Bhyoa7455_1718	Cell division protein FtsZ	76.8	4.7	87.8	3	3	/	5.1
Bhyoa7455_1737	aspartate aminotransferase	45.8	5.8	266.0	5	4	/	19.1
Bhyoa7455_1743	**hypothetical protein**	65.4	4.2	221.0	4	4	/	13.0
Bhyoa7455_1745	hypothetical protein	23.0	4.7	101.0	2	2	/	11.1
Bhyoa7455_1753	OmpR	26.2	4.9	123.2	3	3	/	22.4

Bhyoa7455_1822	pyruvate ferredoxin oxireductase	82.9	6.2	1083.2	23	22	9	44.0
Bhyoa7455_1866	hypothetical protein	88.0	5.1	154.1	5	3	/	6.1
Bhyoa7455_2024	hypothetical protein	14.8	4.8	282.5	6	6	/	59.1
Bhyoa7455_2073	hypothetical protein	107.0	6.4	342.2	8	7	/	10.8
Bhyoa7455_2078	AMP-activated protein kinase (AMPK) beta subunit glycogen binding domain (GBD)	29.4	4.8	245.6	5	5	/	31.7
Bhyoa7455_2091	Phosphoribosylamidoimidazole-succinocarboxamide synthase	27.3	6.0	109.6	2	2	/	13.4
Bhyoa7455_2097	alpha-amylase 1 (1,4-alpha-D-glucan glucanohydrolase)	82.5	5.4	170.5	8	6	4	13.6
Bhyoa7455_2102	hypothetical protein	25.3	4.9	138.8	3	2	/	23.5
Bhyoa7455_2217	bifunctional methionine sulfoxide reductase	23.3	6.6	120.3	3	3	/	22.8
Bhyoa7455_2288	hypothetical protein	15.4	8.9	109.3	4	4	4	30.5
Bhyoa7455_2322	Leucine-rich repeat containing protein	32.6	9.2	65.5	3	3	/	9.0
Bhyoa7455_2337	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	39.3	7.7	102.8	3	3	/	7.0
Bhyoa7455_2368	SpoIIAA, Anti-anti-sigma regulatory factor	12.2	5.2	181.9	2	2	/	26.4
Bhyoa7455_2371	putative methyl-accepting chemotaxis protein B	67.4	4.9	208.4	5	4	/	14.3
Bhyoa7455_2380	hypothetical protein	47.7	4.2	105.6	2	2	/	5.1
Bhyoa7455_2408	hypothetical protein	55.9	9.2	556.1	11	7	6	29.9
Bhyoa7455_2429	hypothetical protein	143.4	6.7	186.3	4	4	/	5.1
Bhyoa7455_2454	hypothetical protein	37.9	4.6	539.4	12	9	3	37.8
Bhyoa7455_2455	TPR domain-containing protein	26.2	5.5	140.2	3	3	/	13.9
Bhyoa7455_2467	preprotein translocase	34.3	8.8	278.4	4	3	3	13.7
Bhyoa7455_2523	hypothetical protein	37.5	5.8	66.5	3	3	/	5.4
Bhyoa7455_2532	methyl-accepting chemotaxis protein McpB	66.0	4.6	178.0	5	3	/	8.5
Bhyoa7455_2544	hypothetical protein	88.4	4.6	220.6	6	5	3	15.6
Bhyoa7455_2567	periplasmic-iron-binding protein BitB	15.4	4.7	178.3	2	2	/	44.6
Bhyoa7455_2634	putative inversin protein alternative isoform	64.3	4.2	339.7	6	4	4	17.5
Bhyoa7455_2645	sodium:alanine symporter family protein	50.0	9.6	154.2	3	3	3	8.8
Bhyoa7455_2653	hypothetical protein	45.7	4.4	538.4	13	11	5	29.0
Bhyoa7455_2657	ankyrin repeat-containing protein	27.0	4.0	214.6	4	4	3	21.2
Bhyoa7455_2678	**variable surface protein – VspA**	22.3	6.2	91.7	2	2	2	9.0

6.4 2D- gel analysis of whole cell extract of *B. hyodysenteriae*

In order to investigate the response of virulent and avirulent *B. hyodysenteriae* to iron-limitation, 2D gels of cell protein extracts, using ASB-14 to solubilize proteins, from exponentially growing bacteria in iron-replete and iron-limited conditions were performed.

Proteins were visualized by Colloidal coomassie blue (**Figure 6.4** and **Figure 6.5**) and quantified by the use of the software Image Master 2D Platinum 7.0. Analysis of the gels was performed by comparing the 2D maps of each group (iron-replete vs. iron-restricted). The analysis, followed by manual verification and filtering of detected spots, revealed 117 and 128 spots on the 2D gels from iron-rich and iron-restricted samples respectively, with 105 spots common to both conditions in the avirulent *B. hyodysenteriae* isolate P7455. Spots were considered only when they were present in 2 out of the 3 biological replicates.

The whole cell protein extracts of the virulent isolate P8544 resulted in very poor quality gels using the EttanDALTsix system (data not shown). Particularly, the pH range of 5.0 to 7.0 revealed hardly any spots. Proteins spots which could be detected in that area appeared as horizontal streaks and were thus not clear and distinctive. Therefore the sample extracts of P8544 were separated by using the Multiphor II Electrophoresis System for the second dimension resulting in higher quality gels compared to the EttanDALTsix. Due to several earlier optimization attempts to obtain sufficient gels using the EttanDALTsix system and decreasing sample stock of P8544, only two biological replicates for each condition of the virulent isolate were separated by the Multiphor II Electrophoresis System.

Analysis of the 2D gels of P8544 showed that 139 spots were identified on the iron-replete 2D gels and 166 spots on the iron-restricted gels, with 116 spots in common to both conditions. Only matched spots were considered when they were present in 2 out of the 2 biological replicates.

To test for significant differences in protein expression between the two groups, one-way analysis of variance (ANOVA) was performed within the software Image Master 2D Platinum 7.0 at a significance level of 0.05 or less. Following this, the data was filtered using the average ratio calculated by the spot intensity in the iron-replete group divided by the spot intensity of that same spot in the iron-limited group. A ratio of 1.5 and above was

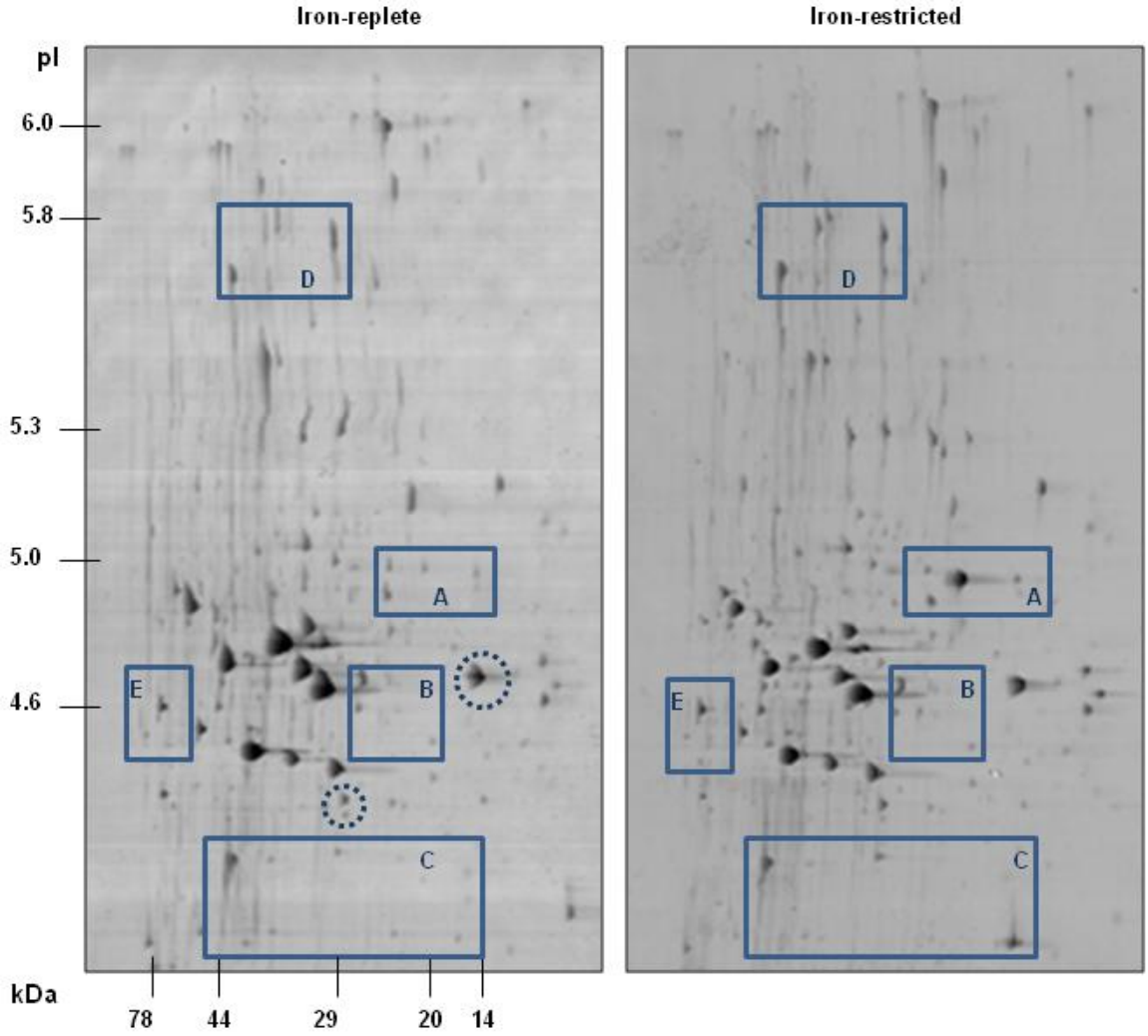


Figure 6.4: Comparison of the 2D gels of *B. hyodysenteriae* P8544 under iron-replete and iron-restricted conditions. The images are representative for the two biological replicates of each condition carried out with the Multiphor II Electrophoresis System. In the sample from iron-replete conditions 139 protein spots were identified whereas 166 spots were detected in the sample from iron-restricted conditions. One hundred and sixteen (116) proteins spots were in common in both tested conditions. The blue boxes A and B contain protein spots which were significantly differentially expressed ($P< 0.05$ and 1.5 fold expressed) between each of the conditions. These boxes have been enlarged in **Figure 6.6** to show the individual areas of interest in more detail together with relevant protein spot identification. Spots circled in blue were used as a landmark.

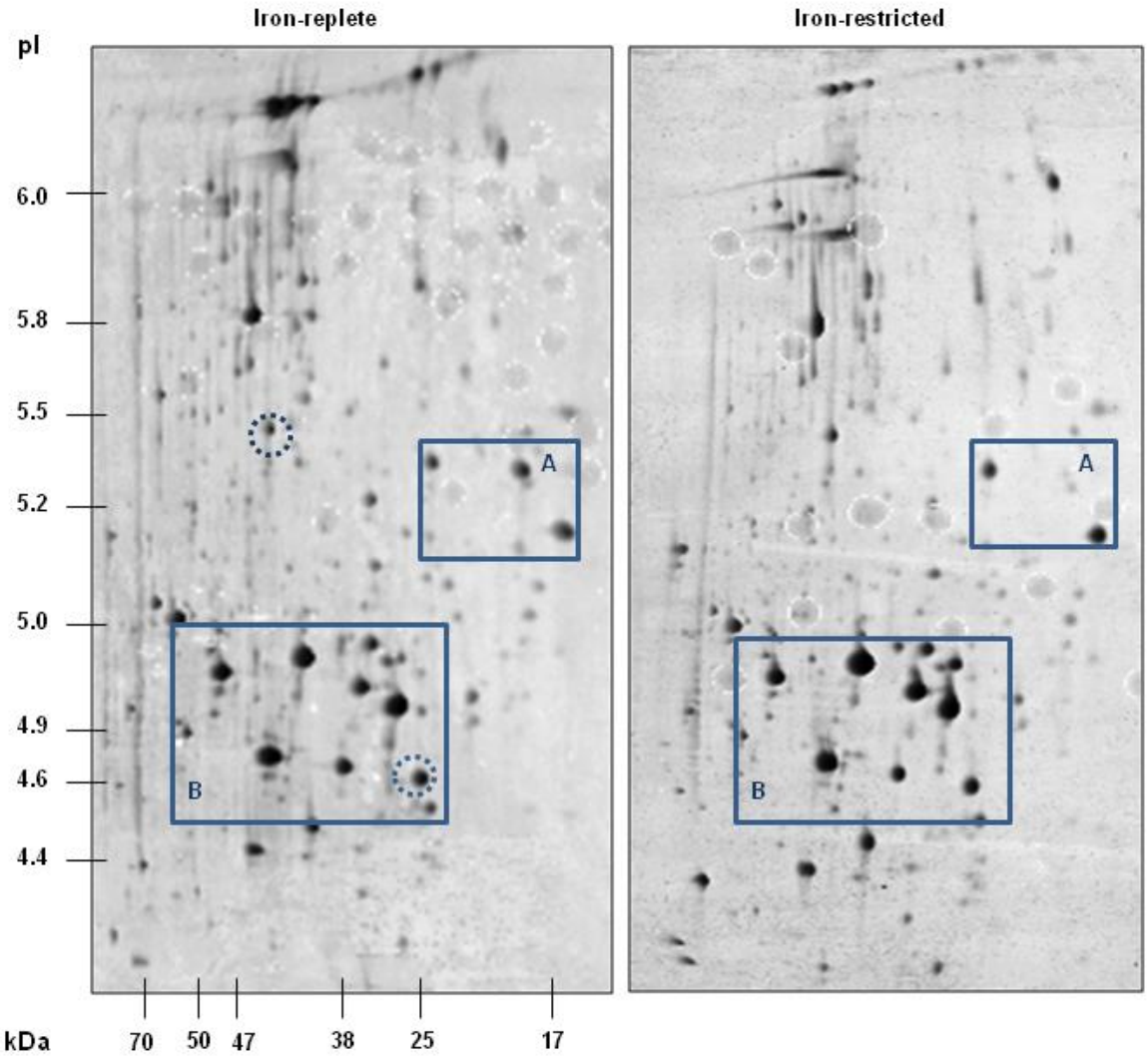


Figure 6.5: Comparison of the 2D gels of *B. hyodysenteriae* P7455 under iron-replete and iron-restricted conditions. The images are representative for the three biological replicates of each condition carried out by the EttanDALTsix system. In total, 117 proteins spots were detected in the iron-replete fraction, while 128 spots were identified in the iron-restricted fraction with 105 protein spots in common in both tested conditions. The blue boxes A and B contain protein spots which were significantly differentially expressed ($P < 0.05$ and 1.5 fold expressed) in of the conditions. These boxes have been enlarged in **Figure 6.6** to show the individual areas of interest in more detail together with relevant protein spot identification. Spots circled in blue were used as a landmark.

considered to be significant for these gels. Spots matching these two criteria were assigned spots of interest and excised from the gel and identified by MALDI (**Table 6.6**).

In the avirulent *B. hyodysenteriae* isolate P7455 a total of three protein spots had significantly increased intensity in the iron-rich gels and 5 spots had significantly increased intensity in the iron-restricted gels. However, a number of these spots were then manually filtered out of the analysis as the expression intensity was less than 1.5 fold difference between the conditions. The alkyl-hydrogen peroxide reductase (Bhyoa7455_0997) was 7.53 fold more expressed under iron-restricted condition while the two flagella filament core proteins FlaB2 and FlaB3 (Bhyoa7455_2357, Bhyoa7455_1918) were 2.3 to 2.8 fold more expressed under conventional growth condition compared to iron-limited (**Table 6.6**). Compared to the avirulent isolate, more proteins were found to be significantly higher expressed under iron-restriction in the virulent isolate P8544. A total of 15 spots identified had significantly increased intensity (2 in iron-replete and 13 in iron-restriction) while only 8 of these proteins were identified to be significantly more expressed (1.81 to 19.82 fold) under iron-restriction than under iron-rich conditions. The hypothetical protein Bhyov8544_0313 was the only protein identified to be significantly more expressed (1.61 fold) under iron-replete conditions in P8544.

The non-haem iron containing ferritin (Bhyov8544_1528) was with 19.82 fold the most significant differentially expressed protein detected under iron-limitation in the virulent isolate (**Table 6.6** and **Figure 6.6**). Additionally two hypothetical proteins (Bhyov8544_1066; Bhyov8544_2473) were shown to be 1.81 to 3.34 fold more expressed under iron-restricted conditions. Further analysis of the identified hypothetical proteins was performed by InterProScan and revealed that the hypothetical proteins (Bhyov8544_0313 and Bhyov8544_1066) contained a Signal peptide while no specific hits were found for the hypothetical protein Bhyov8544_2473.

The non-haem iron containing ferritin protein was also identified in the OM enrichment fraction of P8544 and was also only present in the detergent phase obtained from iron-restriction (**Table 6.4**). Moreover the high abundant alkyl-hydrogen peroxide reductase on the iron restricted 2D-gel of P7455 (**Table 6.6** and **Figure 6.6**) was only detected in the detergent phase of bacteria cultured in iron-restricted conditions. (**Table 6.4**).Thus, the 2D-results confirmed the data obtained from the LC/ESi-MS/MS method. Nevertheless the flagella filament core protein

Table 6.6: Overview of the significant differentially expressed protein of *B. hyodysenteriae* P7455 and P8544detected by 2D-gel electrophoresis.

Locustaq	Protein	MW	pI	Score	No of peptides	SC	Fold change
Increased expression of proteins under iron-replete conditions							
Bhyoa7455_2357	flagella filament core protein FlaB2 *	28.2	5.4	86	15	66.8	2.80
Bhyoa7455_1918	flagella filament core protein FlaB3	30	4.7	153	20	59.7	2.30
Bhyov8544_0313	hypothetical protein	78	4.6	224	39	40.7	1.61
Increased expression of proteins under iron-restricted conditions							
Bhyoa7455_0997	alkyl-hydrogen peroxide reductase *	20.9	5.3	166	21	72.5	7.52
Bhyov8544_1528	Non-haem iron containing ferritin *	20	5.0	161	18	89.5	19.82
Bhyov8544_0605	electron transfer flavoprotein beta subunit	28.6	5.8	321	24	82	1.81
Bhyov8544_1066	hypothetical protein	23.2	4.4	105	11	43.6	3.34
Bhyov8544_2473	hypothetical protein	18.5	4.4	74.7	13	46	2.17
Bhyov8544_1831	intracellular protease	18.5	4.6	75	8	40.8	1.67
Bhyov8544_0903	elongation factor Tu	44.4	5.8	165	23	52.7	2.85
Bhyov8544_0814	REC, Signal receiver domain protein	13.9	4.9	77.2	6	66.7	1.87
Bhyov8544_2608	translation elongation factor P (EF-P)	21.3	4.7	138	11	61.5	2.43

*Detected in the OM enrichment fraction obtained under iron-limited growth conditions presented in Table 6.4.

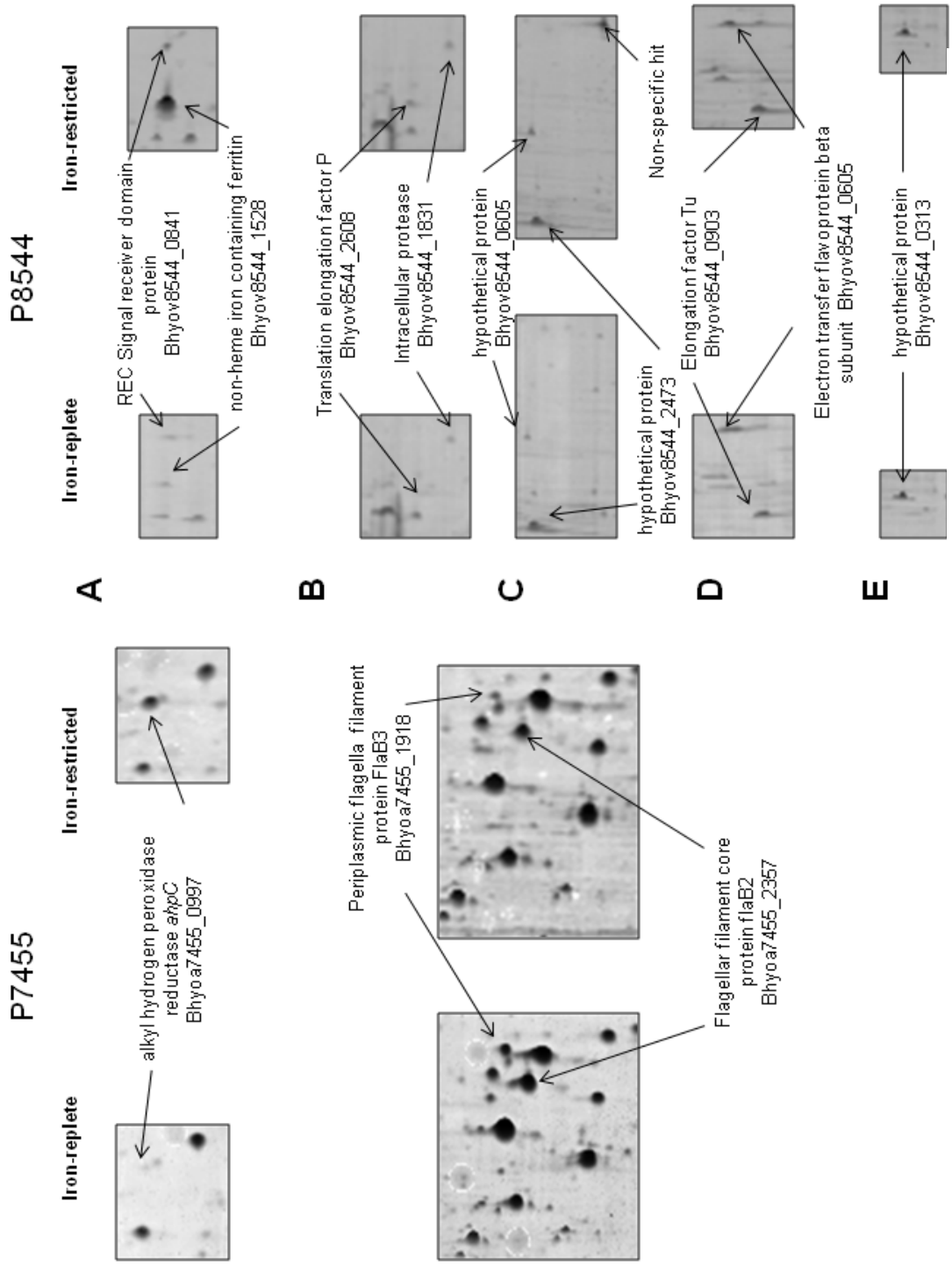


Figure 6.6: Enlarged images of the boxed sections of the 2D-gels shown in Figure 6.4 and Figure 6.5

FlaB2 in P7455 (Bhyoa7455_2357) only detected in the OM profile under iron-restricted conditions (**Table 6.4**) was shown to be 2.8 fold more expressed under iron-replete conditions than under iron-limited conditions by 2D-gel analysis suggesting that the protein should have been detected in either both conditions or iron-rich conditions due to its higher abundance as shown by 2D-gel electrophoresis. The raw data obtained from the three biological replicates of P7455 contained hits for that protein although the protein did not match the criteria of containing four "y" or "b" ions in a row and was thus not included. Therefore using these criteria it cannot be concluded if the protein was present in the sample set or not.

6.4.1 Investigation of insoluble/ hydrophobic proteins

Hydrophobic proteins, particularly membrane proteins, are underrepresented in 2D-gels due to their low solubility (Monteoliva & Albar, 2004).

In this study insoluble proteins obtained after extraction of *B. hyodysenteriae* cells grown under iron-replete and iron-restricted conditions were not included in the 2D-gel analysis. Therefore to identify proteins of the insoluble fractions of *B. hyodysenteriae* P8544 and P7455, protein extracts were boiled for 5 min and separated on a 12.5% SDS-PAGE as described previously **(2.7.2)**.

The heated samples resolved into similar series of high molecular mass protein complexes with a main protein pattern in 40 kDa in size observed in all tested isolates and conditions **(Figure 6.7**). The pattern was excised from the gel of *B. hyodysenteriae* P8544 obtained under iron-restricted conditions and identified as VspF by MALDI and known as one of the variable surface proteins in *B. hyodysenteriae*.

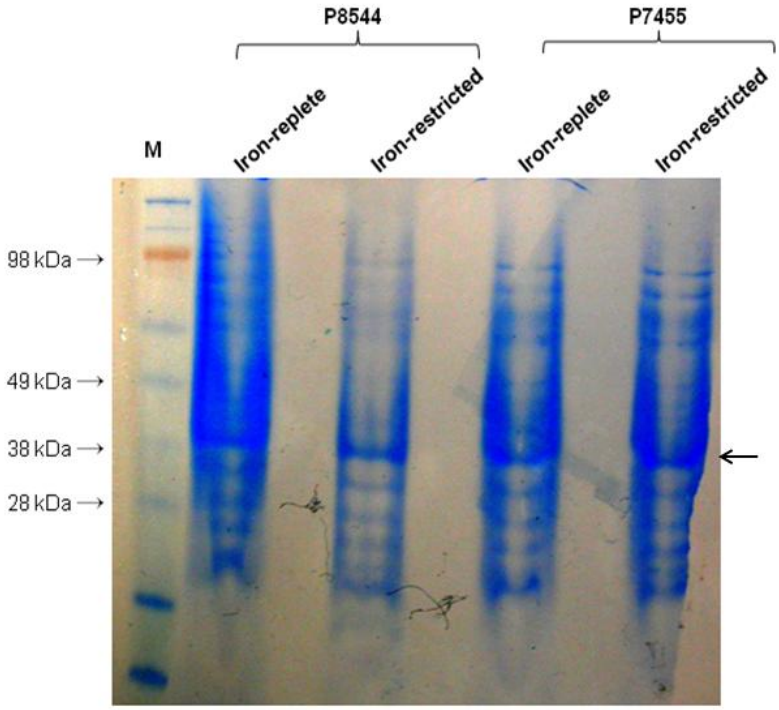


Figure 6.7: Simply Blue stained SDS-PAGE gel of insoluble proteins of *B. hyodysenteriae* P8544 and P7455. The insoluble protein pellets obtained after extraction of *B. hyodysenteriae* P8544 and P7455 was boiled for 5 min and separated by SDS-PAGE as described before. The arrow highlights the band at approximately 40 kDa which was selected and analysed by MALDI.

Table 6.7: Identification of the excised abundant protein band of the separated insoluble fraction of *B hyodysenteriae* P8544.

Isolate	Growth condition	protein	MW	IP	Score	No of peptides	SC
P8544	iron-restriction	VspF	41.3	6.2	91.8	11	30.5

6.5 Discussion

As described previously, iron is an essential element for growth of many organisms including *B. hyodysenteriae.* It was shown in **Chapter 5** that isolates of virulent and avirulent *B. hyodysenteriae* were able to adapt to an iron-restricted environment containing up to 0.1 mM of dipyridyl resulting in an extended lag phase of between 30 and 32 hours compared to 15 and 17 hrs for untreated cultures. Moreover, several genes were shown to be differentially expressed under iron-restricted conditions in both isolates indicating that virulent and avirulent isolates may respond differently in the host which could influence their ability to cause infection in pigs. Therefore, the response to iron stress in the proteome of virulent and avirulent *B. hyodysenteriae* isolates was investigated in cells during exponential growth phase in order to detect differences in their protein profile which could be used as a potential protein marker to distinguish between virulent and avirulent strains

6.5.1 Alteration of the predicted OMP of virulent and avirulent *B. hyodysenteriae* in response to iron-limitation

Identifying OMPs expressed during infection is a prerequisite for the analysis of the host-pathogen interaction. In order to identify differently expressed proteins in *B. hyodysenteriae,* the proteome profile was compared of proteins detected under conventional and iron-limited conditions to mimic *in-vivo* host conditions. Of particular interest was the identification of proteins found in an environment simulating low iron conditions as encountered within host, as these proteins likely contribute to the adaptation process and potentially virulence in *B. hyodysenteriae*.

Studies about the OM composition of *B. hyodysenteriae* are still limited. Therefore, the OM of one virulent and one putative avirulent strain was analysed using shotgun proteomics which has been used to identify proteins in complex mixtures (Gygi *et al.*, 1999). However, shotgun proteomics encounters limitations which affect the data interpretation. In some cases it is not possible to discriminate between proteins that are products of different genes from the same gene family (gene paralogues) (Nesvizhskii *et al,* 2003). Besides determination of the correct peptide sequence which shares high homology with several other peptide sequences is difficult and can result in incorrect protein identification (Nesvizhskii *et al,* 2003). Additionally, methodology used in this

study did not allow quantification of proteins detected in the OM-enriched phase. Therefore, shotgun proteomics gives a global overview of proteins which were found to be expressed in each sample set without determination of relative protein expression levels between strains and growth conditions and is thus not alone sufficient to comprehensively characterize the proteome (Michalski *et al.*, 2011). Accurate quantitation of protein abundance in OM could have been achieved when combined with labeling strategies such as modifying isobaric tags for relative and absolute quantitation (iTRAQ) (Ross *et al.*, 2004), labeling proteins with isotope-coded affinity tags (Gygi *et al.*, 1999), or metabolically labeling proteins by incorporation of stable isotope labels with amino acids in cell culture (SILAC) (Ong *et al.*, 2002). However, these techniques are costly and designed for systematic analyses –intensive (Eshghi *et al.*, 2009). Quantitation of protein expression among *B. hyodysenteriae* strains and growth conditions has been therefore focused on the more budget-priced comparative 2-DGE approach.

Due to their significant proportion of cholesterol, the OM of *Brachyspira* has been shown to be unique compared to other pathogenic spirocheates (Plaza *et al.*, 1997). Studies also indicated that the OM of *Brachyspira* is more susceptible to osmotic stress and higher concentrations of detergents shown by the rapid loss of their OM integrity (Trott *et al.*, 2004). These unique features will influence the biological functions of the OM of *Brachyspira* compared to other pathogen spirochaetes and will also have an impact on the selective removal of OM using the standardized methods described for *Leptospira* and other spirochaetes.

The OM content of virulent and avirulent *B. hyodysenteriae* isolates revealed several cytoplasmic-related proteins including periplasmic flagella proteins indicating that the detergent phase did not contain only membrane-associated proteins. Due to the predictable composition of the outer-membrane of Gram-negative bacteria, the software BOMP was used to predict OMPs out of the detergent fractions on the basis of beta-barrel motifs typically found in proteins associated with that cellular location. The software predicted only a small percentage of proteins (11-14%) to be potential OMPs which was already expected due to the presence of many other proteins related to different cellular compartments. As the OM of *B. hyodysenteriae* appears to be more fragile than in other spirochaetes, lower concentrations of Triton-X-114 (less than 1%) may have enhanced the enrichment of OM in the detergent phase and could be the basis for future study. Although the OMP enrichment method using Triton-X-114 has been successful for spirochaetes

(Cunningham *et al.*, 1988; Haake, 2000), other studies reported the technique to be insufficient manifested by the increased presence of cytoplasmic proteins (Radolf *et al.*, 1995). Due to the fragility of the OM of *Brachyspira,* proteins anchored to the inner membrane are likely to solubilze and are thus being detectable in the detergent phase. Therefore, alternative methods including membrane vesicle fractionation have been suggested and performed for extraction of the OMP in spirochaetes (Cullen *et al.*, 2003a; Trott *et al.*, 2004). The visual observation of the response of *B. hyodysenteriae* OM fraction to different concentrations of the detergent under the electron microscopy described by Li *et al.* (1995) would be useful to identify satisfactorily a concentration to extract the OMPs but prevent the cells from lysis. Therefore, optimization regarding the known OM extraction method would improve the selective isolation and thus identification of OMPs in *B. hyodysenteriae*.

Identification of surface-associated cytoplasmic proteins has been reported regularly in previous studies. Proteomics analysis of surface-associated proteins in various bacteria including *Rickettsia, Chlamydia* and *Campylobacter* detected several cytoplasmic proteins such as ribosomal proteins and heat shock proteins (Molestina *et al.*, 2002; Qi *et al.*, 2013; Cordwell *et al.*, 2008)) as observed in the OM of *B. hyodysenteriae*. Therefore, specifically designed experiments would be mandatory to elucidate transport and anchoring mechanisms to the OM of these proteins routinely detected in the outer-surface of pathogens. This was, however, not a primarily aim of this study.

Even though this study detected a high number of proteins which were not recognized as OM-related, the data provided a novel insight in the expression profile of proteins under two different growth conditions. The KEGG analysis indicated that a higher number of proteins obtained from the iron-replete OM fraction in both isolates were categorized by KO orthologues than proteins of the iron-deplete fraction. Interestingly, the percentage of identified proteins assigned no function by KEGG increased approximately by 13-19% in virulent and avirulent isolates under iron-restricted growth conditions highlighting the fact that biological function of the majority of these expressed proteins in such an environment remains unknown.

The classes of protein showing most obvious reductions in response to iron restriction were seen in the classes of Membrane Transport and Energy Metabolism. While several transporter units identified under conventional growth conditions were absent under iron-

depletion, the preprotein translocase subunit SecG was exclusively expressed. Export of most proteins occurs via a multi-subunit complex termed preprotein translocase and is well studied in *E. coli* (Kontinen & Tokuda, 1995). The genome sequence of *B. hyodysenteriae* showed that the pathogen possess 6 genes associated with the translocase complex termed *secA, secD, secF secY, secE and secG*. The proteins SecA and SecF were found to be expressed in P8544 under both conditions while SecD was detected in P7455 under the two growth (**Appendix 6**) conditions suggesting active transport of proteins under the tested conditions to maintain cell function and viability. Increased expression of SecY and SecE was also seen in *N. gonorrhoeae* under iron-limitation (Ducey *et al.*, 2005). Even though SecG expression has not been associated with iron depletion, different studies showed that SecG is essential for cell growth and protein translocation particularly under challenging conditions as low temperatures (Kontinen & Tokuda, 1995) suggesting that this protein plays a similar important role in various stress situation like iron starvation.

However, although some transport systems detected in the iron-rich fraction did not occur in the iron-deplete conditions it might be that similar transport mechanisms were still expressed but could not be classified as such as their function is not further characterized and thus not available in the KEGG database as shown by the higher percentage of unclassified proteins under iron starvation.

Moreover two further proteins involved in the motility of *B. hyodysenteriae* were assigned to Environmental adaptation, particularly the flagella filament core proteins FlaB2 were found in both isolates under iron-depletion. The FlaB2 (Bhyoa7455_2357) was identified as significantly more expressed under conventional than under iron-restriction in P7455 by 2D gel analysis and is thus not in agreement with the findings of the OM fraction showing that the same protein was not detected under iron-replete conditions. The method LC-ESI-MS/MS used for identification of OMP in this study does not distinguish between up- and down-regulation and therefore no conclusion about expression intensity of proteins using this method can be made. However, as FlaB was detected and verified it was concluded that these protein was higher abundant under iron-restriction than under conventional conditions. Nevertheless, the 2D-analysis proved this protein to be clearly present under both growth conditions also it was 2.80 fold more expressed under iron-replete conditions in P7455. Although cells were always harvested under the same OD, differences between the biological replicates especially of samples analyzed by 2D and LC-ESI-MS/MS may

have occurred among the avirulent isolates causing this problem. Unfortunately, the discrepancy between the two methods remains unknown.

The 2D gel data was indicating that the proteinsFlaB2 and FlaB3 are down-regulated in the avirulent isolate under iron-limitation. Decreased expression levels of flagella proteins have been shown in other bacteria under iron starvation (Chao *et al.*, 2005). Down-regulation of flagella proteins would save the bacteria energy which might be needed to adapt to an iron-restricted environment. Therefore, it might be possible that some genes involved in the flagella machinery are up-regulated like the identified proteins FliC (Bhyoa7455_1135) and FlaB (Bhyov8544_2088) while others are down regulated. Alteration of expression levels of FlaA and FlaB were shown to be dependent on growth-phase stages as well as iron-concentration in *H. pylori* (Merrell *et al.*, 2003) supporting the hypothesis that flagella proteins are differentially expressed under conventional and iron-limited conditions in *B. hyodysenteriae.* As transcription and thus expression levels of these subunits seem to alter within growth it may also explain the detection of FlaB under iron-restriction only in P7455 by LC-ESI-MS/MS. Variation in the expression and thus concentration of flagella subunits may enhance the adaptation process of *B. hyodysenteriae* under these environmental conditions. Thus, the data is suggesting that the motility of *B. hyodysenteriae* is influenced by the availability of iron. However, if a lower iron-content increases or decreases the motility of the pathogen is unclear. Studies in have shown that a decreased number of cells of *H. pylori* lost motility when subjected to an iron-limited environment (Merrell *et al.*, 2003). Nevertheless, further experiments including swarm plate assays and video microscopy of cells of *B. hyodysenteriae* exposed to conventional and iron-restricted media would determine whether iron starvation has a similar effect as observed in other bacteria and provide better insight in the motility behavior of this pathogen.

Lipoproteins have been shown to be important in pathogenicity in spirochaetes particularly *Leptospira* (Cullen *et al.*, 2003a; Guerreiro *et al.*, 2001). A total of 55 and 74 lipoproteins were identified using SpLiP software in the virulent and avirulent isolates respectively under both tested growth conditions. The software has been developed by Setubal *et al* (2006) as it was shown that the lipobox differed significantly from other Gram-negative bacteria. The conventional lipoprotein prediction tools are based on the Heijne consensus lipobox of *E. coli* containing Gly or Ala in the -1 position of the liporotein signal peptide (Heijne., 1989). However, deeper analysis of the lipobox of spirochaetes revealed that a

high percentage of lipoprotein signal peptides contained a Ser at the -1 position resulting in false prediction by Psort (Setubal *et al.*, 2006). Moreover, Asn and Cys were also found to occur in this position which may be unique to spirochaetes causing additional insufficient prediction using the conventional software tools. Therefore, the SpLiP program was designed to accept Ala, Gly, Ser, Asn, and Cys in the -1 position for the prediction of lipoproteins in spirochaetes.

The three proteins MglB, BmpB and BitC expressed under both conditions and strains have been already identified as lipoproteins in the literature. The software SpLip predicted these three proteins as lipoproteins indicating that the results in this study conform to earlier investigations. However, more lipoproteins have been identified by SpLip in this chapter which has not been described in *B. hyodysenteriae* so far. Some of these new identified lipoproteins are reported as lipoproteins in other spirochaetes like the ABC-type oligopeptide transport system, periplasmic component (OppA) in *Borrelia*. Especially lipoproteins predicted under iron-restricted conditions may be of interest for further research including OppA identified in P8544 (Bhyov8544_2611). The three lipoproteins OppA1-3 identified in *Borrelia* share significant similarity with peptide-binding proteins of ABC-type transporters (Kornacki 1998) which are recognized to be involved in cell signalling (Dunny *et al.*, 1995), adhesion, antimicrobial resistance in *Streptococcus* and thus virulence (Alloing *et al.*, 1990; Alloing *et al.*, 1994; Cundell *et al.*, 1995). Identified OppA proteins in *B. hyodysenteriae* shared 35% amino acid sequence similarity with OppA proteins in *Borrelia*. The presence of six copies of genes encoding for the OppA proteins in the genomes of *B. hyodysenteriae* leads to the conclusion of a wide functional repertoire for these proteins which might differ from the function reported in other bacteria. Therefore, it can be inferred that the OppA proteins also contribute to the virulence of *B. hyodysenteriae*.

Additionally the *B. hyodysenteriae* genomes contain two copies of the thiol-disulfide interchange protein DsbD-like proteins which were assigned as a lipoprotein and detected under iron-limitation conditions. DspD participates in the protein-folding machinery and thus important for the correct folding of a great number of proteins that are exported to the cell envelope of bacteria (Nakamoto & Bardwell, 2004). Misfolded proteins are subsequently degraded by periplasmic protease or cannot perform their function correctly leading to reduced virulence in pathogens (Rahme *et al.*, 1997). The primary function of the DsbD-like proteins particularly under iron starvation has not been studied so far but it

is likely that it contributes to the stress response network to ensure the survival of *B. hyodysenteriae* cells. As these proteins play a critical role in protein-folding and were so far not investigated in spirochaetes, it would be worthwhile to study these proteins in more detail to discover their main role in *B. hyodysenteria*.

Although examples of genes encoding for the lipoproteins described above are present in the avirulent *B. hyodysenteriae* isolate the absence of these expressed predicted lipoprotein in the iron-replete fraction of the avirulent P7455 may be another indication of the non-pathogenicity of these isolate but clearly further work needs to be carried out to prove their host-pathogen function *B. hyodysenteriae*.

6.5.2 Proteins expressed as a response to iron-limitation in *B. hyodysenteriae*

To date only one study addressing iron-related response of *B. hyodysenteriae* has been published (Li *et al.*, 1995). However, no information about iron-regulated protein expression has been reported. As described in **chapter 5** avirulent and virulent *B. hyodysenteriae* isolates have been shown to adapt to an iron-limited environment. Nevertheless, the adaptation mechanism and proteins involved to overcome this restriction are not known. The current study revealed expression of 11 proteins which were detected under iron-restricted conditions in the virulent and avirulent *B. hyodysenteriae* isolate. As these proteins were expressed in both strains under stress-like conditions it could be that that these proteins may contribute to the adaptation and survival process of this pathogen in a host.

The gene *feoB* has been already shown to be more transcribed under iron-restricted conditions in both isolates. These findings were confirmed by the detection of FeoB in OM enriched fractions under iron-limited conditions in the virulent and avirulent isolate supporting the assumption that FeoB plays an important role in the acquisition of ferrous iron in *B. hyodysenteriae*.

The alkyl-hydrogen peroxide reductase AhpC was highly abundant in P7455 grown under iron depletion and additionally detected in the OM enriched fraction of both isolates. In *C. jejuni* AhpC was also found to be expressed at a higher level in cells grown under iron-restricted conditions than iron-sufficient as well as in *B. subtilis* when cells were exposed

to a high salt concentration or heat. Besides, knockouts in *C. jejuni* have shown that cells lacking the *ahpC* were more susceptible to oxidative stress and oxygen levels indicating that the protein is an important factor for the pathogen to survive in stress situations and thus likely to contribute to the higher tolerance levels in *B. hyodysenteriae*. The increased production of AhpC in avirulent *B. hyodysenteriae* under iron-restricted condition might be due to the activation of iron-transport system like the FeoB resulting in a higher intracellular concentration of iron leading to a raised level of reactive oxygen intermediates which could damages to the DNA. The alkyl-hyperperoxide reductase as well as the superoxide dismutase (SOD) are known mechanisms which a number of microorganism have evolved to withstand oxidative stress. Therefore, it might be possible that the expression of AhpC might be coupled with the activation of iron-uptake systems functioning as a survival strategy. Similar mechanisms have been demonstrated in *E. coli* showing that the superoxide dismutase SodA and SodB are regulated by the ferric uptake system (Niederhoffer *et al.,* 1990). The NADH oxidase is known to be a virulence factor in *B. hyodysenteriae* enabling the pathogen to tolerate low levels of oxygen (Stanton *et al.*, 1999). Additionally, the higher aerotolerance as described in other Gram-negative bacteria provided by the increased production of AhpC would be another protective response and thus survival mechanism by *B. hyodysenteriae* when transmitted from one host to another.
Even though AhpC was not confirmed to be significantly expressed in an iron-limited condition in P8544 by 2D-gel analysis, this might be due to the different separation system used as discussed later; the protein was identified in the iron-restricted OM fraction of P8544 indicating that the AhpC contributes to the survival mechanism especially under host-like conditions.

The non-haem iron-containing ferritin was also identified under iron-limitation in both isolates while the protein was significantly more expressed in P8544 when cells were grown in a low iron-containing environment. The protein is known as an iron storage protein and was described to be up-regulated under stress situations including acidic conditions in *H. pylori* (Huang *et al.,* 2010) and has been also shown to be essential in the protection of intracellular iron overload and oxidative stress in *C. jejuni* (Wai *et al.,* 1996) suggesting that the protein is crucial for *B. hyodysenteriae* under the tested conditions.

The variable surface proteins have been already described throughout the thesis to be an important virulence factor in *B. hyodysenteriae.* The gene encoding for VspI was shown to be highly conserved among virulent and avirulent isolates (**chapter 3**) and was also

expressed under iron-restricted condition in P8544 and P7455 suggesting that this protein is up-regulated in response to iron-starvation and may be important in virulence. Antigenic variation is proposed to function in the adaptation process and survival to host environments in other spirochaetes and other bacteria (Burgos *et al.*, 2012; Haake, 2000). However, the function of VspI in the antigenic variation process in *B. hyodysenteriae* remains still to be remained.

6.5.3 Possible biomarkers

Biomarkers can be defined as parameters that are measured and evaluated as an indicator of normal biological process, pathogenic process, or pharmacological response to a therapeutic intervention (Paone et al., 1980). For this study, the goal was to identify possible biomarker proteins which were expressed differently between strains differing in pathogenicity.

Studies of the OM enrichment fractions of *B. hyodysenteriae* P8544 and P7455 already detected proteins which were found in the virulent or the avirulent isolate under one of the tested growth conditions (**6.5.2**). However, this method is not able to study the expression intensity of these detected proteins. Therefore, 2D-gel electrophoresis was performed in order to detect proteins which were differentially expressed under both tested conditions and ideally showed a significant fold differences between virulent and avirulent *B. hyodysenteriae*.

However, it needs to be emphasized that two different electrophoresis systems were used for the separation of the protein extracts of P8544 and P7455 making conclusion from the 2D-gels obtained from both isolates not comparable. Unfortunately, proteins samples of the virulent P8544 did not resolve properly using the EttanDALTsix system. Especially proteins in the pH range of 5.0 to 6.0 appeared as horizontal streaks while in the pH range of 4.0 to 5.0 just a few but very highly abundant spots were observed for all three biological replicates obtained under both conditions. As previous whole cell protein extracts of *B. hyodysenteriae* P8544 were successfully separated by the Mulitiphor II system using IPG strips with a pH range between 3-10 and the exact same settings for the first dimension (data not shown) the new obtained samples were separated by conventional SDS-PAGE and compared to the whole cell protein extract of the avirulent sample set which was successfully electrophoresed by the EttanDALTtwelve system. The 1D gel did

not reveal obvious differences in the abundance of protein patterns between the virulent and avirulent strains which might have be an explanation for the insufficient 2D-gels of the P8544 samples. Further attempts have been made in loading lower protein concentration and using fresh stock of IPG buffer resulting in similar 2D images as described before. As the separation of proteins samples of P8544 have been sufficient using the flatbed system, samples were henceforth separated using the Multiphor II system resulting in better resolved protein spots. However, the replicates of the iron-restricted samples resulted in more distinctive protein spots on the entire gel than spots from the samples grown under iron-replete conditions. Spots, particularly settled in the area between the pH of 5.5 and 7.0 on the gels were again not clear and distinctive and were thus not included in the further analysis which consequently affected the number of spots detected in each group. Although the Multiphor II system showed a better resolution than the vertical system, other optimization steps will need to be carried out to obtain single and distinctive spots and the higher pH range in any future studies. Other attempts should be done in extending the focusing time or different composition of the extraction buffer. The reason for the poor quality of the 2D-gels generated by the EttanDALTsix system remains still unsolved.

The 2D-gel analysis did not reveal any shared proteins in the virulent and avirulent isolates to be significantly abundant or underrepresented although 11 proteins could be detected to be common among both strains under iron-restricted conditions as well as iron-replete conditions. The different systems may be a reason for the detection of distinct proteins of interest in both isolates. The most abundant and thus significant protein spots in the iron-restricted samples were the alkyl-hydrogen peroxide reductase in P7455 and the non-haem iron-containing ferritin in P8544. Both proteins were discussed earlier to be detected under iron-limitation only. As these proteins were significantly more expressed under iron-limited condition than under conventional growth conditions they might be a potential marker for the detection of *B. hyodysenteriae* infection. However, further work is required to prove if these proteins are expressed *in-vivo* and also found in a wider panel of isolates and hence have potential as a biomarker.

Unfortunately, the 2D gel data did not show a protein which was more expressed under iron-restriction in one of the isolates which could have potential as biomarker to distinguish between virulent and avirulent *B. hyodysenteriae* isolates.

6.5.4 Insoluble proteins

Limitations in 2-DE are reported regarding low-abundant proteins and very hydrophobic proteins. In this study a narrow pH range from 4.0 to 7.0 was choosen in order to obtain the best possible resolution of proteins. In case of optimazation, smaller pH ranges have the advantage over greater ranges e.g 3-11 as proteins similar characteristics regarding solubilty and charge.

Hydrophobic proteins, mainly membrane proteins, are known to be under-represented on 2D-gels or even likely to absent due to their low solubility or the possibility of not getting transferred onto the second dimension (Rabilloud *et al.*, 2008). Therefore the insoluble protein extracts from each isolate and growth condition was separated by SDS-PAGE in order to identfy proteins which were present but could not be included in the 2D-analysis.
The main protein band observed on the 1D-gel was identified as VspF containing a β-barrel motif predicted by BOMP indicating that it is outer-membrane associated as described previously (Gabe *et al.,* 1998). As the same protein band was observed in all other tested samples it can be considered that VspF was also expressed in P7455 which was already shown by the data of the OM enrichment fraction indicating that VspF was present in both isolates and conditions (**Appendix 6**). The protein VspF was identified by the extraction using Triton-X-100 and was reported to be heat-modifiable (Plaza *et al.*, 1997; Witchell *et al.*, 2011). Studies by Witchell *et al.* (2011) showed that VspF is the most predominant protein in the outer-membrane of *B. hyodysenteriae*. Additionally heated samples of VspF resolved into a 39 kDa protein pattern using SDS-PAGE as seen in this study, while unheated samples appeared as a series of higher molecular mass proteins. Compared to the native form, heat-denaturated VspF was not able to be detected by Immunoblotting (Witchell *et al.,* 2011) confirming that confirmation plays a crucial role in antigenicity and needs to be considered by the development of recombinant protein vaccines.

In conclusion, the work described in this chapter allowed to catalogue proteins which were specifically expressed under conventional and host-like conditions in virulent and avirulent *B. hyodysenteriae* isolates. Previous chapters suggested that both isolates share high similarity with each other while transcriptional profiling and further investigation of their proteome discovered differences in their respond to an iron-limited environment which may affect their pathogenicity potential and persistence in the host. The impact of detected

variations in differential protein expression among the isolates clearly requires more work as most of the potential proteins have not been further studied in *B. hyodysenteriae* in respect of virulence. Therefore, the catalogued proteins generated in this chapter would serve as a good starting point in the investigation of other unknown virulence mechanism in *B. hyodysenteriae* leading to a better understanding of the pathogenicity itself which then would enhance screening studies in order to identify possible marker to distinguish between virulent and avirulent isolates.

Chapter 7: General Discussion

The intestinal spirochaete *B. hyodysenteriae* is the aetiological agent of SD, causing a severe mucohaemorrhagic diarrheal disease affecting pigs of all ages around the world. Therefore, efforts have been made towards the development of a vaccine; so far without any effective success hence control still depends on expensive antibiotic treatments as well as time-consuming and costly restocking of affected pigs.

Since 1982, non-disease-causing isolates of *B. hyodysenteriae* have been reported occasionally but no significant research has been conducted (Achacha *et al.*, 1996; La *et al.*, 2011; Lyson, 1982). Nevertheless, the occurrence of avirulent *B. hyodysenteriae* isolates in healthy status herds in the UK, not showing any clinical signs of SD, presents a major hazard to pig farmers and the industry because of expensive medical treatment and a high mortality rate. Preliminary attempts have been made to find a discriminatory marker(s) to distinguish between virulent and avirulent isolates, although at the time this study commenced no complete genome sequence of *B. hyodysenteriae* was available, hampering the analysis and leading to false-assumptions about these strains.

The work described in this thesis is the first broader investigation regarding pathogenic and non-pathogenic isolates of *B. hyodysenteriae*. The main focus of the work was to identify molecular differences between virulent and putative avirulent isolates using genomics and proteomics as the basis in order to define possible strain markers.

In the present study an avirulent *B. hyodysenteriae* strain was classified as an isolate that was recovered from a pig which did not show any typical sign of SD; therefore, the classification as avirulent was only based on clinical history. Furthermore, some of the work depended upon a DNA panel from 26 virulent and 23 avirulent isolates collected from different geographical regions of EU and non-EU countries and for which only a very limited number of viable bacteria were available. Five isolates in the avirulent panel were re-confirmed by the Scottish Agriculture College Veterinary Services (Jill Thomson, personal communication) as non-pathogenic in experimental challenges and thus not causing SD in pigs. Unfortunately, none of these confirmed isolates were available for culturing and primarily genome sequencing which would have added support to the data obtained in this thesis. Therefore, out of the available *B. hyodysenteriae* isolate panel, the virulent isolate P8544 and the avirulent isolate P7455 were chosen as representatives for further study as they both showed the same phenotypic profile including biochemical test

and carriage of 10 screened target genes but exhibited different severity of SD in pigs. From this, it was hypothesized that variation in genes or proteins was responsible for their differences in disease-causing potential.

7.1 Genome comparison of virulent and avirulent *B. hyodysenteriae*

Genomes of *B. hyodysenteriae* are rare in the public domain and thus limiting the understanding of *B. hyodysenteriae* pathogenicity. At the early stage of this project, the genome of the Australian strain WA1 was the only complete *B. hyodysenteriae* genome sequence available (NC_012225.1). Since the end of 2012, another genome sequence of *B. hyodysenteriae* ATCC 27164 has been registered on NCBI (ARSY00000000.1) and is about to be become publically available. It can be assumed that more complete and incomplete genome sequences of *B. hyodysenteriae* are about to be publically available in the near future due to the advanced and low cost next-generation sequencing technology and will therefore contribute to extensive knowledge in the life-style of that pathogen. However, at the beginning of this project, the NCBI database did not contain a genome of an avirulent or clinically-atypical isolate of *B. hyodysenteriae* that was accessible for this study. Therefore, this project involved sequencing of one avirulent and one virulent field isolate of *B. hyodysenteriae* and included the annotation and prediction of genes and protein functions. The genome sequence of the *B. hyodysenteriae* P7455 is, at this stage of writing, the first one reported for an atypical isolate of *B. hyodysenteriae* and provides a clearer insight into the mechanism for the disease and possible therapeutics.

For the purposes of this project, genomes were not sequenced to completion. Genome finishing is a very time-consuming process. Automatic annotation tools are reducing the workload and manual labor drastically but, on the other hand, are not error-free processes. Although the system used – xBASE (http://www.xbase.ac.uk/) – is able to automatically assign protein identifications and functional classes based on homology to other known protein families and similar sequences, it tends to over- or under-predict functions. For instance, misannotations have been identified within the two newly sequenced genomes of *B. hyodysenteriae* (**Chapter 4)**. Completed genome sequences would have contributed to a more straight forward comparison by removing false-positive and false-negative predictions however this was not the primary aim of the project. The genomes sequences

of P8544 and P7455 will be made available via NCBI. The majority of genomes now being deposited in NCBI are incomplete and completeness and reliability of gene predictions need to be kept in mind for analyses.

Genome sequences of at least ten avirulent and ten virulent *B. hyodysenteriae* isolates would have made comparison much more sufficient regarding defining genes and other genomic features typifying these strains. Although sequencing of another available putative avirulent isolate P7377/3 was considered this was not feasible due to the cost of sequencing at the outset of this project and limitations for funding. Sequencing of further isolates from the panel used in this study would also enable future studies investigating differences which might be related to heterogeneity (including single nucleotide polymorphisms – SNPs) within genes. Analysis of global genomic abnormalities among virulent and avirulent *B. hyodysenteriae* using SNPs would detect possible variation such as deletion, frame-shifts and inversion which might have a major effect on pathogenicity. In particular, SNPs in the flagella apparatus and MCPs (methyl-accepting chemotaxis proteins) would be of interest as microscopic observations revealed that avirulent strains were frequently less abundant in the crypt than virulent isolates thus suggesting that they may differ in their chemotactic and motility behavior. Although the avirulent isolate P7455 did not differ in the carriage of the structural and core genes compared to P8544 and WA1, conclusions regarding sequence polymorphisms cannot be made since numbers of genomes representing virulent and avirulent strains are insufficient.

7.1.1 Presence and absence of genes

The comparative genome analysis of virulent and avirulent *B. hyodysenteriae* revealed that the isolates P8544 and P7455 shared high homology with each other and the virulent Australian isolate WA1. In the first instance, differences were investigated on a presence/absence basis of genes. Although the genomes were highly homologous, genes were detected to be distinct by identifying 35 genes in P8544 and 27 genes in P7455 which have not been reported in *B. hyodysenteriae* before and are likely to result in intra-species variations and may have an impact on the severity of the outcome of disease. The literature suggests that the VSH-1 element is the only known mechanism by which *B. hyodysenteriae* is able to exchange genetic material. Preliminary studies have been conducted in order to investigate the composition of this bacteriophage and its activation

but no further studies have been performed. The current study suggests that the VSH-1 like element is ubiquitous among *B. hyodysenteriae* isolates due to the results of the comparative genome analysis and discovery of unique genes predicted in the newly sequenced genomes and presence of some of these tested genes within the DNA panel consisting of 49 isolates. It also shows that the avirulent strain is able to acquire and transfer genes which enables it to adapt to environment changes. This element is considered to be widespread among *Brachyspira* spp. and thus likely to contribute to the recombination of the more frequently reported intra-species variation leading to complications on the diagnostic level. The acquisition of new virulence genes is a known process necessary for adaptation and response to selective pressure caused by the host as well as other bacteria.

One of the new acquired genes found only in the virulent *B. hyodysenteriae* P8544 isolate was the UDP galactopyranose mutase (UGM) which shared the best protein match to *C. jejuni*. The UGM is a flavoprotein which catalyzes the reaction of UDP-galactopyranose to UDP-galactofuroanose which is the known as the precursor of the galactofuranose (Galf) (Nassau *et al.*, 1996). Galactofuranose is a component of the cell wall and glycoproteins of the cell surface of several pathogens including bacteria and parasites (Pan *et al.*, 2001; Poulin *et al.*, 2010) and considered to play a role in parasite pathogenesis (Oppenheimer *et al.*, 2011). Besides the absence in humans which makes Galf to a potential drug target (Oppenheimer *et al.*, 2012). In *Mycobacteria* Gal*f* has been demonstrated to be crucial for cell growth and maintaince of the pathogen (Pan *et al.*, 2001). Therefore, it can be suggested that the UGM in P8544 contributes towards cell viability and possible adds to the cell surface structure leading towards its persistence in the large intestine as described in *C. jejuni* (Poulin *et al.*, 2010). Furthermore, the gene encoding the Methyltransferase type 11 (pBhyoa7455_25) was only detected in avirulent P7455 and showed initial potential as a distinguishing marker as it was significantly ($P<0.05$) more conserved among avirulent than virulent isolates (PCR survey, **Chapter 4**). However, this gene was only just significantly ($P=0.049$) associated with avirulent isolates, hence a larger sample set needs to be screened to validate its potential as genetic marker. Further genomes sequencing studies or PCR screenings of these new 27-35 identified genes would give insight of how conserved these genes are in *B. hyodysenteriae* spp. and if there is a significant correlation between their presence/absence in virulent and avirulent strains which may affect the disease outcome.

7.2 The intestinal microflora as a prevention of *B. hyodysenteriae* infections

Intra-species variations in virulence have been reported in many pathogens (Heithoff *et al.*, 2012; Melhus *et al.*, 1998) and have been shown to be host-tolerance related. So far, the approaches employed in this project and the previous study carried out by Walker (2001) addressed differences in the outcome of the severity of SD by focusing on aspects of variations in gene or protein expression within isolates and neglecting the possibility of host defending mechanisms against *B. hyodysenteriae* infections such as the indigenous intestinal microflora serving as a barrier. Several studies have shown that the gut microflora plays an important role in the immune system of mammals and that alteration within the composition of this ecosystem caused by changes in the environment or diet and the usage of antibiotics contributes to sensitivity/resistance to infections (Papst *et al.*, 1988; Tsai *et al.,* 2012; Kim *et al.*, 2012; Liu *et al.,* 2012). In this study no precise data was available of the diet or history of antibiotic usage of the pigs which suffered from SD and the pigs which appeared to be healthy. Nevertheless, as the two studied strains exhibited high similarity regarding gene carriage, the possibility of differing susceptibility of pigs would be another key aspect for further investigation. Changes in the colonic microbiota could either enhance or inhibit colonization of *B. hyodysenteriae* which would be reflected in the severity of SD. Future studies involving metagenomic approaches of faecal samples of corresponding pigs would uncover the composition of the gastrointestinal network and would be of great importance to understand host physiology and might reveal answers to the question regarding virulent and avirulent *B. hyodysenteriae* strains.

As mentioned in **Chapter 1** pig's diet containing, for example, inulin (Hansen *et al.*, 2011) has been shown to affect the colonization ability of *B. hyodysenteriae* due to changes in the microbial gut flora which could be an explanation why presumably avirulent strains are less seen in the crypt. Thus, it might be the case that some pigs are more resistant towards *B. hyodysenteriae* infections leading to the assumption of the presence of avirulent strains. Although pigs were fed standard commercial food rations (**Table 2.1**) nothing is known about the defined diet composition of the affected pigs from which the tested *B. hyodysenteriae* panel was assembled. Therefore, it might be interesting to get information about the food constellation between pigs from which virulent and avirulent isolates have

been isolated to investigate possible differences which might be associated with the severity of SD in single pigs.

7.3 Alteration in expression of genes involved in chemotaxis and motility

The phenotypic and genotypic characteristics of the avirulent isolates suggests that the observation of avirulent isolates being frequently less present in the crypt might be rather due to lack of chemotactic response or alteration expression of flagella associated genes or genes involved in metabolism than to differences in the flagella apparatus and presence or absence of the corresponding genes. Therefore, chemotaxis and motility assay should be considered for future studies.

As described in **Chapter 1** avirulent strains of *B. hyodysenteriae* have been described to be significantly less chemotactic than virulent strains of *B. hyodysenteriae*. However, the same number (n=55) of genes involved in chemotaxis in *B. hyodysenteriae* WA1 were identified in P8544 and P7455, thus showing no difference in gene carriage. The high number of MCPs reflects the need and ability to transduce chemotactic signals in both virulent and avirulent *B. hyodysenteriae* isolates. Multiple chemotaxis genes in *B. hyodysenteriae* and other spirochaetes have been suggested to contribute to adaptation capability in a diverse and changing environment like the intestine (Bellgard *et al.*, 2009). Therefore, differences in chemotactic behaviour between pathogenic and non-pathogenic isolates may be related to differential gene expression rather than gene carriage as mentioned earlier. A chemotaxis assay using gastric mucin as described by Milner & Sellwood (1994) could be carried out to study and compare the chemotactic response of P8544 and P7455. At the beginning of the project an attempt of a swarm plate assay as performed by Li *et al.* (2000) was carried out to compare the extent of motility using virulent isolates (P8544, P8226/7) and avirulent isolates (P7455, P7377/3). However, the swarm plate assay as well as plating the bacteria on BA plates never revealed that avirulent isolates were not less motile than virulent isolates as they were spreading over the plates to the same extent as the virulent ones, observed and measured by the expanding haemolytic regions. Indeed, the motility of avirulent strains might be affected when isolates are exposed to a more similar *in-vivo* environment like mucus.

7.4 Metabolism of virulent and avirulent isolates

Several global regulators of metabolic genes are involved in the expression and control of virulence determinants (Deutscher *et al*., 2005; Poncet *et al.,* 2009). Genome comparison between related pathogenic and non-pathogenic strains of the same species showed only a few conspicuous changes in the metabolic genes (Fuchs *et al.,* 2011). This analysis mostly ignores minor differences and changes in the core genome which could be caused by adaptive mutation or altered gene expression and can lead to substantial changes in substrate affinity and/or reaction rate (Ferrenci, 1996). Such alterations have been shown to be crucial for the import as well as metabolism of important nutrients and thus pathogenic potential in various bacteria (Wang *et al.,* 2010). For instance, the entero-invasive *E. coli* (EIEC) is the only known strain among other pathogenic *E. coli* strains which has been shown to be able to replicate in the cytosol of mammalian cells (Zagaglia *et al.,* 1991; Goetz *et al.,* 2001). However, major differences in the metabolic potential of EIEC and non-pathogenic *E. coli* K-12 (Feist *et al.,* 2007) are not detected (Fuchs *et al.,* 2011), suggesting that the ability of EIEC strains to replicate in the mammalian host cells is due to essential differences that influences the activity and/or substrate affinity of important metabolic enzymes (Chang *et al.,* 1999). Variations in the substrate affinity of metabolic components and/or transporters balancing the metabolic requirements might be therefore another reason that some *B. hyodysenteriae* isolates differ in virulence for pigs. Substrate affinity among several rumen bacteria have been tested via the Lineweaver Burks plots of dilution rate versus substrate concentration (Russell *et al*., 1979) and could serve as start point to compare and investigate possible differences within virulent and avirulent *B. hyodysenteriae* isolates.

The occurrence of avirulent *B. hyodysenteriae* might be related to loss or alteration of metabolic pathways and regulation of virulence factors due to less selective pressure to scavenge for nutrient in the gut. Such a balance between self preservation and nutritional competence called SPANC (Ferenci, 2005) is observed in other pathogenic bacteria like *P. aeruginosa*. Infections with *P. aeruginosa* is common in patients suffering from cystic fibrosis, however, isolates of *P. aeruginosa* have been recovered which causes significantly less respiratory outcome (Romling *et al.,* 1994). Major characteristic of *P. aeruginosa* isolated from these chronically infected patients is the loss of a variety of metabolic pathways and loss of expressed virulence factors probably due to the rich

nutrient (Smith *et al.*, 2006). Therefore, it may be suggested that avirulent *B. hyodysenteriae* isolates have adapted to the porcine intestinal by developing nutritional competences giving benefits to intra-intestinal lifestyle.

7.5 Review of *B. hyodysenteriae* identifications methods

The occurrence of atypical *Brachyspira* spp. represents difficulties in the diagnosis of *Brachyspira* infections and also classification of these strains. Diagnostic tests, including the Adiavet® Brachy and Brachyspira Qual PCR Box 1.0, are commercially available. However, the increasing report of the diversity of *B. hyodysenteriae* isolates (Chander *et al.*, 2012; Harding *et al.*, 2012) and the reporting of avirulent strains signifies that the usual diagnostic methods including the 16S rRNA, 23S rRNA and biochemical profiling need to be reviewed and requires an appropriate updating. Moreover, the reported variation within the 16S rRNA reported recently by Burrough *et al*. (2012), indicates that other conserved genes should be considered as diagnostic targets. The distribution studies of genes in this project detected several genes which were highly conserved among *B. hyodysenteriae* isolates including the *mglB* locus and the *clpX* gene and may have potential as further diagnostics e.g. in combination with other established tools, which could be included in the identification procedure.

In particular, the classification of strongly and weakly beta-haemolytic strains turns out to be more and more insufficient as many *Brachyspira* spp. including *B. intermedia* (Harding *et al.,* 2012) have been shown to be strongly beta-haemolytic which causes a huge discrepancy and confusion in the identification of isolated *Brachyspira*. Assumptions of strongly β-haemolytic activity to be associated with the ability of causing serve or mildly SD could not be proved in this study. The capability to disrupt red blood cells is definitely an important factor which enables and supports *B. hyodysenteriae* to persist in the host and might play a possible role in iron-acquisition of the pathogen. However, this potential is not an indicator of strain pathogenicity as all isolates assumed to be avirulent (i.e. from animals without clinical signs of SD) were strongly β-haemolytic on BA plates. Moreover, other known weakly beta-haemolytic *Brachyspira* spp. e.g. *B. piloscoli*, are also able to cause severe disease in mammals.

7.6 The plasmid sequence of *B. hyodysenteriae*

The additional whole genome sequences obtained from P7455 and P8544 enabled the first analysis and comparison of three plasmid sequences of *B. hyodysenteriae* (including WA1) which has previously been lacking due to absence of genome sequences of *B. hyodysenteriae*. The conservation of the plasmid and intra-species variations within the plasmid sequence has not been well studied and this work contributed to a preliminary insight into conserved and unique genes on these plasmids.

The plasmid has been suggested to contribute to virulence in *B. hyodysenteriae* as its lack was associated with non-pathogenicity (La *et al.*, 2011). This hypothesis, however, is in contrast with the findings in the current study as all avirulent isolates possessed plasmid determinants as demonstrated by amplification of a gene which was identified on the plasmid sequence of WA1 (and confirmed as present in the plasmids of strains P7455 and P8544).

The only potential virulence factors identified, so far, on the plasmid is the *rfb* cluster encoding for enzymes involved in attaching O-antigen units to the LPS core structure. This gene cluster was found in the newly sequenced isolates suggesting that these genes are conserved. Genome sequencing and PCR screening performed in this project suggested that the plasmid is ubiquitous among *B. hyodysenteriae* regardless of the strain's association with SD. Additionally, it can be concluded that *B. hyodysenteriae* strains lacking the plasmid seem to occur only rarely and could be classified as another atypical form of this species. However, another question of interest involves how the absence of the plasmid affects *B. hyodysenteriae*. The data obtained from this study cannot confirm that the plasmid is associated with virulence and neither does it seem that the plasmid is essential for survival for *B. hyodysenteriae* as the strain A1 was culturable and survived in the host (La *et al.*, 2011). Nevertheless, many genes specifying virulence factors of pathogenic bacteria are obtained by lateral DNA transfer and may be part of mobile elements such as plasmids or prophages. Since many plasmids have been reported to contribute to virulence in other bacteria, further plasmid-related studies would be useful to uncover the plasmid's main role. The plasmid incompatibility method would be an appropriate approach. During this study attempts have been made to isolate the plasmid of *B. hyodysenteriae* P8544 by using the Cesium Chloride (CsCl)/ ethidium bromide density

gradient centrifugation method (Heilig *et al.*, 2001) and the alkaline lysis and boiling method (Birnboim and Doley *et al.*, 1979). However, none of these attempts have been successful as chromosomal DNA was still detected by screening for plasmid and chromosomal-related genes by PCR. Therefore, the plasmid incompatibility may be an approach for curing *B. hyodysenteriae* of plasmid. This relies on the inability of two different but related plasmids to co-exist in the same host cell in the absence of continued selective pressure. Therefore, introducing a smaller, high-copy number plasmid from the same incompatibility group in *B. hyodysenteriae* by electroporation might eliminate the resident plasmid. A plasmid-cured strain of *B. hyodysenteriae* would offer a great tool for further investigation and would enhance the understanding and key role of the plasmid. Comparison of a plasmid-cured *B. hyodysenteriae* strain versus plasmid-containing strain would be of interest as the *rfb* genes are considered to shape the structure of O-antigens which are likely to be involved in the host-defense mechanism. Protein glycosylation is a common post-translational modification (PTMs) in bacteria and may play a role in adhesion and thus virulence. There are several reports that proteins in bacteria are glycosylated by LPS-like-systems (Power *et al.*, 2003). Since the plasmid of *B. hyodysenteriae* contains up to five (putative) glycosyltransferases, investigation and comparison of PTMs, in particular glycosylation, of plasmid-free and plasmid-containing isolates should be carried out in futures studies. Additionally tissue culture assays comparing the adherence ability of typical *B. hyodysenteriae* strains versus plasmid-free isolates would add to our understanding of the plasmid function.

7.7 Response to iron-limitation in *B. hyodysenteriae*

Due to the fact that the virulent and avirulent isolate shared high sequence similarity between each other it was considered that phenotypic differences may be based on gene expression rather than gene carriage. Therefore, the isolates were exposed to a host-environment-like condition. Results of this study defined a medium supplemented with 0.1 mM of dipyridyl as an iron-restricted environment for *B. hyodysenteriae*. The conducted analysis was the first described in *B. hyodysenteriae* monitoring its growth overtime under various iron-replete and iron-restricted conditions showing that *B. hyodysenteriae* is able to overcome dipyridyl-induced iron-restriction. How *B. hyodysenteriae* is able to acquire remains hypothetical. Due to the ability of *B. hyodysenteriae* to produce a β-haemolysin, it has been suggested that the bacteria acquires iron by completely rupturing red blood cells

which leads to the release of iron. As shown in **Chapter 3** all tested avirulent *B. hyodysenteriae* isolates manifested β-haemolysis; therefore, it can be assumed that virulent and avirulent isolates of *B. hyodysenteriae* cold scavenge for iron by their β-haemolytic capability. However, this determinant is unlikely to confer iron acquisition under the *in vitro* conditions employed.

Bacteria like *E. coli* contain a *ChuA* homologue which encodes for an OMP responsible for haem uptake (Torres and Payne, 1997). A similar homologue could not been detected in the genome of *B. hyodysenteria* and there has been no haem uptake system described in *B. hyodysenteriae* so far. Nevertheless, the gene *hemH* which is part of the haem biosynthesis pathway was identified in all three genomes of *B. hyodysenteriae* whereas no other gene known to be involved in the pathway was detected. It has been shown that *hemH* has ferrochelatase activity which is known to catalyze the insertion of Fe^{2+} into the haem precursor protoporphyrin (Frustaci & O'Brian, 1993). In addition, ferrochelatase has been suggested to be involved in the removal of iron from haem by *Haemphilus influenza* through an inverse ferrochelatase reaction (Loeb, 1995). Therefore, it can be hypothesised that *B. hyodysenteriae* might be able to access haem-bound iron in a similar manner.

As mentioned in section **5.1**, in Gram-negative bacteria the uptake of siderophores is driven by outer-membrane receptors, so-called TonB-dependent receptors, which usually have high affinity and specificity for their Fe-containing substrate. In *E. coli* the TonB dependent receptor, FhuA, is specific for active transport for ferrichrome. Bacteria often possess multiple TonB-ExbB-ExbD-like systems with distinct specificity for the TonB receptor. For example, in *Leptospira* spp. five ExbB and ExbD proteins and three TonB-dependent receptors could be identified (Louvel *et al.*, 2006). However, the analysis of the three *B. hyodysenteriae* genomes revealed only one gene encoding a homologue of the TonB-dependent receptor protein, one gene encoding a homologue of the biopolymer transport protein ExbB and one gene encoding a homologue of the biopolymer transport protein ExbD/TolR each of which are located in different loci. The sequence of the *B. hyodysenteriae* WA1 TonB-dependent receptor homologue actually shares poor similarity (e-value 0.22, total score 104) with the TonB-dependent receptor sequence of *L. biflexa* (LBF_3248). However, protein analysis using the Pfam database showed that the sequence of the TonB-dependent receptor protein contains a β-barrel domain which is typical and highly conserved among TonB-dependent receptors. A domain belonging to the porin

superfamily (SSF56935) and sharing a β-barrel structure was identified which could serve as a channel, acting either as a general diffusion pore or specific-substrate channel. Even though the transcriptional response of the TonB complex in *B. hyodysenteriae* P8544 and P7455 was not investigated it is entirely possible that iron-complexes are imported via this system into the cell, in particular iron released from erythrocytes.

Another possibility to acquire might be through the expression of lactoferrin iron-binding proteins. Other than the TPR-domain-containing proteins, the virulent P8544 also expressed an outer-membrane protein (Bhyov8544_2195) which consisted of a TolC domain and thus shares similarity in functionality to the outer-membrane channel tunnel protein TolC of *E. coli*. Studies in *E. coli* showed that TolC was essential for the import of the siderophore enterobactin into the cell (Bleuel *et al.*, 2005) suggesting that the expressed OM protein in P8544 may contribute to the transport of iron via siderophore binding, although the mechanism is unknown. Lactoferrin has been shown to bind the porins OmpF, OmpC and PhoE in *E. coli* (Erdei *et al.*, 1994). Studies suggested that Lf uses porins as an anchoring site (Sallmann *et al.*, 1999), however, the role of porin-Lf interaction is not fully understood. Due to the ability of Lf to bind to the OM of Gram-negative bacteria it can be hypothesised that Lf facilitates destabilization of the membrane of *B. hyodysenteriae* as demonstrated in *E. coli* (Yamauchi *et al.*, 1993) although no sign of decrease in growth could be observed which would support the theory. Therefore, the data suggests that *B. hyodysenteriae* might acquire iron from Lf with the possible involvement of TPR-domain-containing protein and outer-membrane protein. However, further investigations of the predicted Lf-binding proteins would be necessary as Lf is known to bind non-specifically as show in the current study and elsewhere.

Moreover, it seems that *B. hyodysenteriae* has a possible advantage as an inhabitant of the intestinal tract as iron exists more likely in a soluble ferrous form under anaerobic conditions (Aisen, 1976, Rocha and Smith, 2004), which supports the theory of other scientists that siderophore production might not be involved in iron-uptake in *B. hyodysenteriae*. However, it would present a selective advantage when competing with other intestinal bacteria for iron. Thus, iron-uptake in *B. hyodysenteriae* seems to be mainly driven by the Feo-system.

Exposing virulent and avirulent *B. hyodysenteriae* to two diverse growth conditions revealed differences between the two isolates at transcription level. The data suggested that fewer ABC-transporters were up-regulated in the avirulent isolate under iron-limited conditions compared to the virulent isolate. This may be significant as these systems might have effects on the uptake of other important metals including zinc and manganese which act as cofactor for many genes and proteins. To test if this preliminary result is isolate-specific or could serve as a suitable discrimination factor between virulent and avirulent strains, further experiments needed to be conducted. First of all, a global transcriptional study (microarray or RNAseq) would measure the transcription levels of a far larger number of genes than tested in the current study and would, hence, provide a broad overview of regulation of genes including those encoding for ABC-transporters under iron-restricted conditions in the avirulent isolate. The impact of hypothesized down-regulated of several ABC-transporters and consequently lack of imported metal ions could be then further investigated and confirmed by pulse polarography in virulent vs. avirulent *B. hyodysenteriae* strains, which measures the direct uptake of metals ions in solution by bacteria and was described in *Pseudomonas cepacia* (Savvaidis *et al.*, 2003).

Further differences on mRNA-level between P8544 and P7455 were seen in respect to the HKG *recA*. The gene *recA* is highly conserved among prokaryotes and mainly known as being involved in the repair process of damaged DNA via the SOS pathway (Witkin, 1991). The SOS pathway is induced by various DNA breaks resulting in single-stranded (ss) DNA. RecA binds ssDNA which leads to formation of the nucleoprotein filament termed RecA* (Kowalczykowski, 1991). The activated RecA* is able to act as co-protease promoting self-cleavage of the transcriptional repressor LexA resulting in the induction and up-regulation of several SOS genes (Kowalczykowski *et al.*, 1994). Due to its primary role of repairing and thus assuring functional cell replication, *recA* has been suggested to have housekeeping functions and has been used as a reference gene in several real-time studies (Aravena-Roman *et al.*, 2012; Zhao *et al.*, 2011). However, RecA has been also associated with functions such as adaptation of *Lactococus lactis* to oxygen and high temperatures (Duwat *et al.*, 1995) as well as nutrient starvation and heat shock in *Streptococcus thermophilis* (Giliberti *et al.*, 2002). Even early studies reported an increase of transcripts of *recA* of *E. coli* cells when exposed to DNA damaging treatments (Little & Hanawalt, 1977; Little & Kleid, 1977) suggesting that expression of *recA* does not appear to be constant in different cells under varying conditions. Apparently, the reduction of the

iron-level in the medium causes a stress response in *B. hyodysenteriae* as cells were observed to dwell on a longer lag phase. The genome of *B. hyodysenteriae* reveals that the organism possesses a LexA homologue and RecA which are involved in the SOS machinery in bacteria. In this study the *recA* of the avirulent isolate was differentially transcribed to a greater extent when cells were subjected to an iron-limited environment by exhibiting higher Ct-values under iron-limited conditions than under iron rich conditions. Interestingly, the *recA* was not found to be differentially more transcribed when cells of the virulent *B. hyodysenteriae* isolate P8544 were exposed to iron-restricted conditions. Unfortunately, the mechanism by which *B. hyodysenteriae* is able to cope with iron-limitation remain unclear. Nevertheless, the determination of an appropriate housekeeping gene revealed that the two tested isolates might react differently on mRNA level by higher or lower transcription of different SOS genes such as *recA*. Therefore, the analysis of the *recA* gene was indicating a possible difference regarding the stress response in virulent and avirulent *B. hyodysenteriae* and already signifying that pathogenic and non-pathogenic *B. hyodysenteriae* isolates may be distinguishable at the mRNA level. Indeed, further pathogenic and non-pathogenic needs to be tested to confirm that this observation is not due to intra-species variation.

7.8 Protein expression in *B. hyodysenteriae*

Proteomic approaches detected several proteins which were significantly differentially expressed under iron-limitation. FeoB and AhcP were significantly present in both isolates and were more highly expressed under iron-restriction suggesting that these proteins are typical of the response of *B. hyodysenteriae* to iron-restriction and play a key role in this adaptation process. However, technical issues hampered an accurate comparison between the two isolates and analyses of proteins expression by 2-DGE. Therefore, technical issues need to be solved in order to ensure appropriate comparison between isolates and, thus, advance identification of any strain-differentiating markers. Even though 2D-gel results can be variable among gels, an accurate comparison requires first of all that protein samples are separated via the same electrophoretic system to ensure consistency. The system of 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) (Marouga *et al.*, 2005) is another variant of 2D-electrophoresis and would offer the possibility of including an internal standard making comparison of gels easier and quantification of proteins more accurate. However, inherent drawbacks of 2-DGE would still occur including limited

sample capacity, insufficient detection of low-abundant and hydrophobic membrane proteins as well as low visualization sensitivity (Monteoliva & Albar, 2004). Currently, the non-gel based technique Multidimensional Protein Identification Technology (MudPIT) is used to identify highly complex samples for large scale proteome analysis such as the human plasma proteome (Fujii *et al.*, 2004). This technology was first introduced by Washburn *et al.* (2000), allowing the identification of 1,500 proteins in *S. cerevisiae* including low-abundance proteins as well as highly acidic and basic proteins (Washburn *et al.*, 2001). Thus, MudPIT would offer a great alternative to overcome many 2D-gel limitations. However, MudPIT and similar "shotgun" methodologies are non-quantitative hence for quantification of potential biomarkers for avirulent/virulent *B. hyodysenteriae* isolates quantitative methods based on protein labeling (such as iTRAQ or SILAC) or on unlabelled approaches should be considered for future studies.

In conclusion, this project has contributed significantly to the understanding of *B. hyodysenteriae* gene carriage and protein expression and thus to understanding possible mechanisms by which it colonises hosts and causes disease. Pathogenesis of *B. hyodysenteriae* is not fully understood which makes investigation of avirulent isolates even more challenging. Nevertheless, the genome sequences of P8544 and P7455 enables to study the pathogen in more depth, especially the more undiscovered areas including the plasmid and the VSH-1 element. The genome of P7455 is the first genome sequence available of a strain classed as a non-typical isolate and will therefore contribute to the understanding of the more frequently reported atypical isolates and global virulence factors of *B. hyodysenteriae*.

Additionally, the OM profiling as well as analysis of differentially expressed proteins of the two isolates provided knowledge in the response of *B. hyodysenteriae* under stress situation such as iron-starvation which has not been reported before. The proteins catalogued also are an important resource for the comparison of future proteomics studies in *B. hyodysenteriae*. The identification of these proteins also offers new starting points for research areas including the investigation of predicted lipoproteins, which might have an important function in pathogenesis and could be therefore contribute to the control of, and perhaps even eradication of, SD from the UK and elsewhere.

Appendices

Appendix 1

Common buffers and reagents:

TE-buffer pH 8.0

50 mM Tris-HCl

10 mM EDTA

10% Polyacrylamide Gel

Running gel:

5 ml 1.5 M Tris-HCL, pH 8.8

0.2 ml 10% (w/v) SDS

6.6 ml 30% (v/v) acrylamide

0.2 ml 10% (w/v) APS

0.02 ml TEMED

Make up to 20 ml with ddH_20

Stacking gel:

0.63 ml 1 M Tris-HCl, pH 6.8

0.05 ml 10% (w/v) SDS

0.66 ml 30% (v/v) acrylamide

0.5 ml 10% (w/v) APS

0.005 ml TEMED

Make up to 5 ml with ddH_20

Extraction buffer outer-membrane proteins

1% (v/v) Triton-X-114

150 mM NaCl

10 mM Tris-HCL (pH 8.8)

1mM EDTA

Extraction buffer

4% (w/v) CHAPS

7M Urea

2% (w/v) DTT

2M Thiourea

2% (w/v) ASB-14

Make up to2 .5 ml with HPLC grade H_20

Rehydration buffer

4% (w/v) CHAPS

8 M Urea

1% (v/v) Pharmalytes 4-7 for IEF

2 mg/ml (13 mM) DTT

Add a few grains Bromphenol blue

Make up to 25 ml with HPLC grade H_20

Equilibration buffer 1-stock solution

50 mM TrisCl (pH 8.8, 1.5 M)

6M Urea

30% (v/v) Glycerol (87% (v/v))

2 % (w/v) SDS

Add a few grains Bromphenol blue

Make up to 200 ml with HPLC grade H_20

Equilibration buffer 1-

10 ml Equilibration stock solution 1

100 mg DTT

Equilibration buffer 2

10 ml Equilibration stock solution 2

250 mg Iodoacetamide

Colloidale Coomassie Blue G 250 dye stock solution

50 g Ammounium sulphate

6 ml Phosphoric acid 85% (w/w)

10 ml 5% Coomassie Blue G-250 stock (0.5g in 10 ml)

500 ml double distilled water

Colloidale Coomassie Blue G 250 working solution

400 ml Colloidale Coomassie Blue G 250 dye stock solution

100 ml Methanol

1× NuPAGE®MES Running buffer

50 ml 20 × NuPAGE® MES Running buffer

950 ml ddH_20

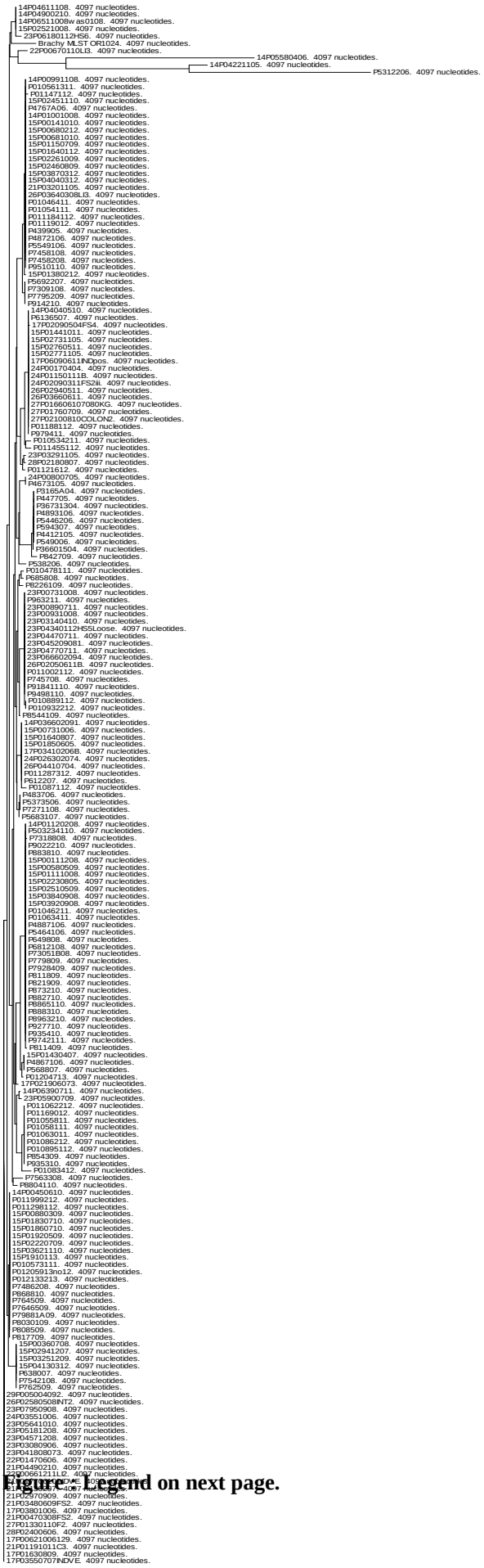
2× NUPAGE® Transfer buffer

10 ml 20× NUPAGE® Transfer buffer

10 ml NUPAGE® Antioxidant (for reduced sample)

10 ml Methanol

Make up tp 100 ml with ddH_20



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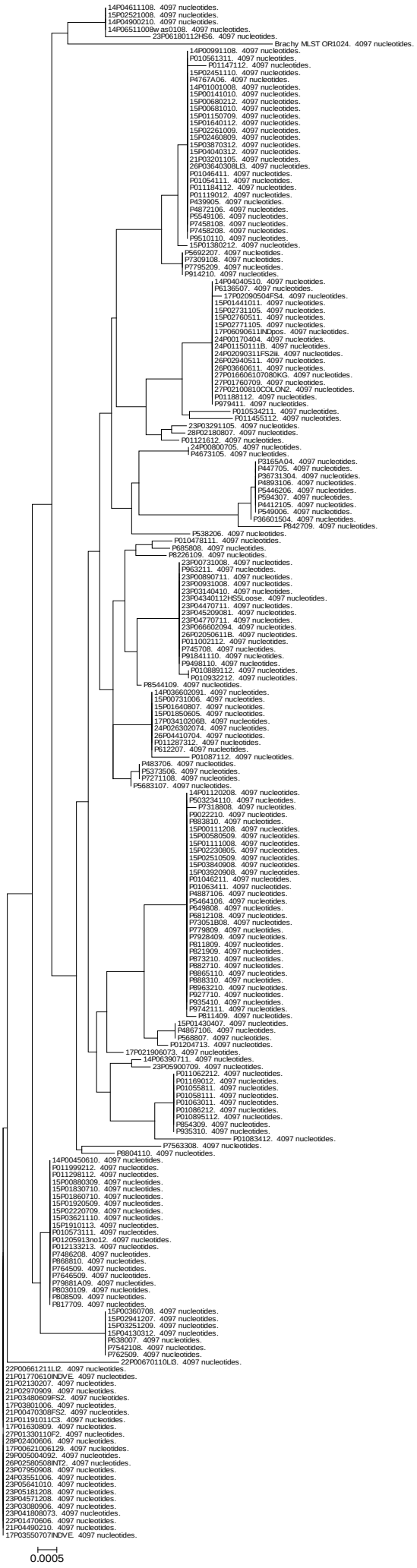

Figure: UPGMA dendogram of depicting genetic similarity of 150 *B. hyodysenteriae* field isolates. The figure represents a section of 150 *B. hyodysenteriae* isolates. The tree was generated from concatenated sequences of the seven loci. Arrows are highlighting the 10 isolates which were used in this study. The project was funded by BPEX and data was analysed and kindly provided by Richard Ellis and Ben Strugnell from Animal Health and Veterinary Laboratories Agency (AHVLA) (Weybridge, Surrey).

Appendix 3

Whole genome sequence of *B. hyodysenteriae* P8544 and P7455 annotated by Xbase. See attached CD- folder **Appendix 3**:Whole_genome vP8544/ aP7455 including Plasmid sequences: P8544_ Plasmid and P7455_Plasmid.

Appendix 4

Novel regions prediction in *B. hyodysenteriae* P8544 using Panseq2.0

Contig	***novel region length (bp)***			**locustaq**
	lenght	***start***	***end***	
	2318	20627	22944	Bhyov8544_1565
104	918	109751	110668	Bhyov8544_0808; Bhyov8544_0809
10	1451	250	1700	Bhyov8544_2402
	2455	25111	27565	Bhyov8544_2427
129	1804	1	1804	no ORF predicted
132	694	1	694	Bhyov8544_1402
	3800	43739	47538	Bhyov8544_1442;Bhyov8544_1443
13	3670	1	3670	Bhyov8544_2612; Bhyov8544_2613
143	16310	548	16857	Bhyov8544_2547- Bhyov8544 _2564
144	1304	1	1304	Bhyov8544_2678
159	528	35403	35930	Bhyov8544_2100
15	694	1	694	Bhyov8544_2698
16	16866	1	16866	Bhyov8544_1612-Bhyov8544_1624
17	1035	39883	40917	Bhyov8544_1712
	1308	50195	51502	Bhyov8544_1721
	3585	3266	6850	Bhyov8544_2460-2462
19	10759	1	10759	Bhyov8544_1976-Bhyov8544_1980
	1863	25512	27374	Bhyov8544_1996; Bhyov8544_1997
	1018	28225	29242	Bhyov8544_2000
	1952	40471	42422	Bhyov8544_2012; Bhyov8544_2013
1	10703	1	10703	Bhyov8544_2630-Bhyov8544_2638
20	8254	1	8254	Bhyov8544_1864-Bhyov8544_1870
	6707	8414	15120	Bhyov8544_1871-Bhyov8544_1873
21	18911	1	18911	Bhyov8544_2492-Bhyov8544_2508
22	7923	1	7923	Bhyov8544_2291-Bhyov8544_2297
	5811	19539	25349	Bhyov8544_2310-Bhyov8544_2314
	1919	29365	31283	no ORF predicted
231	9271	1	9271	Bhyov8544_2639-Bhyov8544_2647
246	1346	1	1346	Bhyov8544_2665
24	1447	16570	18016	Bhyov8544_1093
25	4690	1	4690	Bhyov8544_2619-Bhyov8544_2625
	601	8592	9192	no ORF predicted
26	2556	17253	19808	Bhyov8544_2364-Bhyov8544_2366
	5083	20957	26039	Bhyov8544_2368-Bhyov8544_2370
304	2661	1	2661	Bhyov8544_0302, Bhyov8544_0303, Bhyov8544_0304

	1140	123605	124744	Bhyov8544_0386
30	1013	1	1013	Bhyov8544_2657
34	28262	1	28262	Bhyov8544_2194-Bhyov8544_2217
37	7007	1	7007	Bhyov2649-Bhyov2656
3	32615	1	32615	Bhyov8544_2025-Bhyov8544_2054
41	21907	1	21907	Bhyov8544_1250- Bhyov8544_1272
42	2994	401	3394	Bhyov8544_1743, Bhyov8544_1744
	1904	5527	7430	Bhyov8544_0017, Bhyov8544_0461, Bhyov8544_0747
	1362	10341	11702	Bhyov8544_1749
58	671	1	671	no ORF predicted
59	2471	1	2471	Bhyov8544_2676, Bhyov8544_2677
5	641	32838	33478	no ORF predicted
	755	228096	228850	Bhyov8544_0220
	862	306824	307685	Bhyov8544_0290
	2869	307803	310671	Bhyov8544_0291
63	8593	1	8593	Bhyov8544_0553-Bhyov8544_0559
	815	24802	25616	Bhyov8544_0572
	548	25738	26285	Bhyov8544_0572
	2311	29015	31325	Bhyov8544_0576
	1057	84031	85087	Bhyov8544_0625
	2201	93023	95223	Bhyov8544_0632,Bhyov8544_0633
	1456	133317	134772	Bhyov8544_0671
	1388	138440	139827	Bhyov8544_0673
	1059	149056	150114	Bhyov8544_0683
	1482	157612	159093	Bhyov8544_0691
66	1349	1	1349	Bhyov8544_2673
68	2754	723	3476	Bhyov8544_2579-Bhyov8544__2581
	977	3664	4640	Bhyov8544_2582 -Bhyov8544 _02583
	2310	6032	8341	Bhyov8544_2584-Bhyov8544__02585
	939	8532	9470	Bhyov8544_2586
	2837	10267	13103	Bhyov8544_2587
	903	13447	14349	Bhyov8544_2588
6	4853	1	4853	Bhyov8544_1328-Bhyov8544_1330
	2488	46662	49149	Bhyov8544_1367
	2091	49525	51615	Bhyov8544_2544-Bhyov8544_2546
73	2025	15045	17069	Bhyov8544_2699
74	662	1	662	Bhyov8544_2699
7	5742	1	5742	Bhyov8544_2153- Bhyov8544_2159
8	27071	1	27071	Bhyov8544_0859- Bhyov8544_0883
98	898	12974	13871	Bhyov8544_2124
	1266	14231	15496	Bhyov8544_2126

Novel regions prediction in *B. hyodysenteriae* P7455 using Panseq2.0

Contig	**novel region length (bp)**			**locustaq**
	length	***start***	***end***	
1	85109	1	85109	Bhyoa7455_1294-Bhyoa7455_1358
2	21466	3774	25239	Bhyoa7455_2296- Bhyoa7455_2313
	900	1	900	Bhyoa7455_2294
3	40167	1	40167	Bhyoa7455_2041-Bhyoa7455-2080
5	69522	1	69522	Bhyoa7455_1568- Bhyoa7455_1644
6	42840	1	42840	Bhyoa7455_1963_-Bhyoa7455_1997
7	4421	18920	23340	Bhyoa7455_2376 -Bhyoa7455_2378
7	18832	1	18832	Bhyoa7455_2361-Bhyoa7455_2375
8	11973	62741	74714	Bhyoa7455_1555 -Bhyoa7455_1566
	49221	13031	62525	Bhyoa7455_1510_-Bhyoa74551554
	12764	1	12764	Bhyoa7455_1500-Bhyoa7455_1509
9	17794	33279	51703	Bhyoa7455_1904 -Bhyoa7455_1920
	32703	1	32703	Bhyoa7455_1874 -Bhyoa7455-_1903
10	13578	1	13578	Bhyoa7455_1425-Bhyoa7455-1499
11	159659	1	159659	Bhyoa7455_0848- Bhyoa7455_0994
12	89572	1	89572	Bhyoa7455_0995_Bhyoa7455_1076
	20166	90475	110641	Bhyoa7455_1079_Bhyoa7455_1100
	11916	111192	123108	Bhyoa7455_1102- Bhyoa7455_1111
	28762	123921	152683	Bhyoa7455_1113-Bhyoa7455_1132
14	60500	1	60500	Bhyoa7455_1820-Bhyoa7455_1873
15	26806	1	26806	Bhyoa7455_2264-Bhyoa7455_2293
17	36454	1	36454	Bhyoa7455_2081-Bhyoa7455_2112
18	60996	1	60996	Bhyoa7455_1764-Bhyoa7455_1819
19	7395	1	7395	Bhyoa7455_2602- Bhyoa7455_2610
20	32979	1	32979	Bhyoa7455_2145-Bhyoa7455_2174
21	32116	1	32116	Bhyoa7455_0367-Bhyoa7455_0391
	2138	32215	34353	Bhyoa7455_0392-Bhyoa7455_0687
22	60500	1	60500	Bhyoa7455_2515-Bhyoa7455_2530
23	26806	1	26806	Bhyoa7455_0688-Bhyoa7455_0759
	90855	90056	180911	Bhyoa7455_0760-Bhyoa7455_0847
24	21994	1	21994	Bhyoa7455_1922-Bhyoa7455_1939
	68735	22782	45953	Bhyoa7455_1942- Bhyoa7455_1962
	21994	1	21994	Bhyoa74551921- Bhyoa7455_1939
26	2352	1	2352	Bhyoa7455_2640- Bhyoa7455_2650
27	4321	12128	16449	Bhyoa7455_2459- Bhyoa7455_2461
	12017	1	12017	Bhyoa7455_2452- Bhyoa7455_2458
28	41747	1	41747	Bhyoa7455_1998- Bhyoa7455_2040
29	1495	1	1495	Bhyoa7455_2661
30	8714	1	8714	Bhyoa74552596- Bhyoa7455_2601
31	20488	14334	34822	Bhyoa7455_2124- Bhyoa7455_2144
	1140	13082	14222	Bhyoa7455_2130 -Bhyoa7455_2132
	12945	1	12945	Bhyoa7455_2113- Bhyoa7455_2122
32	36108	27288	63396	Bhyoa7455_1728- Bhyoa7455_1763
	4508	21984	26492	Bhyoa7455_1723- Bhyoa7455_1726
	13472	8410	21882	Bhyoa7455_1713- Bhyoa7455-1722
	8255	1	8255	Bhyoa7455_1706 -Bhyoa7455-1712
33	24523	1	24523	Bhyoa7455_2314- Bhyoa7455_2340

34	23043	1	23043	Bhyoa7455_2379- Bhyoa7455_2399
36	25402	3579	28981	Bhyoa7455_2211- Bhyoa7455_2238
36	3154	1	3154	Bhyoa7455_2208- Bhyoa7455_2210
39	28271	1	28271	Bhyoa7455_2239- Bhyoa7455_2262
40	1268	19568	20836	Bhyoa7455_2433
	18713	1	18713	Bhyoa7455_2416- Bhyoa7455_2431
42	15347	1	15347	Bhyoa7455_2531- Bhyoa7455_2543
43	756	1	756	Bhyoa7455_2504
43				Bhyoa7455_2494- Bhyoa7455_2503
44	12181	744	12925	Bhyoa7455_2544- Bhyoa7455_2554
47	23639	1	23639	Bhyoa7455_2341- Bhyoa7455_2359
48	2049	30250	32299	Bhyoa7455_2207
	24053	5470	29523	Bhyoa7455_2180- Bhyoa7455_2205
	4584	1	4584	Bhyoa7455_2175- Bhyoa7455_2178
49	12397	1	12397	Bhyoa7455_2555- Bhyoa7455_2566
51	1257	1	1257	Bhyoa7455_2663
54	2909	1	2909	Bhyoa7455_2643- Bhyoa7455_2645
62	15794	1	15794	Bhyoa7455_2480- Bhyoa7455_2493
67	833	1	833	Bhyoa7455_2669
68				no ORF predicted
69	6409	367	6776	Bhyoa7455_2627- Bhyoa7455_2633
72	7338	1	7338	Bhyoa7455_2611 -Bhyoa7455_2618
74	1184	1	1184	Bhyoa7455_2664
75	4043	822	4895	Bhyoa7455_2639- Bhyoa7455_2642
76	1205	1	1205	Bhyoa7455_2646
78				no ORF predicted
79	8795	335	9130	Bhyoa7455_2578- Bhyoa7455_2594
80	1167	1	1167	Bhyoa7455_2665 -Bhyoa7455_2666
90	48587	41606	90193	Bhyoa7455_1169 -Bhyoa7455_1214
	1112	40351	41463	Bhyoa7455_1168 -Bhyoa7455_1169
	26047	13194	39241	Bhyoa7455_1148 -Bhyoa7455_1167
	12337	1	12337	Bhyoa7455_113- Bhyoa74553_1146
93	1562	1	1562	Bhyoa7455_2660
99	2548	1	2548	Bhyoa7455_2648
120	454324	1	454324	Bhyoa7455_0001- Bhyoa7455_0365
121	845	1	854	Bhyoa7455_2679
136	587	1	587	Bhyoa7455_2676
151	3843	1	3843	Bhyoa7455_2634 -Bhyoa7455_2635
157	743	1	743	B.hyoa7455_2672
161	733	1	733	Bhyoa7455_2673
163	47007	1	47007	Bhyoa7455_1645-Bhyoa7455_1684
	21757	47142	68899	Bhyoa7455_1685 -Bhyoa7455_1705
165	672	1	672	Bhyoa7455_2674
167	1642	1	1642	Bhyoa7455_2654
199	886	1	886	Bhyoa7455_2670 -Bhyoa7455_2671
235	70812	1	70812	Bhyoa7455_1215 -Bhyoa7455_1279
	7767	70955	78722	Bhyoa7455_1281 -Bhyoa7455_1287
	712	78947	79659	Bhyoa7455_1288
	2047	84072	86119	Bhyoa7455_1291 -Bhyoa7455_1292
282	15126	66786	81912	1Bhyoa7455_1414- Bhyoa7455_1424
	66687	1	66687	Bhyoa7455_1359- Bhyoa7455_1413
289	20921	1	20921	Bhyoa7455_2400 -Bhyoa7455-2415
307	13197	1219	14416	Bhyoa7455_2506- Bhyoa7455_2514

Appendix 5

The prediction of signal peptides and lipoproteins in the genomes of P8544, P7455 and WA1 using the software SpliP, SignalP 4.0 and LipoP 1.0.
See attached CD-**Appendix 5.**

Appendix 6

Entire data set of the outer-membrane enrichment fraction of proteins detected by LC-ESI-MS/MS using the criteria described in **chapter 2** under iron replete and iron-restricted conditions in P8544 and P7455
See attached CD-**Appendix 6.**

Appendix 7

Characterization of the novel genes predicted in the genomes of *B. hyodysenteriae* P7455 and P8544

loqcustaq	size	closest protein match	InterproScan		
			Signalpeptide	Domaim	Family/Superfamily
Bhyov8544_0303	33	hypothetical protein XF0398 [Xylella fastidiosa 9a5c]	yes	/	/
Bhyov8544_0559	675	hypothetical protein HMPREF1122_01040 [Clostridium difficile 002-P50-2011]	no	Domain of unknown function DUF262 (IPR004919)	/
Bhyov8544_0632	469	unnamed protein product Bmur_1538 [Brachyspira murdochii DSM 12563]	no	/	P-loop containing nucleoside tripohosphate hydrolase (SSF525540)
Bhyov8544_0633	205	unnamed protein product [Brachyspira murdochii DSM 12563]	No hits reported		
Bhyov8544_0646	46	hypothetical protein Bint_2751 [Brachyspira intermedia PWS/A]	No hits reported		
Bhyov8544_0795	154	hypothetical protein Bint_2640 [Brachyspira intermedia PWS/A]	yes	/	/
Bhyov8544_0980	80	hypothetical protein Swol_0219 [Syntrophomonas woLfei subsp. woLfei str. Goettingen]	no	Ribonucleotide reductase, stirrup	Uncharacterized protein family (UPF0395)
Bhyov8544_1743	544	conserved hypothetical protein Bp_0177 [Brachyspira pilosicoli 95/1000]	no	Tetratricopeptide-like helical	TPR-like family (SSF48452)
Bhyov8544_1756	31	hypothetical protein SPAP_0864 [Streptococcus pneumoniae AP200]	yes	/	/
Bhyov8544_2013	209	hypothetical protein Bmur_2524 [Brachyspira murdochii DSM 12563]	No hits reported		
Bhyov8544_2093	30	hypothetical protein BP951000_1514 [Brachyspira pilosicoli 95/1000]	No hits reported		
Bhyov8544_2249	65	conserved hypothetical protein Bint_2186 [Brachyspira intermedia PWS/A]	No hits reported		
Bhyov8544_2255	47	truncated Lex2A [Haemophilus influenzae]	No hits reported		
Bhyov8544_2303	89	conserved hypothetical protein [Fusobacterium sp. 1_1_41FAA]	No hits reported		
Bhyov8544_2313	398	conserved hypothetical protein [SuLfurovum sp. NBC37-1]	no	NAD-dependent epimerase/dehydratase (PF1370) NAD(P)-binding domain	NAD(P)-binding Rossman-fold domains (SSF51735)
Bhyov8544_2314	394	FkbM family methyltransferase [Brachyspira murdochii DSM Bmur_2571}	no	Methyltransferase type 12 (PF08242) C-metyltransferase (PF08484)	S-adenosyl-L-methionione-dependent methyltransferase (SS53335)
Bhyov8544_2369	270	dolichyl-phosphate beta-D-mannosyltransferase [Brachyspira pilosicoli 95/1000]	no	Glycosyl transferase, family 2 (PF00535)	Nucleotide-diphospho-sugar-transferase (SS53448)
Bhyov8544_2370	251	Methyltransferase domain. [Synergistetes bacterium SGP1]	no	Methyltransferase type 11 (PF00535)	S-adenosyl-L-methionione-dependent methyltransferase (SS53335)
Bhyov8544_2401	33	ankyrin repeat-containing protein [Brachyspira intermedia PWS/A	No hits reported		
Bhyov8544_2427	786	conserved hypothetical protein [Enterococcus faecalis HH22]	436	/	Triphosphate hydrolases (SSF52540)
Bhyov8544_2430	43	ankyrin Bmur_2481 [Brachyspira murdochii DSM 12563]	No hits reported		
Bhyov8544_2460	77	CDP-Glycerol:Poly(glycerophosphate) glycerophosphotransferase family [Treponema azotonutricium ZAS-9]	yes	/	/
Bhyov8544_2461	428	hypothetical protein J07AB56_02180 [Candidatus Nanosalinarum sp. J07AB56]	no	/	S-adenosyl-L-methionione-dependent methyltransferase (SS53335)
Bhyov8544_2531	66	DNA polymerase beta domain protein region [Brachyspira murdochii Bmur_1349]	No hits reported		
Bhyov8544_2544	155	propionyl-CoA carboxylase, beta subunit [Roseobacter sp. CCS2]	yes	/	/
Bhyov8544_2545	112	hypothetical protein NH8B_1950 [Pseudogulbenkiania sp. NH8B]	No hits reported		
Bhyov8544_2546	372	hypothetical protein SI859A1_00926 [Aurantimonas manganoxydans SI85-9A1]	No hits reported		
Bhyov8544_2597	221	hypothetical protein APT_2250 [Acetobacter pasteurianus NBRC	no	/	

Bhyov8544_2581	269	RES domain protein Bmur_1895 [Brachyspira murdochii DSM 12563]	no	/	RES (PF08808)
Bhyov8544_2585	305	hypothetical protein GCWU000341_02825 [Oribacterium sp. oral taxon 078 str. F0262]	yes		
Bhyov8544_2587	683	putative KAP NTPase P-loop domain-protein [Brachyspira pilosicoli 95/1000]	no	KAP P-loop (PF07693)	P-loop containing nucleoside tripohosphate hydrolase (SSF525540)
Bhyov8544_2652	31	hypothetical protein Bmur_1517 [Brachyspira murdochii DSM 12563]	yes	/	/
Bhyov8544_2676	370	UDP-galactopyranose mutase [Campylobacter jejuni subsp. jejuni 327]	no	UDP-galactopyranose mutase (TIGR00031) UDP-galactopyranose mutas, C-terminal (PF03257)	FAD/NAD(P)-binding domain (SSF51905)

loqcustaq	**size**	**Closest protein match**	**InterProScan**		
			Signalpeptide	Domaim	Family/Superfamily
Bhyoa7455_0003	35	hypothetical protein EAI_14639 [Harpegnathos saltator]	No hits reported		
Bhyoa7455_0608	147	hypothetical protein Bint_2375 [Brachyspira intermedia PWS/A]	yes	/	/
Bhyoa7455_0626	297	Abortive infection bacteriophage resistance protein Bmur_02390 [Brachyspira intermedia PWS/A]	no		Abortive infection bacteriophage resistance-related (PF07751)
Bhyoa7455_0652	191	adenine-specific DNA methylase [[Helicobacter cinaedi CCUG 18818]	no		S-adenosyl-L-methionine – dependent methytransferase (SSF53335)
Bhyoa7455_0654	577	predicted protein [Helicobacter bilis ATCC 43879]	No hits reported		
Bhyoa7455_0742	561	conserved hypothetical protein [Escherichia coli E110019]	no	/	P-loop containing nucleosidetriphosphate hydrolases
Bhyoa7455_0743	282	D12 class N6 adenine-specific DNA methyltransferase [Streptococcus mitis SK1080]	no	D12 class N6 adenine-specific DNA methyltransferase (PR00505)	S-adenosyl-L-methionine – dependent methytransferase (SSF53335)
Bhyoa7455_0746	80	hypothetical protein Bmur_1901 [Brachyspira murdochii DSM 12563]	No hits reported		
Bhyoa7455_0747	221	type I site-specific deoxyribonuclease, HsdR family [Methanococcus voltae A3]	No hits reported		
Bhyoa7455_0748	156	serine/threonine protein kinase [Rhodopirellula baltica WH47]	No hits reported		
Bhyoa7455_0749	80	hypothetical protein Arcve_1381 [Archaeoglobus veneficus SNP6]	no	Uncharacterized protein family (UPF0395)	Ribonucleotide reductase, stirrup (SSF54786)
Bhyoa7455_0887	816	hypothetical protein HMPREF9467_03923 [Clostridium clostridioforme 2_1_49FAA]	no	DNA/RNA helicase, DEAD/DEAH box type, N-terminal (PF00270) DEAD-like helicase (SM00487)	P-loop containing nucleosidetriphosphate hydrolases (SSF52540)
Bhyoa7455_0888	270	hypothetical protein HMPREF9467_03922 [Clostridium clostridioforme 2_1_49FAA]	No hits reported		
Bhyoa7455_0955	39	hypothetical protein Bint_1171 [Brachyspira intermedia PWS/A]	yes	/	/
Bhyoa7455_1266	92	transposase [Thiorhodococcus drewsii AZ1]	No hits reported		
Bhyoa7455_1267	229	conserved hypothetical protein [Helicobacter cinaedi CCUG 18818]	No hits reported		
Bhyoa7455_1341	234	methyltransferase type 11 [DesuLfarculus baarsii DSM 2075]	no	Methyltransferase type 12 (PF08242)	S-adenosyl-L-methionine – dependent methytransferase (SSF53335)
Bhyoa7455_1378	1579	helicase [Bacteroides fragilis YCH46]	no	Helicase,C-terminal (SM00490) UvrABC complex,subunit B (PF04851) DEAD-like helicase (SM00487)	N6 adenine-specific DNA methyltransferase, N12 class (PR00507)
Bhyoa7455_1715	95	hypothetical protein SYNPCC7002_C0007 [Synechococcus sp. PCC 7002]	no	/	Abortive infection bacteriophage resistance-related (PF07751)
Bhyoa7455_1835	46	hypothetical protein Bint_2751 [Brachyspira intermedia PWS/A]	No hits reported		
Bhyoa7455_1976	182	hypothetical protein [Paramecium tetraurelia strain d4-2]	No hits reported		
Bhyoa7455_2005	31	hypothetical protein SPV1_10039 [Mariprofundus ferrooxydans PV-1]	No hits reported		
Bhyoa7455_2084	81	Aida-related Type V secretory pathway adhesin [Neisseria meningitis NM2795]	No hits reported		

Bhyoa7455_2131	274	Methyltransferase type 11 [Paludibacter propionicigenes WB4]	no	Methyltransferase type 11 (PF08241)	S-adenosyl-L-methionine – dependent methytransferase (SSF53335)
Bhyoa7455_2136	78	hypothetical protein HMPREF9369_00085 [Fusobacterium nucleatum subsp. Polymorphum F0401]	No hits reported		
Bhyoa7455_2334	33	conserved hypothetical protein Bmur_1153 [Brachyspira murdochii DSM 12563]	No hits reported		
Bhyoa7455_2601	679	hypothetical protein Cphamn1_1150 [Chlorobium phaeobacteroides BS1]	no	Domain of unknown function DUF262 (IPR004919)	/
Bhyoa7455_2638	124	hypothetical protein Bint_1219 [Brachyspira intermedia PWS/A]	No hits reported		

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