Molecular characterization of the serotype-associated plasmids of *Salmonella enterica.*

Lynda M Browning B.Sc.

A thesis submitted for the Degree of Doctor of Philosophy
Faculty of Medicine, University of Glasgow
October 1996

University Department of Bacteriology
Royal Infirmary
Castle street
Glasgow G4 OSF

Acknowledgements

I would like to thank Dr David Platt for his help and advice during the course of this study.

I wish to acknowledge the staff at The Scottish Salmonella Reference Laboratory, Glasgow.

Special thanks are due to my friend and colleague Shelley Rankin for her constant nagging (encouragement she insists !!) to finally finish.

Finally, thanks are due to my family for all their support and encouragement, especially in the final stages. Thanks Kirsteen, for words of wisdom during many late night, long distance telephone calls.

INDEX

Declaration.....ii
Acknowledgements.....iii
Summary.....viii
Abbreviations.....xi
List of Tables.....xii
List of Figures.....xiv

Chapter 1. Introduction to the Salmonellae.....1
History.....2
Definition.....2
Nomenclature.....3
Typing Methods.....4
Serotyping.....4
Phage Typing.....5
Biotyping.....6
Multilocus Enzyme Electrophoresis.....6
Molecular Analysis.....7
Plasmid Analysis.....7
Pulse Field Gel Electrophoresis.....9
Nucleic Acid Hybridization.....10
Ribotyping.....10
IS200 Profiling.....11
Polymerase chain reaction.....11
Comparison of Different Typing Methods.....12
Pathogenicity.....12
Types of Disease.....12
Virulence Factors.....13
Surface Structures.....14
Cellular Invasion and Survival.....14
Survival Within the Macrophage.....15
Toxin Production.....15
Plasmids.....16
Serotype Associated Plasmids.....16
SAP Homology.....17
Clonality.....18
Aims.....20

Chapter 2. General Materials And Methods.....22
Storage of Strains.....23
Media.....23
Bacterial Strains.....23
Antimicrobial Susceptibility Testing.....23
Buffers And Reagents.....26
Centrifugation.....26
Plasmid Profile Analysis.....26

Preparation of the bacterial call lysate....26
Vertical Gel Electrophoresis....27
Visualisation And Documentation of Plasmids....27
Restriction Endonuclease Fragmentation Pattern Analysis....27
Plasmid Purification For REFP Analysis....27
Restriction Endonuclease Digestion of Purified Plasmid DNA....27
Horizontal Gel Electrophoresis....28
Computer–Aided Analysis of REFP's....29
Incompatibility Testing of SAP's....29
Broth Transfer Method....30
Selection of Transconjugants....30
Characterisation of Potential Transconjugants....30
Southern Blotting....31
Transfer of DNA fron Agarose Gel....31
Extraction of DNA from Agarose Gel....32
Preparation of DEAE Cellulose Paper....32
Elution of DNA....32
Nucleic Acid Hybridization....33
Preparation of DNA Probe From *Pst*I Restriction Fragments....33
Dephosphorylarion of pUC19....34
Ligation of DNA fragment to pUC19....34
Transformation of Competent Cells....35
Histochemical Screening of Transformed Cells....35
Labelling of DNA With Digoxygenin....35
Estimation of DIG–Labeled DNA....36
Preparation of DNA Probe From *Sma*I Restriction Fragments....37
Ready–To–Go™ SmaI....37
Hybridization of Southern Blots....37
Post Hybridization Washes....38
Immunological Detection....38

Chapter 3. Restriction endonuclease fragmentation pattern analysis of the serotype associated plasmids of the salmonellae....39
Introduction....40
Rationale for the inclusion of serotypes....41
Materials and methods....41
Definitive choice of SAP....41
Results....43
REFP analysis of the plasmids of Rostock, Moscow, Blegdam and Antarctica....43
Rostock....43
Blegdam....43
Moscow....43
Antarctica....43
REFP analysis of the serotype associated plasmids of the salmonellae....49

REFP analysis of the plasmid of Abortusovis.....49
REFP analysis of the plasmid of Choleraesuis.....57
REFP analysis of the plasmid of Wangata.....57
REFP analysis of the plasmid of Bovismorbificans.....58
REFP analysis of the plasmids of Gallinarum and Pullorum.....58
REFP analysis of the plasmid of Dublin.....58
HindIII analysis of the SAP's.....59
Hybridization of SAP's with a probe generated from the 2.3kb PstI fragment from pOG660.....59
Discussion.....59

Chapter 4. Incompatibility analysis of the serotype associated plasmids of the salmonellae.....**63**
Introduction.....64
Materials and methods.....66
Results.....66
Incompatibility analysis of the SAP's.....66
Incompatibility analysis of of SAP's or variant plasmids by atypical serotypes.....66
Discussion.....69

Chapter 5. Molecular variants of the serotype associated plasmids of the salmonellae.....**71**
Introduction.....72
Materials and methods.....72
Bacterial strains.....73
Results.....73
Molecular variation among the plasmids of Choleraesuis.....73
Molecular variation among the plasmids of Gallinarum.....76
Molecular variation among the plasmids of Pullorum.....76
Molecular variation among the plasmids of Bovismorbificans.....83
Molecular variation among the plasmids of Dublin.....83
Incompatibility analysis of variant SAP's and other plasmids harboured by Dublin.....96
Antimicrobial resistance.....96
Discussion.....99

Chapter 6. The identification of restriction endonuclease sites on the Typhimurium SAP.....**103**
Introduction.....104
Materials and methods.....105
Digestion of DNA with two enzymes
Anomalies in the literature about the size of the Typhimurium SAP.....106
Results and Discussion.....107
Restriction endonuclease fragment sizes of pOG660.....107

The XbaI site is at map position 0.....107
Map positions of BamHI, BglII, EcoRI, HindIII SalI and XhoI.....107
The virulence region extends from map position 83-91.....107
XhoI restriction sites.....107
BamHI restriction sites.....112
BglII restriction site.....112
HindIII restriction sites.....112
EcoRI restriction sites.....114
SalI restriction sites.....114
The identification of SmaI sites on pOG660.....116
Position of 11kb SmaI fragment.....116
Orientation of SmaI fragment around XbaI.....118
Position of 4.0kb SmaI fragment.....122
Position of 4.7kb SmaI fragment.....122
The identification of PstI sites on pOG660.....124
*Sma*I restriction fragments common to other SAP's.....137
*Pst*I restriction fragments common to other SAP's.....138

Chapter 7. Discussion.....**140**
Overall conclusions.....149

References.....**151**

Appendix 1 Preparation of media.....173

Appendix 2 Buffers and reagents.....176

Summary

The clinical importance of *Salmonella* has been known for more than a century.The control of salmonellosis requires detailed understanding of both pathogenicity and epidemiology. Certain plasmids are involved in the virulence of the salmonellae and their analysis often contributes to epidemiological investigation. Molecular characterization of the serotype associated plasmids of the salmonellae was undertaken. A predefined strategy of restriction endonuclease fragmention pattern (REFP) analysis revealed plasmids previously defined as "serotype specific" were present in different serotypes. Plasmids indistinguishable from or molecular variants of established serotype associated plasmids (SAP's) were detected in other serotypes of serogroup D1. The results showed that related or identical plasmids were present in both strains which varied only slightly in their H antigens e.g. Enteritidis (gm), Moscow (gq) and Blegdam (gmq) as well as a strain of Antarctica which possessed the H antigens gz_{63}. In addition to plasmid similarity within a serogroup, plasmids were identified in strains of Wangata which although a member of serogroup D are outwith the g-complex of flagellar antigens (H = z_4z_{23}). Unexpectedly, these plasmids were closely related to Typhimurium which belongs to serogroup B.

The incompatibility of the plasmids was tested with a cointegrate plasmid pOG669 (a cointegrate of pOG660, the Typhimurium plasmid and pOG670, an IncX R-plasmid) and confirmation of incompatibility to the Typhimurium component of this plasmid was shown by introduction and compatibility with pOG670. Plasmid incompatibility analysis of these plasmids revealed all the SAP's, Except Dublin, were incompatible with Typhimurium and confirmed a family of related plasmids common to but not restricted in their distribution to individual serotypes.

Co-resident plasmids of intermediate size (30 - 40 kb) were observed relatively frequently in certain serotypes of GpD1- notably Dublin, Enteritidis, Moscow, Blegdam and Antarctica. With the exception of Antarctica these plasmids exhibited IncX properties - and although the possibility of dual incompatibility was not investigated, these properties, by inference were impossible as it would have resulted in incompatibility to pOG669 also.

Restriction endonuclease fragmentation pattern analysis of the serotype associated plasmids of the salmonellae revealed a high degree of relatedness between plasmids of Typhimurium, Wangata, Gallinarum and Pullorum and a low degree of REFP similarity with the plasmids of Dublin and Abortusovis and the otherSAP's. The presence of a plasmid thought to be an evolutionary intermediate in the development of Typhimurium and Enteritidis has been suggested. This study demonstrated the presence of plasmid in Dublin which showed more REFP similarity to the plasmid of Gallinarum than to Dublin itself and may be an intermediate in the development of the Dublin plasmid. This was strengthened by the incompatibility analysis of the plasmids. All the SAP's except Dublin were incompatible with the Typhimurium plasmid only; the plasmid of Dublin exhibited dual incompatibility properties with both pOG660 and IncX. The intermediate Dublin plasmid pOG683 showed incompatibility to the Typhimurium plasmid only. The presence of other co-resident plasmids in this serotype which exhibit IncX properties as well as the identification of large cointegrate plasmids which were unstable, suggests that the SAP of Dublin has arisen via a cointegration event with an IncX plasmid.

Molecular variation within serotypes was observed at a higher incidence in host adapted serotypes (23%-Dublin, 47%-Pullorum) than those of broad host range (5% for both Enteritidis and Typhimurium). This was contradictory to the hypothesis that the narrow range of ecological conditions encountered by these serotypes would reduce the possibility of genetic diversity. Chromosomal analysis of these serotypes has previously shown that they were relatively stable and consisted of a single world-wide clone and minor sub-clones.

The location of restriction sites for *Pst*I and *Sma*I were determined for the plasmid of Typhimurium and fragment similarity toother SAP's in relation to existing maps suggested.

A 2.3 kb PstI fragment was demonstrated to be present in the plasmids of Typhimurium, Wangata, Gallinarum, Pullorum, Bovismorbificans, Dublin and the Dublin variant pOG683. Smaller fragments of Abortusovis and Choleraesuis hybridized and indicated partial sequence homology. No homology was detected in the plasmid of Enteritidis.

Not only do these results confirm a family of related plasmids within the salmonellae, they indicate much more of the plasmid is conserved. These analyses suggest molecular divergence of the plasmids from a common ancestor (Typhimurium) has arisen by loss of DNA. The population genetics of the SAP's of the salmonellae parallel the findings of chromosomal analysis in as much as they demonstrate the presence of a common world wide clone. However, they also demonstrate that the rate of evolution of the plasmid is much higher than previously thought.

Abbreviations

DNA	**Deoxyribonucleic acid**
DIG	**Digoxygenin**
EDTA	**Ethylenediaminetetraacetic acid**
REFP	**Restriction endonuclease fragmentation pattern**
PP	**Plasmid profile**
PPA	**Plasmid profile analysis**
SSRL	**Scottish Salmonella Reference Laboratory**
LPS	**Lipopolysaccharide**
kb	**kilobase**
MLEE	**Multilocus enzyme electrophoresis**
ET	**Electrophoretic type**
PFGE	**Pulsed field gel electrophoresis**
RNA ase	**Ribonuclease A**
spv	***s**almonella **p**lasmid **v**irulence genes*
IS	**Insertion sequence**
rRNA	**Ribosomal RNA**
SAP	**Serotype associated plasmid**
λ	**Phage Lambda DNA**
UV	**Ultraviolet light**
DEAE	**Diethylaminoethyl**
dNTP	**Deoxynucleotide triphosphate**
IPTG	**Isopropylthio-β-D-galactoside**
X-gal	**5-bromo-4-chloro-3-indolyl-galactoside**
CIAP	**Calf intestinal alkaline phosphatase**
≡	**equivalent to**
Inc	**Incompatibility group**
PCR	**Polymerase chain reaction**
TBE	**Tris Borate EDTA buffer**
TE	**Tris EDTA buffer**
SDS	**Sodium dodecyl sulphate**
TGE	**Tris Glucose EDTA buffer**

List of Tables

Table 1.1 Examples of genes found in some serotypes of *Salmonella*.....19

Table 2.1 Reference strains for plasmid analysis.....24

Table 2.2 Strains of *E.coli* used in the study.....25

Table 3.1 Plasmid size,designation and associated serotype.....44

Table 3.2 Dice coefficients of similarity between plasmids of Enteritidis, Blegdam, Moscow and Antarctica.....48

Table 3.3 Fragment sizes of SAP's after digestion with *Pst*I.....52

Table 3.4 Fragment sizes of SAP's after digestion with *Sma*I.....53

Table 3.5 Dice coefficients of similarity between plasmids of Abortusovis, Choleraesuis, Enteritidis, Typhimurium, Wangata, Gallinarum, Pullorum, Bovismorbificans and Dublin.....56

Table 4.1 Incompatibility analysis of the SAP's of the salmonellae with pOG669 and pOG670.....67

Table 4.2 Incompatibility analysis of the SAP's or variant plasmids harboured by atypical serotypes.....68

Table 5.1 Molecular characterization of Choleraesuis plasmids.....74

Table 5.2 Molecular characterization of Gallinarum plasmids.....77

Table 5.3 Dice coefficients of similarity of variant plasmids of Gallinarum...79

Table 5.4 Strain designation, source and plasmid profile of Pullorum strains analysed.....80

Table 5.5 Dice coefficients of similarity of variant plasmids of Pullorum.....82

Table 5.6 Strain designation, source and plasmid profile of Bovismorbificans strains analysed.....85

Table 5.7 Dice coefficients of similarity of variant plasmids of Bovismorbificans.....87

Table 5.8 Characteristics of Dublin strains studied.....88

Table 5.9 Dice coefficients of similarity of variant plasmids of Dublin.....91

Table 5.10 Incompatibility analysis of Dublin plasmids.....97

Table 5.11 Incidence of molecular variants of SAP's.....98

Table 6.1 Restriction fragment sizes of the Typhimurium SAP digested with various enzymes.....108

Table 6.2 Orientation of restriction fragment on the Typhimurium SAP.....126

Table 6.3 *Sma*I restriction sites on the Typhimurium SAP.....127

Table 6.4 Map positions of *Sma*I fragments.....128

Table 6.5 *Pst*I restriction sites on the Typhimurium SAP.....135

Table 6.6 Map positions of *Pst*I fragments.....136

List of Figures

Figure 3.1 REFP's of plasmids of *Salmonella* Enteritidis, Moscow, Blegdam and Antarctica digested with *Pst*I and *Sma*I...45

Figure 3.2 REFP's of plasmids of *Salmonella* Enteritidis, Moscow, Blegdam and Antarctica digested with *Eco*RV.....46

Figure3.3 Computer generated printout of the REFP's of plasmids of *Salmonella* Enteritidis, Moscow, Blegdam and Antarctica digested with *Pst*I and *Sma*I.....47

Figure 3.4 *Pst*I digestion of the plasmids of *Salmonella* Abortusovis, Choleraesuis, Enteritidis, Typhimurium, Wangata Gallinarum, Pullorum, Bovismorbificans and Dublin.....50

Figure 3.5 *Sma*I digestion of the plasmids of *Salmonella* Abortusovis, Choleraesuis, Enteritidis, Typhimurium, Wangata Gallinarum, Pullorum, Bovismorbificans and Dublin.....51

Figure 3.6 Computer generated REFP analysis of the plasmids of Abortusovis, Choleraesuis, Enteritidis, Typhimurium, Wangata,Gallinarum, Pullorum, Bovismorbificans and Dublin digested with *Pst*I and *Sma*I.....54

Figure 3.7 *Hind*III digestion of the of the plasmids of Abortusovis, Choleraesuis, Enteritidis, Typhimurium, Wangata, Gallinarum, Pullorum, Bovismorbificans and Dublin.....55

Figure 3.8 Hybridization of a DIG-labeled probe generated from the 2.3 kb *Pst*I fragment of the Typhimurium plasmid pOG660 with a REFP southern blot of the of the plasmids of Abortusovis, Choleraesuis, Enteritidis, Typhimurium, Wangata, Gallinarum, Pullorum, Bovismorbificans and Dublin.....60

Figure 5.1 Computer generated REFP analysis of the plasmids of Choleraesuis.....75

Figure 5.2 Computer generated REFP analysis of the plasmids of Gallinarum.....78

Figure 5.3 Computer generated REFP analysis of the plasmids of Pullorum.....81

Figure 5.4 Computer generated REFP analysis of the plasmids of Bovismorbificans.....86

Figure 5.5 REFP analysis of plasmids of Dublin digested with *Sma*I.....89

Figure 5.6 Computer generated REFP analysis of the plasmids of Dublin....90

Figure 5.7 REFP analysis of the cointegrate plasmids of Dublin....95

Figure 6.1 Map positions of the sequenced regions on the Typhimurium SAP....109

Figure 6.2 *Xba*I, *Xho*I, *Hin*dIII, *Bgl*II, *Bam*HI and *Sal*I restriction sites on the Typhimurium SAP....110

Figure 6.3 Double digest REFP's of pOG660 with *Hin*dIII/*Bgl*II, *Hin*dIII/*Bam*HI, *Hin*dIII/*Sal*I, *Bgl*II/*Sal*I, *Bam*HI/*Sal*I *Bgl*II/*Bam*HI, *Xba*I/*Bgl*II, *Xho*I/*Bgl*II, *Xho*I/*Bam*HI *Xba*I/*Hin*dIII, *Xho*I/*Hin*dIII, *Xho*I/*Sal*I, *Xba*I/*Sal*I....113

Figure 6.4 Double digest REFP's of pOG660 with *Hin*dIII/*Eco*RI *Xho*I/*Eco*RI and *Sma*I/*Eco*RI....115

Figure 6.5 Double digest REFP's of pOG660 with PstI/SalI *Pst*I/*Bgl*II and *Pst*I/*Hin*dIII....119

Figure 6.6 *Hin*dIII fragment extraction and redigestion with *Pst*I and *Sma*I....121

Figure 6.7 Identification of *Sma*I restriction sites on the Typhimurium plasmid....125

Figure 6.8 Double digest REFP's of pOG660 with *Pst*I/*Sal*I and *Pst*I/*Bgl*II....130

Figure 6.9 Extracted *Bgl*II fragments redigested with PstI and *Sma*I....131

Figure 6.10 *Sma*I fragments extracted and redigested with *Pst*I....133

Figure 6.11 Identification of *Pst*I restriction sites on the Typhimurium plasmid....134

Figure 7.1 Possible framework for the evolution of the serotype associated plasmids of the salmonellae....150

CHAPTER 1

Introduction

History

The salmonellae were named after DE Salmon, an American bacteriologist who, in 1885, isolated and identified a micro-organism which was at the time described as the causative agent of hog cholera (Salmon and Smith, 1886). Although the aetiological agent of this disease was later proved to be viral and the bacillus isolated subsequently recognised as a secondary infectious agent, the generic designation *Salmonella* was introduced to honour the work of Salmon. This bacillus was named *Salmonella choleraesuis* and was found frequently to be a secondary invader in cases of hog cholera (Weil and Saphra, 1953). In the years that led up to the turn of the century, organisms were identified that caused both human (Gaertner, 1886) and animal disease (Loeffler, 1892) and although differed culturally and immunologically, were sufficiently related epidemiologically, serologically and culturally to be considered a single genus. Although primarily an intestinal parasite of man and other animals salmonellae are also found in soil, rivers, sewage and food.

Definition

The genus *Salmonella* belongs within the family *Enterobacteriaceae* and consists of gram negative, aerobic, non-sporing, rod-shaped organisms which are serologically related (Le Minor, 1984). Most strains are motile by means of peritrichous flagella and will grow on defined media without special growth factors. In general, they do not ferment lactose, sucrose or adonitol but do produce acid and gas from glucose, mannitol, sorbitol and a variety of other carbohydrates. In addition, they can utilise citrate as a sole source of carbon, rarely form indole, do not hydrolyse urea and produce H_2S from triple sugar iron agar. In addition to cultural properties, all members have an antigenic structure by which they can be recognised – although neither of these characteristics is wholly exclusive to the genus (Kauffmann 1954). These characteristics are not always stable features of the salmonellae and has made classification and nomenclature somewhat controversial.

Nomenclature

Before the recognition of the genus *Salmonella*, the causative agents of disease were generally referred to by the type of illness caused e.g. *Bacillus typhi* was or were given names associated with the animal from which they had been isolated e.g. *Bacillus pullorum* and *B. abortus-equi* (Weil and Saphra, 1953). Although the genus was originally created by medical bacteriologists to include organisms that gave rise to a certain type of illness in man and animals and were related antigenically it soon was apparent that salmonellae had many common biochemical characters and subsequently more emphasis was placed on biochemical activity than antigenic structure in their definition (Kauffmann, 1960). Further classification was introduced by PB White and F Kauffmann who divided the salmonellae into sub-groups based on their antigenic properties (Kauffmann, 1966). However, rather than refer to each type by its antigenic formula, the convention was established that each new type should be named after the place in which it was first isolated. In 1925 only 25 *Salmonella* serotypes were known. Currently there are 2375 serotypes with new types being discovered every year (Popoff *et al* 1994). However it soon emerged that some salmonellae were not host–adapted and others did not cause the disease that first inspired their name and this procedure for naming salmonellae was abandoned. As a result efforts were taken to limit the plethora of "species". Various unsuccessful efforts were made to clarify the situation. Ewing (1972) succeeded with the proposal that there were only three species of *Salmonella* : *S.choleraesuis*, *S.typhi* and *S.enteritidis*; the latter of which included all other serotypes. Under that method *S. typhimurium* became *S. enteritidis* serotype Typhimurium.

The advent of modern methods e.g. DNA–DNA reassociation techniques clearly indicated that all serotypes of *Salmonella* belonged to one group which comprised seven sub-species (Crosa *et al* 1973, Le Minor *et al* 1982a): subsp I included most of the serotypes responsible for widespread disease in man and animals; the other six sub-species comprised mainly parasites of cold-blooded animals or were found in the natural environment. It therefore followed that the species should have a single name. Following the rules of the Bacteriological Code, Le Minor *et al* (1982b) proposed *Salmonella choleraesuis* as the type species – it

being the first type described – with serotypes given in Roman type after the sub-species name e.g. *S.typhimurium* became *S.choleraesuis* subsp *choleraesuis* ser Typhimurium. However this provided yet more ambiguity by using a name previously used to designate a serotype. As a result a new epithet was proposed – *S.enterica* (Le Minor and Popoff 1987). In this way *S.typhimurium* became *S.enterica* subsp *enterica* serotype Typhimurium which although taxonomically correct, is too complicated for general use. A much simpler designation was *Salmonella* Typhimurium or just Typhimurium. The proposal has generally been accepted and although it is being widely used (Ewing 1986, Le Minor and Popoff 1987, Old 1990, Crichton and Old 1990, Browning *et al* 1995, Browning and Platt 1995, Rankin and Platt 1995) it has not yet acquired universal acceptance.

Typing Methods

Serotyping

In 1929 serological studies by White introduced the terminology by which the salmonellae are referred to today. The Kauffmann-White scheme is based on the recognition of bacterial surface antigens – the thermostable polysaccharide cell wall or somatic antigens (O antigens) and the thermolabile flagellar proteins or H antigens (Kauffmann, 1966). Those bacteria which possess capsules or envelopes also have a third variety of antigen (Vi or K). The Kauffmann-White scheme divided the salmonellae into serotypes based on the O and H antigens present.

The typical *Salmonella* possess both somatic (O) and flagellar (H) antigens. The Kauffmann-White scheme (Report 1934) classified salmonellae into groups and sub-groups designated by the letters A, B, C1, C2, C3, D, E1, E2, E3, E4 to Z. Each group was characterised by one or more antigenic factors which was common to all members of the group but which was not represented in members of other groups. The O antigens were designated numbers and to identify individual organisms within each group in was necessary to determine the H antigens. Most salmonellae have two forms of H antigen (Phase I and II) the expression of which are genetically determined and an individual cell may possess one or other form. A culture may therefore be composed of organisms all of which have the same H

antigen or may be a mixture of both. Phase I antigens were identified by lower case letters and Phase II by arabic numbers 1–7. The scheme arranged the salmonellae into serotypes based on antigens present in the envelope, cell wall and flagella. This method only recognised those antigens of primary diagnostic importance and is not a record of the complete antigenic structure of the organism.

By convention the four subgenera (I-IV) established by Kauffmann (1960, 1966) were recognised on the basis of biochemical tests only. However, detailed phenotypic and numerical-taxonomic studies revealed that most of the serotypes in these four subgenera were indistinguishable biochemically (Veron and LeMinor 1975).

Phage-typing

Bacteriophages are viruses that are capable of infecting bacteria resulting in either lysis of the cell or lysogeny in which the phage persists within the cell. All strains of *Salmonella* are susceptible to infection by bacteriophages.

Strains in particular serotypes can be further differentiated into phage types by their susceptibility to a series of bacteriophages. Phages with different specificities produce a pattern of lysis which when interpreted give rise to the phage type. However phage typing schemes have only been developed for a few serotypes e.g. Typhi (Craigie and Felix 1947), Typhimurium (Anderson *et al* 1977), Infantis (Kasatiya *et al* 1978), Hadar (de Sa *et al* 1980) Enteritidis (Ward *et al* 1987), and Virchow (Chambers *et al* 1989).

Phage typing is a phenotypic method of sub-dividing certain serotypes of *Salmonella* and is dependent on the presence of phage receptor sites on the bacterial cell surface. Chart *et el* (1989) showed that the loss of LPS in strains of Enteritidis resulted in change of phage type from 4→7 (with a concomitant loss of virulence). This resulted from the loss of six phage reactions for which the receptors are obviously found on the LPS. There have also been various reports of a change in phage type due to the acquisition of a plasmid. Frost *et al* (1989) showed that the acquisition of a plasmid resulted in the conversion of strains of Enteritidis from PT4 to PT24. An ampicillin resistant plasmid has been reported to be responsible for the increase in Enteritidis phage type 6a in Greece (Vatopoulos

et al 1994). Elimination of a similar sized plasmid (40kb) by Rankin and Platt (1995) resulted in the conversion of phage type 6a to 4 and also demonstrated the conversion of several different phage types of Enteritidis by the addition of temperate phages from the typing scheme. Although it is not known if this inter-conversion occurs readily in nature, it indicates that phage type results should be interpreted with considerable caution.

Biotyping

Biotyping is a method of discriminating within a serotype on the basis of biochemical properties. The typing scheme of Duguid *et al* (1975) was initially devised for Typhimurium and was based on 15 biochemical tests. 32 possible biotypes were defined by the combination of positive and negative reactions obtained with certain substrates (D-xylose, meso-inositol, L-rhammnose, d- and m-tartrates). Sub-types within these primary biotypes are characterised after reactions with a further 10 secondary tests. Full biotypes were designated a primary number and secondary letter. Although modified versions of the Typhimurium typing scheme were applied to other serotypes e.g. Paratyphi B (Barker *et al* 1988), Montevideo (Old *et al* 1985), Agona (Barker *et al* 1982) this system remains limited to a few serotypes. Biotyping is of most use when combined with other typing methods (Barker and Old 1989).

Multilocus Enzyme Electrophoresis

The typing methods mentioned previously either used individually or in combination provide phenotypic discrimination, which although useful in an outbreak or epidemiological situation, provides no information as to the population genetics of the salmonellae and does not readily allow the determination of relationships between serotypes. Multilocus enzyme electrophoresis (MLEE) was initially used in the field of eukaryotic population genetics. A modified method was developed by Selander and co-workers for the analysis of bacterial populations. This method characterises isolates by the relative electrophoretic mobility of a large number of cellular enzymes in starch or polyacrylamide gels (Selander *et al* 1986). Between 20–30 basic metabolic enzymes (which were expressed in all isolates of a

species) were chosen based on their neutrality under selection and hence were minimally subject to convergence through adaptive evolution. Mobility variants of enzymes were termed electromorphs. Each isolate was defined by its combination of electromorphs over the number of enzymes assayed. In this manner distinctive profiles of electromorphs were designated electrophoretic types (ET's). The relative mobilities of each enzyme can be equated with alleles at a corresponding gene locus. This method provided an indirect method of genotypic analysis whereby the amino acid sequence of the resultant proteins determines the result and has been of most use in the study of the population genetics of bacteria. Beltran *et al* (1988) demonstrated that certain multilocus enzyme types were of global distribution within the salmonellae. However, it is possible for genetic change to occur without any change either in the amino acid sequence or sufficient change to affect enzyme mobility. Hence subtle changes in DNA sequence may remain undetected. This method provided no discrimination of strains within an outbreak.

Molecular analysis

The phenotypic nature of serotyping, phage typing and biotyping does not allow the determination of evolutionary relationships between serotypes. The application of MLEE to bacterial population genetics demonstrated the clonal nature of the salmonellae (Beltran *et al* 1988, Selander *et al* 1990). These methods relied on the identification of surface structures or metabolic activities of the organisms. The recent advances in the field of molecular biology have enabled a greater understanding of the salmonellae. A number of techniques have been developed which have been applied to the salmonellae.These include plasmid analysis, pulsed field gelelectrophoresis, nucleic acid hybridization, ribotyping, IS200 analysis and polymerase chain reaction studies. These will be dealt with in the next section.

Plasmid analysis

Plasmids or extrachromosomal genetic elements have been found in almost all bacterial genera so far investigated. Takahashi *et al* (1969) showed that the

relative molecular length of plasmids could be determined by their mobility in agarose gel after electrophoresis whilst Aaij and Borst (1971) showed that the electrophoretic ability of DNA was dependent on its conformation in the gel matrix; closed circle DNA migrated faster than the duplex form. The development by Birnboim and Doly (1979) of a rapid simple method to isolate plasmid DNA has enabled plasmid analysis to be undertaken in any laboratory using simple equipment. Many wild–type strains of *Salmonella* carry plasmids which differ in size and number, and can be separated on agarose gels by electrophoresis. The investigation of outbreaks of salmonellosis was one of the first situations in which plasmid analysis was able to make a unique contribution in the identification of sources of infection (Spika *et al* 1987, Taylor *et al* 1982, Riley *et al* 1983). Widespread outbreaks of salmonellosis had in the past only been recognised if they were due to unusual serotypes e.g. *S.eastbourne* in chocolate (Craven *et al* 1975), or unusual phage types of Typhimurium (Cowden *et al* 1989). By using plasmid analysis Taylor *et al* (1982) were able to accurately distinguish a number of strains of *S.muenchen* from others isolated in the United States.

However plasmids of identical size but different in sequence and function can exist in many bacteria. This problem can be overcome by restriction endonuclease fragmentation pattern analysis of the plasmids whereby digestion with enzymes that recognise specific sequences in double stranded DNA produces a characteristic series of linear DNA fragments when separated by electrophoresis in agarose gels. The resultant pattern comprises the plasmid fingerprint (Platt 1983). The choice of enzyme depends on the size of the plasmid and also on the number of fragments generated. There should be sufficient fragments generated for specificity but not so many that coincidental matching occurs (Platt *et al* 1986). Interpretation of such plasmid fingerprint gels in an epidemiological situation requires a prerequisite knowledge of the plasmid pool of the organism.

It is often assumed that plasmids are accessory DNA elements and that their presence in bacterial strains is ephemeral. However, where a plasmid contributes to the virulence of an organism it is maintained over considerable periods of time by selection in infected persons or animals. Many strains of Typhimurium, Dublin, Enteritidis carry virulence plasmids (Woodward *et al* 1989)

which may reside by chance in epidemiologically unrelated strains (Platt *et al* 1986). The demonstration that several strains harbour an indistinguishable plasmid cannot always be taken as evidence of their relatedness.

Chromosomal analysis

Pulsed Field Gel Electrophoresis

The genome of Typhimurium (and other salmonellae) is approx 4.5 x 10^6 bp and is thought to code for about 3000 genes. About 4% of the genome encodes functions neccesary for virulence.

Because of its large size, great care must be taken when isolating chromosomal DNA in order to avoid physical shearing. One of the most successful techniques involves extraction *in situ*, where intact bacterial cells are embedded in agarose, lysed, deproteinised and digested and the agarose plugs loaded directly into the wells of an agarose gel for electrophoresis (Schwartz and Cantor 1984). Conventional electrophoresis does not resolve fragments larger than about 50 kb and restriction enzymes that cut the bacterial genome into fragments smaller than 50 kb tend to generate too many similar sized fragments. Pulsed field gel electrophoresis (PFGE) was derived from conventional agarose electrophoresis to enable the resolution of large fragments >200 kb – the electric field was alternated between spatially fixed pairs of electrodes. In this way, DNA fragments were able to re-orientate and move more easily (Carle *et al* 1986).

PFGE has proved of little value in epidemiological investigation. A study by Arbeit *et al* (1990) revealed that PFGE was useful in the determination of the evolutionary divergence of E.coli but could not discriminate epidemiologically related and unrelated strains. Similarly, Thong *et al* (1996) reported 29/32 sporadic cases of Enteritidis were indistinguishable from the outbreak-related cases. Olsen and Skov (1994) used PFGE to demonstrate four different genomic lineages of Dublin, one of which predominated throughout the world and paralleled the MLEE results of Selander *et al* (1992), plasmid analysis (Browning *et al* 1995) and whole cell REFP (Platt *et al* 1995) of this serotype. PFGE analysis is therefore open to mis-interpretation if the operator is unaware of the population genetics of the

organism being studied; within particular geographical areas a common clone often exists.

Nucleic Acid Hybridisation

Nucleic acid hybridisation takes advantage of the ability of double-stranded DNA/RNA molecules to be separated (by heat or alkali treatment) and the subsequent introduction of a probe fragment will allow the re–formation of H–bonds between complementary sequences that exist. The probe is labelled with a detectable marker

DNA hybridisation

DNA hybridisation is most often combined with REFP or chromosomal fingerprint gels. The DNA fragments are transferred to nylon or nitrocellulose membranes and then hybridised with a probe. Probes can be either specific e.g. *spv* – a probe from a region of the Typhimurium virulence plasmid (Woodward *et al* 1989, Poppe *et al* 1991) which demonstrated the presence of a virulence associated region in plasmids of different serotypes, or random sequences (Tompkins *et al* 1986) which served to highlight sequence heterogeneity.

Ribotyping

This method, introduced by Grimont and Grimont in 1986, uses ribosomal RNA or the corresponding sequence as a probe. This method relies on the fact that rRNA genes are organised into operons –16S, 23S and 5S and that several (4–8) copies are found in a typical bacterial cell. Used individually or collectively these probes will hybridise with fragments in a chromosomal fingerprint that contain the corresponding gene or part thereof. It has been found to be useful primarily when combined with other typing methods e.g. plasmid analysis and phage typing and has been used to study Enteritidis (Martinetti and Altwegg, 1990), Berta (Olsen *et al* 1992), Typhi (Nastasi *et al* 1993), Reading, Senftenberg and Typhimurium (Esteban *et al* 1993). All researchers basically found that within a serotype isolates can be sub–divided into smaller groups based on their ribosomal patterns. The discrimination of strains achieved by this method was generally low.

IS200 analysis

Insertion sequences are genetic elements that can insert themselves into different sites in a genome and as a result can often cause chromosomal rearrangements (inversions, deletions) and thereby alter gene expression. In 1983 a *Salmonella*–specific insertion sequence was discovered by Lam and Roth and was designated IS200. This 700bp element and has been found to be present in different copy numbers in different serotypes of salmonellae. IS200 profiles have been used in the area of population genetics and Stanley *el al* (1991) identified three clonal lineages of Enteritidis based on the distribution of IS200 although the conclusions reached by the authors, that IS200 analyses "underline the value of phage typing which maximises strain discrimination in epidemiological studies of *S.enteritidis*" are inaccurate. Given that the authors found all isolates within a phage type had the same IS200 profile, it would appear that in an epidemiological context, the method is of no more benefit than other typing methods. A recent study concluded that IS200 analysis was not applicable to Choleraesuis (Weide-Botjes, 1996). Thus although beneficial in the area of population genetics, IS200 profiles are of most use when combined with other methods.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) enables the amplification of a few copies of target DNA to a level that can be detected by gel electrophoresis or hybridization (Saiki *et al* 1988). PCR has been used for the detection of different pathogenic organisms in clinical samples and foodstuffs (Widjojoatmodjo *et al* 1991) and semi-quantitative assays have been developed for the detection of *Salmonella* virulence plasmid genes (Mahon and Lax 1993, Rexlach *et al* 1994). Although this method is extremely sensitive, it is subject to certain constraints. In epidemiology it is currently limited by reproducibility. False positive reactions can occur and contamination is a major consideration in the analysis of diagnostic results. However when used correctly PCR can provide an important research technique

Comparison of different typing methods

There have been many reports on the merits and drawbacks of different typing systems. Barker and Old (1989) found biotyping afforded excellent strain discrimination whereas phage typing provided good discrimination of epidemic strains. Brunner *et al* (1983) however found biotyping was of no use in the analysis of Typhimurium but phage typing was as good as plasmid analysis. Holmberg *et al*, (1984) and Tompkins *et al* (1986) also found plasmid analysis comparable to phage typing. However, these studies relied on the determination of plasmid size as an indication of their relatedness, and as indicated by Farrer (1983) identity of plasmid size does not indicate plasmid identity. For this reason the use of plasmid fingerprint analysis should be employed in the analysis of plasmids, although it should be pointed out that the choice of restriction enzyme is important. Many workers have used enzymes that cleave infrequently which generate only few fragments and may not reveal sequence variations which reduces the information content of a fingerprint.

Each laboratory, perhaps understandably, advocates their own particular method of analysis. In epidemiological investigation, the best results were achieved when a combination of methods were implemented (Riley *et al* 1983, Brunner *et al* 1983, Mayer *et al* 1988, Holmberg *et al* 1984, Platt *et al* 1987).

Pathogenicity

Types of Disease

The salmonellae cause a wide variety of disease in man and animals. In its natural setting salmonellosis is acquired by oral ingestion of the organism. Thereafter there are three common clinical manifestations -- gastroenteritis, systemic infection (enteric fever) or the asymptomatic carrier state. The most common clinical manifestation is gastroenteritis in which the organism passes through the stomach to the intestine where it is engulfed by the epithelial cells of the ileum. There is rarely penetration beyond the basal epithelium. This non-systemic infection of the intestinal tract and regional lymph nodes results in

diarrhoea and the disease is usually self-limiting. Enteric fever occurs when the bacteria cross the intestinal epithelia to the reticulo-endothelial system where systemic infection occurs as the bacteria multiply in mononuclear phagocytes in the liver, spleen, lymph nodes and Peyers patches. Bacteraemia occurs when the patient develops fever minus the manifestations of enteritis. Asymptomatic carriers can excrete organisms more than a year after the symptoms of salmonellosis have disappeared. In this instance the bacteria reside in the gall bladder and are excreted into the intestine with the bile (Christie 1974).

The salmonellae are primarily intestinal parasites of man and animals. A few serotypes are host adapted e.g. Gallinarum – fowl, Abortusovis – sheep, Typhi – man etc. The virulence of the salmonellae is associated with the organisms ability to invade and persist in target organs during systemic infections (Kawahara *et al* 1988). However, the factors responsible for virulence of the salmonellae are ill–defined. The LPS is the most extensively characterised virulence determinant and although its exact mechanism of action is not known it may be involved in attachment and invasion (Finlay and Falkow, 1988). It is unknown if any effects on the host by the Lipid A component are essential for virulence but they certainly contribute to the overall disease. Rough strains of *Salmonella* are avirulent. The underlying cause probably involves loss of chemostatic motility as well as diminished resistance to gastric acidity (Finlay and Falkow, 1988).

Virulence Factors

Most of the experimental analysis of the virulence of *Salmonella* has been carried out in the mouse model and although the effects in man and animals will vary slightly (e.g. mice infected with Typhi do not display clinical signs of typhoid) it is a useful method for understanding the basis of pathogenesis.

Before it can cause disease, *Salmonella* must overcome a variety of host defence mechanisms. Typhimurium has long been the model system for studying the virulence factors by which the salmonellae are able to cause disease – invasion, survival and replication in host cells.

Surface Structures

In order to avoid elimination by peristalsis, the salmonellae must first adhere to the intestinal epithelial cells. Type 1 pili, or fimbriae, are filamentous surface structures involved in the adherence to epithelial cells. Although the exact role of such structures in the virulence of the organism appears limited – oral challenge of piliated and non-piliated Typhimurium strains resulted in minor differences in virulence in the mouse model (Duguid *et al* 1976). The composition of type 1 pilin proteins from Typhimurium and Enteritidis varies considerably (Purcell *et al* 1987, Feutrier *et al* 1986). More recently a study by Baumler *et al* (1996) demonstrated that plasmid encoded fimbrial genes (*pef*) mediated adhesion to the murine small intestine. This operon, although necessary, did not mediate fluid accumulation and suggests that in the mouse model, the *pef* operon enhances the organisms ability to bind to the epithilium of the small intestine and acts in conjunction with other virulence factors to cause fluid secretion.

Another surface structure shown to contribute to the virulence of *Salmonella* is the flagellum – although neither functional motility nor chemotaxis is required. Carsiotis *et al* (1984) demonstrated that flagella were not required for colonisation of the gastro-intestinal tract but were needed for survival and growth of the organism in the liver and spleen. Again, the exact mechanism of virulence is unclear although it is thought that the flagellum is necessary for either intracellular multiplication in the macrophage or to contribute to enhanced resistance to macrophage killing (Weinstein *et al* 1984).

Cellular invasion and survival

Invasion of the gastrointestinal mucosa is an essential step in the pathogenesis of *Salmonella* (Gianella *et al* 1973). Strains that are unable to invade are avirulent. The exact mechanism of entry into the host cell remained unknown for a long time but advances in the area of molecular genetics has provided some answers. Galan and Curtiss (1989) identified a chromosomally encoded locus *inv* which is essential for entry of *Salmonella* into cultured epithelial cells. So far 15

genes have been identified in this locus termed *inv*A, B,C, D – O. The protein InvA is a member of a family of proteins involved in either the secretion of virulence determinants of flagellar biosynthesis (Ginoccio and Galan 1995).

Survival within macrophage

Once inside eukaryotic cells, salmonellae have a number of strategies by which they avoid host defences. Phagocytosis is the first line of defence against invading organisms. Fields *et al* (1986) constructed a series of Typhimurium mutants and showed that those unable to survive within the macrophage were avirulent. Mechanisms for survival within macrophage include inhibition of the respiratory burst (the process in which the cell produces toxic metabolites including hydrogen peroxide and superoxide), inhibition of lysosomal fusion, escape from the phagolysosome and resistance to granular antimicrobial peptides. The exact mechanisms of these survival strategies are unknown, although further characterisation of Typhimurium mutants will elucidate such bacterial virulence properties. One such genetic locus *pho*P has been identified that controls the expression of genes encoding factors that protect *Salmonella* from the bactericidal action of macrophage-derived cationic peptides or proteins (Fields *et al* 1986, Groismann *et al* 1986).

Toxins

Salmonella produce three types of toxin, each of which can contribute to virulence during infection. The Lipopolysaccharide (LPS) or endotoxin – intact O–antigen side chains of the LPS protect the bacteria from host attack. The unique sugars that constitute the O–antigen limit the activation of the Complement system, and thereby reduce the ability of macrophage to ingest and kill the organism (Luderitz *et al* 1986). The heat-labile enterotoxin shows immunological cross-reactivity with cholera toxin (i.e. it increases levels of cAMP) (Wallis *et al*, 1986). The cytotoxin is localised to the bacterial outer membrane and inhibits protein synthesis in epithelial cells and results in extensive detachment of intact vero cells in experimental infection (Koo and Peterson, 1983).

The exact role of the three toxins has not been clearly defined although their involvement in the pathogenesis of experimental infection has been clearly demonstrated.

Plasmids

The unique property often conferred by plasmids to bacterial pathogenesis is virulence, although these organisms may also harbour plasmids encoding ancilliary functions e.g. antimicrobial resistance, bacteriocinogeny. However, those plasmids mediating essential virulence factors *per se* are generally present in all wild-type strains of a pathogenic species (Brubaker 1985).

In the 1970's a large molecular weight plasmid was identified in Typhimurium (Spratt *et al* 1973, Smith *et al* 1973). This plasmid was termed "cryptic" since no phenotype could be attributed to it. Jones *et al* (1982) described this plasmid as being associated with virulence; loss of this plasmid led to reduced virulence in mice and the loss of ability to adhere to and invade HeLa cells. Reintroduction of the plasmid restored these properties. Further studies demonstrated plasmids with similar properties in other serotypes e.g. Dublin (Terakado *et al* 1983), Enteritidis (Nakamura *et al* 1985), Gallinarum (Barrow *et al* 1987), Pullorum (Barrow and Lovell 1988) and Choleraesuis (Kawahara *et al* 1988). The observation that particular plasmids were associated with certain serotypes of salmonellae led Helmuth *et al* (1985) to introduce the term "serotype-specific" plasmid (SSP). Although this designation is widely used it was based on size and distribution alone without the additional data provided by REFP analysis. As shall be shown later, it is incorrect. Plasmids previously described as SSP's of Enteritidis and Dublin have been demonstrated in different, although closely related serotypes. For this reason the term "serotype-associated plasmid" has been proposed (Browning and Platt 1995) and adopted throughout.

Serotype-associated plasmids of the salmonellae

Serotype–associated plasmids have been identified in certain serotypes of *Salmonella* including those most commonly isolated from man (Typhimurium and

Enteritidis) as well as host adapted serotypes (Dublin, Gallinarum, Pullorum and Choleraesuis). However, although this is not a universal feature of the salmonellae the incidence of SAP carriage in such serotypes is between 87–89% (Helmuth *et al* 1985).

Homology between SAP's

Comparison of SAP's of different serotypes by REFP analysis and DNA homology by Popoff *et al* (1984) used the entire Typhimurium plasmid as a probe and showed that the plasmids of Paratyphi C, Enteritidis, Newport, Dublin and Abortusovis shared a high degree of sequence homology with Typhimurium (73–90%). As mentioned previously, these plasmids are important in the virulence of the organism. Physical and genetic analyses of these plasmids have determined that not all of the plasmid is required for virulence (Michiels *et al* 1987). The essential virulence region has been identified as being 8kb in size (Williamson *et al* 1988b) and was found to be common to eleven serotypes of *Salmonella* : Abortusovis, Blegdam, Choleraesuis, Dublin, Enteritidis, Gallinarum, Moscow, Paratyphi C, Pullorum, Rostock and Typhimurium (Williamson *et al* 1988a). This region was later found to encode the salmonella plasmid virulence gene operon - *spv* and consists of the regulator *spv*R and four structural genes *spv*ABCD (Gulig *et al* 1993). Prior to this nomenclature different groups used designations such as *vir*, *vag*, *mkf*, *mka*, *vsd* and *mba* for the same genes. The exact mechanism of action of these genes remains largely unknown but a number of functions have been discovered for different loci e.g. resistance to complement action, plasmid replication and partition. Table 1.1 shows some examples of genes identified so far.

Popoff *et al* (1984) and Williamson *et al* (1988a) have suggested different mechanism for the evolution of SAP's. The results of both their studies conflict with each other, not as written but in the conclusions drawn from them. The former suggests a family of related plasmids which have diverged from a common ancestor; the latter implies that a particular sequence has either been transposed between plasmids or conserved during plasmid evolution. Incompatibility studies by Platt *et al* (1988) revealed that the SAP's of Typhimurium, Enteritidis and

Dublin belong to the same incompatibility group which strengthens the arguement that the salmonellae contain a family of related plasmids.

Clonality

In the strict sense a bacterial clone consists of "a single cell and all its descendants representing a monophyletic branch on an evolutionary tree" (Whittam 1994) although in the field of population genetics its definition is slightly less rigid and generally refers to a sub–group of bacteria within a species derived from a common ancestor – and that have many similarities not shared by other organisms in the species. A clonal lineage is a genetic system whereby any differences occur with a single cell e.g. point mutation, deletion, inversion.

The concept of clonality was first applied in bacteriology by Kauffmann and Orskov in 1956 when they noticed that most enteropathogenic *E.coli* O:H serotypes had characteristic biotypes regardless of isolation or geographic origin. The first indication of clonality was provided by the observation of a stable world-wide association of several specific O:K:H serotypes and biotypes.

The genetic structure of Salmonella is basically clonal. This conclusion was reached based on OMP electrophoretic patterns and plasmid analysis (Helmuth *et al* 1985) and MLEE (Beltran *et al* 1988). For each serotype analysed, except Derby and Newport, there is one predominant world–wide clone with minor clones probably recently derived from them. Both Derby and Newport have two divergent clone clusters based on MLEE analysis, which differ in the relative frequencies with which their clones cause disease in birds v animals (Derby) and humans v animals (Newport).

Clonal expansion is also evident from the plasmid population. A study of clinical isolates from wide geographical sources of *Salmonella* Wein revealed that the plasmid contant had remained uniform over a decade and also in the late stages of the epidemic history of the strain (Casalino *et al* 1984). Similarly, plasmid analysis revealed 89% of Dublin isolates from 5 continents to harbour virulence plasmids. These data, combined with whole cell REFP analysis extented the findings of Selander *et al* (1992) – that the population of Dublin consisted of two clones Du1 which was of worldwide distribution and Du3 which was restricted to

Table 1.1

Examples of genes found in some serotypes of *Salmonella*

GENE	LOCATION	FUNCTION	REFERENCE
*spv*RABCD	plasmid	virulence	Gulig *et al* 1993
*pef*A	plasmid	fimbrial biosynthesis	Friedrich *et al* 1993
traT	plasmid	enhanced serum resistance	Rhen and Sukupolvi 1988
rsk	plasmid	enhanced serum resistance	Vandenbosch *et al* 1987
rck	plasmid	complement resistance	Heffernen *et al* 1992
omp	chromosome	porin production	Dorman *et al* 1989
*lpf*ABCDE	chromosome	fimbrial biosynthesis	Baumler and Heffron 1995
rpoS	chromosome	transcription of *spv* genes	Heiskanen *et al* 1994
*inv*A-O	chromosome	invasion of epithelial cells	Galan and Curtiss 1989
rfb	chromosome	O-antigen biosynthesis	Brahmblatt *et al* 1988

Europe (Platt *et al* 1995) by including Du3 to beyond Europe. These results demonstrated once geographically separated members of the same clone diverged in response to local selection pressure, and will be discussed more fully later. All Scottish strains of Dublin were fully sensitive to all antimicrobial agents tested. In contrast 74% of strains from outwith the UK were resistant to between one and five antimicrobial agents (Browning and Platt 1995). This is an example of a sub-clone within a localised geographical area.

AIMS

The aims of this study were as follows

1) To determine the relatedness of the serotype associated plasmids of the salmonellae firstly by the comparison of their restriction endonuclease fragmentation patterns and secondly to determine their incompatibility with a naturally occuring cointegrate plasmid that comprised pOG660 (the Typhimurium SAP) and pOG670 (and IncX plasmid).

2) To determine the extent of molecular variation of serotype associated plasmids both within and between serotypes.

3) To determine whether the plasmids harboured by host adapted serotypes exhibites more or less molecular variation than those of broad host range serotypes.

4) To determine the location of *Pst*I and *Sma*I restriction sites on the Typhimurium Sap and thereby identify potential regions on the plasmid which may be common to other SAP's but not associated with virulence functions. From these data it was envisaged that a number of fragments that were common to all or some of the

SAP's would be chosen with which to construct DNA probes and hybridize to Southern blots of REFP's.

5) To consider the serotype associated plasmids of the salmonellae within a possible evolutionary framework based on the combined data.

CHAPTER 2

General Materials and Methods

Storage of Strains

Presumptive isolates of *Salmonella* were sent to the Scottish Salmonella Reference Laboratory (SSRL) where confirmation of serotype was carried out by standard methods (Kauffmann, 1954). A single colony was inoculated onto a Dorset's egg slope and incubated overnight at 37°C. Thereafter long term storage of strains was at room temperature. Duplicate cultures were prepared – the growth from approx 1/2 a nutrient agar plate was resuspended in a 3ml solution of glycerol--peptone (8% glycerol in 1% protease peptone) and stored at -80^0C.

Media

Short term maintenance of bacterial strains was by sub–culture on cystine–lactose–electrolyte–deficient (CLED) agar plates (Mast DM110).

Nutrient agar (Nutrient broth, Oxoid CM1, that contained 1% Bacteriological agar, Oxoid L11) was used for the growth of bacteria from which DNA was extracted for plasmid profile analysis (PPA).

Isosensitest agar (Oxoid CM471) was used for antimicrobial susceptibility tests.

Brain heart infusion (BHI) broth (Oxoid CM225) was used for the growth of organisms before extraction and purification of plasmid DNA for restriction endonuclease fragmentation pattern (REFP) analysis.

Bacterial strains

Bacterial reference strains are shown in Table 2.1 together with strain designation, antigenic structure and source. Strains of *E.coli* used in this study are shown in Table 2.2.

Antimicrobial susceptibility testing

Bacterial susceptibility to a range of antimicrobial agents was determined by disk diffusion assay on isosensitest agar with antibiotic disks (Mast) impregnated with individual antimicrobial agents -- amikacin (Ak;10μg), ampicillin (Ap;10μg), carbenicillin (Cb;100μg), cephamandole (Ma;30μg), cephazolin (Kz;30μg), chloramphenicol (Cm;10μg), colistin sulphate (Ct;25μg), gentamicin

Table 2.1

Reference strains for plasmid analysis

Serotype	Strain Designation	Antigenic Structure	Source
Abortusovis	**GR7594**	**4,12 : c : 1,6**	**NK**
Bovismorbificans	**GR6389**	**6,8 : r : 1,5**	**NCTC 5754**
Choleraesuis	**GR6489**	**6,7 : c : 1,5**	**NCTC 5735**
Dublin	**GR34285**	**9,12 : g,p : –**	**Platt *et al* 1988**
Enteritidis	**GR16485**	**9,12 : g,m: –**	**Platt *et al* 1988**
Gallinarum	**GR6589**	**9,12 : – : –**	**NCTC 9240**
Pullorum	**GR6689**	**9,12 : – : –**	**NCTC 10706**
Typhimurium	**NCTC 73**	**4,5,12 : i :1,2**	**NCTC 73**
Wangata	**NCTC 8276**	**9,12 : z_4,z_{23} : 1,7**	**Denmark, 1953**
Blegdam	**NCTC 5769**	**9,12 : g,m,q : –**	**Copenhagen, 1939**
Moscow	**NCTC 10480**	**9,12 : g,q : –**	**Copenhagen, 1939**
Rostock	**NCTC** 5767	**9,12 : g,p,u : –**	Copenhagen, 1939
Antarctica	**NCTC11342**	**9,12 : g, z_{63}**	**NCTC 11342**

NK – Not Known

NCTC – National Collection of Type Cultures

Table 2.2

Strains of *E.coli* used in the study.

K12 Strain Designation	Purpose in Study	Phenotype
39R861 (NCTC 50192)	**Source of plasmids for PPA molecular standards**	**lac^+**
J53-2	**Recipient strain for plasmid transfer**	**lac^+, pro^-, met Rif^r**
J53-2 pOG669	**Donor plasmid used in incompatibility studies**	**lac^+, pro^-,met^- Rif^r, Ap^r, K^r**
J53-2 pOG670	**Donor plasmid used in incompatibility studies**	**lac^+, pro^-,met^- Rif^r,Ap^r, K^r**

lac^+ lactose fermenter

pro^- proline auxotroph

met^- methionine auxotroph

(Gm;10μg), kanamycin (Km;30μg), nalidixic acid (Nal;30μg), rifampicin (Rif;50μg), sulphamethoxazole (Su;25μg), streptomycin (Sm;10μg), tetracycline (Tc;10μg), tobramycin (Tb;10μg) and trimethoprim (Tp;1.23μg).

Buffers and Reagents

All buffers and reagents are detailed in Appendix 2.

Centrifugation

Unless otherwise stated all microcentrifugation steps were carried out at 13000 rpm in a Heraeus microcentrifuge.

Plasmid Profile Analysis

Plasmid DNA was examined initially as a crude lysate to determine the plasmid profile of the bacterial strain.

Preparation of cell lysates

The growth from approx 3/4 of a nutrient agar plate, incubated overnight at 37°C, was suspended in 600μl Tris Borate–EDTA (TBE 89mM Tris, 89mM Boric acid, 1.25mM EDTA pH 8.2) buffer in an eppendorf microcentrifuge tube using a sterile swab. 400μl of sodium dodecyl sulphate (SDS, 10% solution in TBE buffer) was added and the eppendorf inverted gently a few times to mix. The suspensions were then placed in a heating block at 55°C for 10 minutes, or until lysis was complete and the solution cleared. The lysates were then centrifuged for 10 minutes and the resultant pellet of cell debris removed with broken swab or a pipette tip.

Vertical Gel Electrophoresis

To 100μl of cleared cell lysate 5μl of tracking dye (25 % sucrose, 0.06 % sodium acetate, 0.1 % SDS, 0.05 % bromophenol blue) was added and electrophoresed in a vertical agarose gel (0.7% w/v in TBE buffer) at 100V for 1 hour followed by 200V for 4 hours. Plasmids of known molecular weight (*E.coli*

K12 39R861 – 147:63:36:7 kb) were included in each gel as markers for the estimation of plasmid size.

Visualisation of Plasmids

After electrophoresis the gel was stained in ethidium bromide (6µg/ml) in TBS (50mM Tris, 50mM NaCl, 5mM EDTA pH 8.0) buffer for 15 minutes. The plasmid bands were visualised under ultraviolet light (302nm) and photographed on Polaroid Type 665 film with a Polaroid MP4 land camera.

Plasmid purification for REFP analysis

Purified DNA for REFP analysis was prepared by the method of Platt *et al* (1988). A 10ml overnight culture in BHI broth was centrifuged for 10 minutes at 4500 rpm in a benchtop centrifuge. The bacterial pellet was resuspended in 400µl TGE (25mM Tris, 10mM EDTA, 50mM glucose pH 8.0) buffer. Each sample was then divided into two eppendorf tubes and centrifuged for 30 seconds. The supernatant fluid was discarded and the pellet resuspended in a solution of lysozyme in TGE (5mg/ml). The samples were incubated on ice for 5 min, then 400µl of alkaline SDS (1% SDS, 1% NaOH) was added, the tubes mixed gently by inversion and incubated on ice for a further 5 min. 300µl sodium acetate (3M) was added, the tubes inverted a few times until flocculation occured and then vortexed. The samples were placed on ice for 5 min after which time they were centrifuged for 2 min. The supernate was transferred to a clean eppendorf tube and 500µl phenol–chloroform (1:1w/v) added and the tubes vortexed. After centrifugation for 2 min the upper aqueous layer was transferred to a clean eppendorf, 500µl of propan–2–ol added and the tubes vortexed. After at least 5 minutes at room temperature, the DNA was recovered by centrifugation for 10 min, the supernate discarded and the pellet resuspended in 100µl TE (10mM Tris, 1mM EDTA pH 8.0) buffer. Duplicate tubes were pooled, 100µl of ammonium acetate (7.5M) added, vortexed and 600µl ethanol added. The tubes were vortexed and stored overnight at –20°C. After centrifugation for 10 min, the supernate was discarded

and the pellet resuspended in 160µl TE. 18µl of RNAase (1 mg/ml) was added and incubated at 37°C for 30 min. 20µl of NaCl (2.5M) was added, mixed and 500µl of phenol–chloroform added. The tubes were vortexed and centrifuged for 2 min. The upper aqueous layer was transferred to a clean eppendorf, 500µl propan–2–ol added and mixed thoroughly. After at least 5 min at room temperature the samples were centrifuged for 10 min and the resulting pellet resuspended in 100µl TE buffer. 100µl ammonium acetate (7.5M) was added, mixed and then 600µl ethanol added. The tubes were vortexed and placed at –20°C overnight. After centrifugation for 10 min, the pellet was allowed to air dry and then resuspended in 60µl TE buffer and stored at 4°C.

Restriction endonuclease digestion of purified plasmid DNA

DNA was digested with a range of restriction endonucleases (Life Technologies) used according to the manufacturers instruction. The equivatent of 20 units of enzyme was used (where one unit of enzyme is defined as the amount required to digest 1µg of lambda DNA completely in 1 hour under specified conditions). Reaction mixtures were prepared as follows:

20µl sample DNA

5µl REact™ buffer

2µl enzyme

23µl sterile distilled water

The tubes were vortexed gently and centrifuged for a few seconds to ensure thorough mixing. The samples were then incubated at 37°C (30°C for *Sma*I) for 4 hours. Control samples were included of phage lambda (λ) DNA digested with the same enzyme as the sample DNA to determine full enzymatic activity occured. λ DNA digested with *Pst*I and also λ DNA digested with *Kpn*I were included as markers for the calibration of fragment sizes.

Horizontal Gel Electrophorsis

A 0.8% agarose gel was prepared (0.8g agarose in 100ml TBE buffer containing ethidium bromide). After enzymatic digestion was complete, 5µl of gel

loading buffer (25% Ficoll, 0.25% bromophenol blue in TBE containing ethidium bromide) was added to each reaction tube. The gel was placed in a horizontal gel tank (Life Technologies Model H3) and submerged in TBE containing ethidium bromide and the samples loaded. The current was applied (18mA for the 10 x 14 cm gel tank and 32mA for the 20 x 20 cm tank) and electrophoresis carried out overnight (or approx 16 hours). The following morning the gel was viewed and photographed as previously described.

Computer aided analysis of REFP's

Restriction fragment mobility in ethidium bromide stained agarose gels was recorded on Polaroid film and input to a computer using a digitiser and commercially available software (Platt and Sullivan, 1992). Each gel was calibrated with restriction fragments from both *Pst*I and *Kpn*I digests of λ DNA. The molecular weights of these fragments was fitted to a robust modified hyperbola (Plikaytis *et al*, 1986) from which fragment sizes in adjacent tracks were estimated by interpolation. The numerical values (kb) were stored for subsequent calculation of similarity co-efficients (Dice 1945) and graphical output (logarithmic scale). The calculation of Dice coefficients of similarity was based on the formula

$$S_D\,(\%) = [2m/(a+b)] \times 100$$

where 'm' was the number of restriction fragments common to two plasmids (A and B) and 'a' and 'b' were the total number of fragments generated from each plasmids after digestion with the same enzyme.

Incompatibility Analysis of Plasmids

Bacterial strains and their plasmid designations are detailed in Table1.1.

BHI broth was used for growth of donor and recipient organisms for plasmid transfer.

Antibiotic impregnated discs were supplied by Mast at a concentration of 30μg per disc.

Minimal Media (Appendix 1) was used for the selection of transconjugants. Inhibition of the recipient *Salmonella* was by antibiotic selection.

Certain serotypes were unable to synthesise essential vitamins and amino acids which therefore had to be incorporated in the media. Dublin had a nutritional requirement for nicotinic acid and so for this experiment was incorporated into the minimal media at a concentration of 20µg/ml. Similarly Pullorum, Gallinarum and Choleraesuis had nutrititional requirements for cystine which was incorporated at a concentration of 0.2mg/ml. Abortusovis required the addition of nicotinic acid (20µg/ml), cystine and methionine (0.2mg/ml) (Stokes and Bayne 1958)

Broth transfer method

An overnight BHI broth culture of both donor and recipient organism was mixed in the ratio of 1:5 i.e 1ml donor (pOG669) + 4ml recipient (Salmonella) plus 5ml fresh BHI and incubated for 6 hours at 37°C. The cultures were then centrifuged, washed and resuspended in saline (3ml). A sterile swab was used to spread the suspension on a minimal media plate and ampicillin and kanamycin discs were placed 1 cm apart on the plate. A purity plate (CLED) was also sub-cultured from the mating mixture to confirm the presence of both organism types. The plates were incubated overnight at 37°C.

Selection of transconjugants

Colonies that grew within the normal zone of inhibition of the antibiotic were selected onto CLED agar plates and ampicillin and kanamycin discs added. If no colonies were present then the plates were re-incubated overnight. The selection pressure was maintained for the incoming plasmid for three successive subcultures. Thereafter single colonies were subcultured to nutrient agar for plasmid profile analysis.

Characterization of potential transconjugants

Confirmation of plasmid transfer was carried out by plasmid profile analysis as described previously. Each plasmid profile gel contained ten potential transconjugants, pOG669 (or pOG670) and the parental SAP.

REFP analysis of the transconjugants was performed as described previously, to confirm the presence of pOG669/pOG670 in its entirety and loss of the SAP where the plasmids were incompatible.

Southern Blotting

This technique, devised by EM Southern in 1975, involved the transfer of DNA fragments from an agarose gel to a nitrocellulose or nylon membrane. The DNA fragments in the gel were denatured and a replica of a plasmid fingerprint was then suitable for hybridisation with a labelled probe (Maniatis *et al* 1982).

Transfer of DNA from agarose gel

After electrophoresis, the DNA fragments were transferred to nitrocellulose membranes (Sigma, pore size 0.2μm) for subsequent hybridisation with a DNA probe. This was achieved by vacuum transfer which was much more efficient and quicker than conventional capillary transfer and did not involve pre-treatment of the gel – all the steps were performed in the vacuum blotting unit. The nitrocellulose membrane was pre-wet with sterile distilled water and subsequently immersed in 20×SSC (3M NaCl, 0.3M Sodium Citrate pH 7.0) for 30 minutes. The vacuum blotting unit (Pharmacia LKB 2016 VacuGene XL) was set up in accordance with the manufacturers instructions, the nitrocellulose membrane put in place (with one corner marked for orientation) and the gel placed on top. The vacuum was stabilised at 50mBar pressure. The gel was depurinated with 0.25 M HCl by flooding the gel without leakage (approx 30ml) for 4 min, after which time it was removed and replaced with the denaturation solution (1.5M NaCl, 0.5M NaOH). After 3 minutes this solution was removed and replaced with a neutralisation solution (1.0M Tris, 2.0M NaCl) which was left on for a further 3 minutes and finally replaced with 20×SSC to twice its depth and the transfer carried out for 45 minutes. The nitrocellulose membrane was then air–dried, the wells and λ control lanes marked with a pencil by visualisation under UV light and the DNA fixed by baking at 80°C for 2 hours. The membrane was stored in an airtight plastic bag.

Extraction of DNA from agarose gels

The extraction of DNA fragments from agarose was attempted by many methods. These included spin-bind columns (Flowgen), glass wool extraction (Heery *et al* 1990) and commercially available kits such as Geneclean (Stratech Scientific Ltd). However the only satisfactory method was found to be electroelution of the fragment onto DEAE cellulose paper (Schleicher & Schuell NA-45, 0.45µm pore size). However, one limitation of this method was a low yield of DNA. To compensate for this, multiple tracks of the same fragment were extracted in parallel. In this manner 12 × 20µl of plasmid DNA was digested and electrophoresed overnight.

Preparation of DEAE strips

Gloves were worn to prevent contamination of DEAE paper. Strips of DEAE were cut approx 4 cm x 1cm and were pretreated by immersion in 10mM EDTA for 10 min followed by 5 min in 0.5M NaOH and several washes in distilled water. The strips were then stored in distilled water at 4°C.

Elution of DNA

A cut was made, with a scalpel, in front of the fragment of interest and a DEAE strip inserted. One strip was used to extract 6 fragments, therefore 2 strips were used per gel. To prevent elution of the fragment immediately following, another DEAE strip was inserted behind the one of interest. Electrophoresis was carried out at 18mA for 1 hour after which time both the gel and the DEAE strips were examined under UV light to confirm the DNA had eluted onto the paper.

The DEAE strip was washed in NET (0.15M NaCl, 0.1mM EDTA, 20mM Tris pH 8.0) buffer to remove any residual agarose. Each strip was placed in a microcentrifuge tube and 250µl of high salt NET (1.0M NaCl, 0.1mM EDTA, 20mM Tris pH 8.0) buffer added. The tubes were placed in a heating block at 60°C for 45 min after which time the buffer was removed to a clean eppendorf and the strip washed with a further 50µl of high salt NET buffer. The strip was discarded. To remove the ethidium bromide from the solution 500µl of n-butanol was added

mixed, allowed to separate and the upper layer discarded. 600µl of ethanol was added and the tubes placed at -20^{0}C overnight. The tubes were centrifuged for 10 min and the pellet washed in 80% ethanol. One of each duplicate tube was resuspended in 20µl TE buffer and this transferred to the corresponding duplicate tube. This DNA was then ready for subsequent ligation to the vector DNA.

Nucleic Acid Hybridization

A non-radioactive digoxigenin (DIG) system (Boehringer Mannheim) was used for hybridization and subsequent colour detection. Random primed DIG–labelled DNA probes were generated by the hybridization of random oligonucleotides to a denatured DNA template. A complementary strand was synthesised by Klenow enzyme which used the $3^{/}$ OH termini of random oligonucleotides as primers and a mixture of deoxyribonucleosides containing DIG–11–dUTP. The vector pUC19 (Life Technologies) was chosen because as well as the possession of a selectable marker (ampicillin resistance) it contained a segment of DNA derived from the *lac* operon of *E.coli* that codes for the amino terminal fragment of β-galactosidase. Synthesis of this enzyme can be induced by IPTG (isopropylthio-β-D-galactoside) and resulted in the formation of blue colonies when plated on media that contained the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D galactoside). DNA inserted into this polycloning site of pUC19 abolished α-complementation and bacterial colonies that contained recombinant plasmids produced white colonies.

Preparation of nucleic acid probes from *PstI* restriction fragments

The first step in this process was the ligation of the extracted DNA fragment to the vector.

Dephosphorylation of pUC19

The first step in the preparation of the probe was the linearisation of pUC19. 10µl of pUC19 (≡ 5µg) was digested with *Pst*I (10µl pUC19, 25µl React 2 buffer, 20µl *Pst*I, 194µl distilled water) for 1 hour and complete digestion confirmed by electrophoresis of 5µl of the reaction mixture in a minigel system

(Life Technologies Horizon 58, Model 200). The reaction mixture was purified by the addition of 500µl of phenol–chloroform, the tubes mixed and centrifuged for 2 min. The upper aqueous layer was removed to a clean eppendorf and 500µl of ethanol added. This was placed on ice for 15 min and then centrifuged for 10 min. The pellet was resuspended in 90µl TE buffer and a 10µl aliquot removed and stored at –20°C (this served as a linearised pUC19 control for use later)

1µl of Calf Intestinal Alkaline Phosphatase (CIAP, Life Technologies) was diluted in 6µl of dilution buffer. 1µl of this dilution was added to the 80µl of linearised pUC19 DNA and 10µl of dephosphorylation buffer (10mM $ZnCl_2$, 10mM $MgCl_2$, 100mM TrisCl pH 8.3) added. The reaction mixture was incubated at 37°C for 30 min and after dephosphorylation was complete the CIAP was denatured by heating to 75°C for 10 min and removed by extraction with 50µl phenol–chloroform (centrifugation for 2 min). The upper aqueous layer was removed to which 10µl of sodium acetate (3M pH 7.0) and 250µl ethanol was added. This was placed on ice for 15 minutes. After centrifugation for 10 min the pellet was resuspended in 50 µl TE buffer. This was then stored at –20°C in aliquots of 5µl.

Ligation of extracted DNA fragment to pUC19

The extracted DNA fragments (stored in ethanol at –20°C) were centrifuged for 10 minutes, the pellet washed in 100µl 70% ethanol, resuspended in 10µl TE buffer and the duplicate tubes pooled. Thereafter ligation and control reactions were set up to include :

2.5µl insert DNA + 2.5µl water + 2.5µl dephosphorylated pUC19

2.5µl dephosphorylated pUC19 + 2.5µl water

2.5µl linear pUC19 + 2.5µl water

2.5µl pUC19 control (supplied) + 2.5µl water

These mixtures were then heated at 45°C for 5 minutes to melt any cohesive ends that may have formed, and chilled on ice. 1µl of T4 DNA ligase (Life Technologies, diluted 1 in 10 in the dilution buffer) was added to each reaction

mixture in addition to 2µl of buffer. The tubes were then incubated for 1 hour at 16°C.

Transformation of competent cells

Falcon tubes were placed on ice prior to use and 100 µl DH5α™ competent cells (Life Technologies) added to each. 1µl of each ligation mixture was added and the reactions placed on ice for 30 minutes. The tubes were then heated for 45 seconds at 42°C and then placed on ice for 1 minute. 900µl SOC (Appendix 2) medium was added and the tubes shaken at 37°C for 1 hour. Transformed cells were cultured on LB agar (Luria Broth base, 1% Bacteriological agar) (Life Technologies) plates under ampicillin selection (Sigma, 100µg/ml).

Dilutions of each transformation reaction (1/100) and an undiluted sample were cultured on on LB plates under ampicillin (100µg/ml) selection. The plates were incubated overnight at 37°C. Control strains that were ampicillin resistant and sensitive were also set up to confirm ampicillin selection.

Histochemical screening of transformants of vector plus insert

IPTG (Life Technologies, 200mg/ml) and XGal (Life Technologies, 20mg/ml in dimethyl formamide) were incorporated into LB plates which also contained ampicillin. Approximately 100–150 colonies of the transformed cells were touched/spotted onto a plate and the plates incubated overnight. White colonies (i.e. colonies that contained the insert) were inoculated into Terrific Broth (Appendix 1) broth that contained 100µg/ml ampicillin, sub–cultured for purity and incubated overnight. From the BHI broths, the DNA was extracted and purified. The growth from the purity plate was harvested and a crude plasmid lysate used to confirm the presence of the insert in the vector on a minigel. Control tracks were included of pUC19 and the fragment DNA alone.

Preparation of DIG labelled probe

In order to avoid the reduction in DNA yield that would result from the re–extraction of the cloned fragment from pUC19, the vector + insert was digested with *Pst*I and both fragments were labelled. 30µl of labelled DNA was digested

with *Pst*I (2µl enzyme plus 5µl REact™ buffer) for 1 hour. The template DNA was denatured at 100°C for 10 minutes and chilled rapidly to remove any cohesive termini that may have formed. Reaction mixtures were set up :

30µl template DNA (digested)

4µl Hexanucleotide mixture

4µl dNTP mixture

2µl Klenow enzyme

The tubes mixed, centrifuged briefly and incubated overnight at 37°C. The reaction was stopped by the addition of 4µl 0.2M EDTA (pH 8.0) and the labelled DNA was precipitated by the addition of 5µl lithium chloride (LiCl, 4M) and 150µl ice cold ethanol. After 30 minutes at –80°C the tubes were centrifuged for 10 minutes and the pellet washed in 50µl of 70% ethanol. The pellet was air dried and resuspended in 50µl TE. The probe was then available for immediate use or storage at –20°C.

Estimation of the yield of DIG labelled DNA

The yield of labelled probe was determined by comparison to a DIG labelled DNA control (pBR328, supplied) in a dot-blot followed by direct immunological detection with the colour substrates NBT (nitroblue tetrazolium salt, 75mg/ml in dimethyl formamide 70%w/v) and X-phosphate (5–bromo–4–chloro–3-indolyl phosphate, toluidinium salt, 50mg/ml in dimethyl formamide). Ten–fold serial dilutions (in dilution buffer : 50mg/ml herring sperm DNA in 10mM TrisCl, 1mM EDTA pH8.0) of the labelled DNA and the control DNA were prepared (Neat – 1/100,000) and spotted onto a nitrocellulose membrane (pre–treated in 20×SSC for 30 minutes). This was then UV cross–linked for 3 min to fix the DNA and each dilution marked lightly with a pencil for identification. The membrane was washed briefly in maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5) and incubated for 30 min in 100ml of blocking solution (1% blocking solution in maleic acid). This was discarded and the membrane incubated in 20ml of anti–DIG-alkaline phosphatase (1/5000 in blocking solution) for 30 min and then washed twice for 15 min in 100ml maleic acid buffer. The membrane was

equilibrated in 20ml detection buffer (0.1M Tris-HCl, 0.1M NaCl, 50mM $MgCl_2$, pH 9.5). This was discarded and 10ml of freshly prepared colour substrate added (10ml detection buffer, 45µl NBT, 35µl X–phosphate solution). Colour development was carried out in the dark overnight after which time the reaction was stopped by the addition of water. Comparison of the intensity of the control and test dilutions enabled the estimation of the concentration of the probe DNA.

Preparation of nucleic acid probes from *Sma*I restriction fragments

The availability of commercially available kits (Ready-To-Go™ Pharmacia) for the ligation of blunt ended DNA fragments to vector DNA made the preparation of *Sma*I fragments simple.

*Sma*I fragments were extracted as previously described. The amount of DNA per fragment was calculated with the knowledge that a 10ml BHI culture yielded ~0.5 µg of DNA (results not shown). Therefore the quantity of DNA per fragment was estimated as a fraction of the total plasmid DNA in kb.

An equivalent amount of vector and insert DNA was used for the ligation reactions.(The vector DNA was supplied at 100ng).

The *Sma*I fragments were resuspended in TE buffer and added to the Ready–To–Go™ pUC18 *Sma*I vial, left at room temperature for 5 minutes and mixed by a gentle pipette action. The tubes were centrifuged for a few seconds, incubated at 16°C for 45 minutes then 70°C for 10 minutes. Transformation of competent cells and selection of vector plus insert was carried out as described previously.

Hybridization of Southern blots

Prehybridization of the nitrocellulose membranes was carried out. 20ml of DIG Easy Hyb™ (Boehringer Mannheim) was heated to 42°C. The nitrocellulose membrane to be probed was pre-wet in 2×SSC (0.3M NaCl, 30mM sodium citrate pH 7.0) and placed on top of a nylon mesh cut to the same size. The membrane and the mesh were rolled up (mesh outermost) and placed in a hybridization bottle. 10ml 2×SSC was added and the bottle rolled gently until the mesh stuck to the bottle – with no air bubbles. The mesh allowed full contact of the probe solution

and the membrane. The 2×SSC was poured off and replaced with the pre-warmed DIG Easy Hyb™ solution, the bottle placed in the rotisserie hybridization oven (Hybaid) and incubated for 30 min at 42°C.

The DIG labelled probe was denatured (100°C for 5 min and cooled rapidly on ice) and the appropriate dilution prepared. In this case 1/1000 was optimal therefore 1µl was diluted in 10ml DIG Easy Hyb. The prehybridization solution was discarded, the probe solution added and incubated overnight.

Post-hybridization washes

The probe solution was decanted and stored at −20°C for further use and the membrane washed 2×5min in 50ml 2×SSC, 0.1% SDS at room temperature followed by 2×15min in 0.1×SSC, 0.1% SDS at 68°C with constant agitaton.

Immunological detection procedure

After the post hybridization stringency washes the membrane was rinsed in 50ml maleic acid buffer. Thereafter the detection procedure was as before. The reaction was stopped and the membrane stored in a bag in TE buffer. Results were documented by photography of the membrane.

CHAPTER 3

REFP analysis of the serotype associated plasmids of the salmonellae

Introduction

The association of virulence plasmids with particular serotypes of *Salmonella* is well recognised (Woodward *et al* 1989). These plasmids differ in size with respect to the host serotype and range from 50 kb in Choleraesuis (Kawahara *et al*, 1988) to 95 kb in Typhimurium (Jones *et al*, 1982). Such plasmids have also been identified in Abortusovis (Colombo et al 1992), Enteritidis (Nakamura et *al*, 1985), Dublin (Terakado *et al*, 1983), Gallinarum (Barrow *et al*, 1987) and Pullorum (Barrow and Lovell, 1988). The REFP of each plasmid is characteristic of the serotype (Helmuth *et al*, 1985). Methods such as heteroduplex analysis and nucleic acid hybridisation have shown these plasmids to be related (Montenegro *et al* 1991, Popoff *et al* 1984, Williamson *et al* 1988a). An 8kb region has been identified in the plasmids of Abortusovis, Blegdam, Choleraesuis, Dublin, Enteritidis, Gallinarum, Moscow, Paratyphi C, Pullorum, Rostock and Typhimurium that is responsible for the virulence of the organism (Williamson *et al* 1988a). This region has been variously termed *mba* (mouse bactericidal action, Matsui *et al* 1990), *mka* (mouse killing action, Tiara and Rhen 1989), *mkf* (mouse killing factor, Norel *et al* 1989a), *vsd* (virulence *Salmonella* Dublin, Krause *et al* 1990) and *spv* (*Salmonella* plasmid virulence, Gulig and Chiodo 1990). It is the latter of these designations which has been universally accepted and encompasses five genes *spv*RABCD. This region has been fully sequenced in Typhimurium (Norel *et al* 1989 a,b, Gulig *et al* 1992), Dublin (Krause *et al* 1990) and Choleraesuis (Matsui *et al* 1990).The extent of sequence identity between these plasmids showed no significant difference (Gulig *et al* 1993). Therefore although it is well demonstrated that a small portion (8kb) of the virulence region is common to certain plasmids, the extent of sequence similarity outwith the virulence region is unknown. Friedrich *et al* (1993) sequenced a 14kb region of the Typhimurium plasmid involved in flagellar biosynthesis whilst Cerin and Hackett (1993) sequenced a 4.3kb region of the plasmid responsible for incompatibility and partition functions. However analysis of the other SAP's has not been undertaken. The application of REFP analysis allows relatively detailed comparison of the entire plasmid.

Rationale for inclusion of serotypes

This study included plasmid analysis of most of the serotypes of *Salmonella* previously shown to harbour a virulence plasmid namely Typhimurium, Enteritidis, Dublin, Gallinarum, Pullorum, Choleraesuis and Abortusovis. In addition routine epidemiological monitoring of Bovismorbificans indicated this serotype to harbour a plasmid related to those of other serotypes. A recent report has suggested relationships exist between certain serotypes on the basis of chromosomal markers (IS200 and ribotype) (Stanley *et al* 1994). However these authors extended their conclusions to relationships between plasmids on the basis of demonstration of the presence of *spv* (virulence) genes and an estimate of plasmid size. The evidence presented in the study by Stanley *et al* (1994) similarly does not exclude such a possibility. Therefore in order to extend previous observations (Platt *et al* 1988, Rankin and Platt 1995) and clarify uncertainties (Stanley *et al* 1994) representatives of serogroup D1 (Blegdam, Moscow, Rostock and Wangata) were included in the study. The results of this analysis of plasmids are presented and the relatedness of the plasmids discussed.

Materials and Methods

PP and REFP analyses were performed as detailed in Chapter 2.

Salmonella serotypes antigenic structures plasmid sizes and designations are given in Table 2.1.

The construction of a probe from the 2.3 kb *Pst*I pOG660 fragment was achieved as detailed in Chapter 2.

Definitive choice of SAP

Although SAP's have been identified in certain serotypes, in the absence of detailed sequence analysis, the identification has been made purely on the basis of plasmid size and homology to an *spv* probe (Williamson *et al* 1988a). In 1986 Brown *et al* demonstrated a predominant plasmid REFP in Typhimurium isolates. Similar results were obtained for the plasmids of Enteritidis and Dublin (Platt *et al* 1988). The existance of molecular variants of plasmids within a serotype has been

documented (Browning and Platt 1995, Rankin and Platt 1995) and will be dealt with in the next chapter. Where possible the predominant REFP was used for all serotypes (and the oldest strain when available). In some cases e.g. Choleraesuis the rarity of the serotype limits the number of strains that can be analysed. Early work by Kawahara *et al* (1988) led to the correlation between the plasmid in *Salmonella* Choleraesuis and virulence in the mouse model. Part of this work involved *Eco*R1 digestion of the plasmid and although it only generated 7 fragments, this pattern was consistent with the reference plasmid used in this study which was obtained from the National Collection of Type Cultures. Although only one strain of Abortusovis was available for analysis, it was included in the study after REFP analysis of the 50kb plasmid revealed it to share a high degree of similarity with other SAP's. Similarly with Gallinarum and Pullorum the limitations of the population size of these serotypes led the selection of the SAP to be based on the identity of a plasmid found in reference strain obtained from the National Collection of Type Cultures. In the case of Gallinarum, the archetypal SAP was originally chosen as pOG676 – obtained from NCTC. However, analysis of an international collection of strains revealed pOG642 to be the dominant plasmid type. This is discussed in the Chapter 5 – Molecular Variants of the SAP's. The serotype Wangata was included for analysis because although relatively uncommon, a plasmid similar in REFP to other SAP's was identified. Blegdam, Moscow, Rostock and Antarctica were rare serotypes, with only a few examples of each serotype available. However REFP analysis revealed a high degree of similarity to other SAP's. Previous work by Popoff *et al* (1984) failed to establish the presence of a virulence plasmid in Bovismorbificans. However routine epidemiological monitoring of this serotype revealed the presence of a high molecular weight plasmid albeit at a much lower incidence than othe SAP's (39%). The plasmid identified was indistinguishable with the enzymes used from that of the NCTC 5754 strain which was isolated in 1939. These results are discussed and the presence of an SAP in Bovismorbificans is reported for the first time.

It was anticipated that the identification of restriction fragments of the same size in different SAP's would lead to the construction of a series of probes and their use in hybridization to Southern blots. Unfortunately, a combination of

technical difficulties and time constraints meant that this work was not carried out to completion. However the preliminary results generated in this study have enabled further analysis of the salmonellae and provided scope for future work.

Results

Unless otherwise stated all strains of *Salmonella* were fully sensitive to the antimicrobial agents tested.

Plasmid sizes and designations are shown in Table 3.1.

REFP analysis of the plasmids of Rostock, Moscow, Blegdam and Antarctica

The results of plasmid digestion with *Pst*I and *Sma*I are shown in Fig 3.1 and digestion with *Eco*RV in Fig 3.2. The computer generated output of the REFP's from *Pst*I and *Sma*I is shown in Fig 3.3 and the Dice coefficients of similarity in Table 3.2.

***Salmonella* Rostock**

The plasmids present in both strains of Rostock examined were indistinguishable from pOG675 the reference plasmid of Dublin.

***Salmonella* Blegdam**

Both strains of Blegdam contained a plasmid previously identified as a molecular variant (designated pOG704) of the Enteritidis reference plasmid pOG674. Digestion with *Pst*I revealed the 2.8 kb fragment of pOG674 to be absent with an additional 3.2 kb fragment generated. Digestion with *Sma*I revealed none of the SAP fragments to be missing but an additional 2.4 kb fragment was present.

***Salmonella* Moscow**

One strain of Moscow harboured a plasmid indistinguishable from pOG674 with *Pst*I, *Sma*I and *Eco*RV. Four strains of Moscow examined contained two

Table 3.1

Plasmid size, designation and associated serotype.

SEROTYPE	PP [Designation] kb
Abortusovis	50 [pOG645]
Choleraesuis	50 [pOG678]
Enteritidis	54 [pOG674]
Blegdam	54 [pOG704] (2)*
Moscow	54 [pOG674] 54 [pOG680] : 40 (3)* 54 [pOG704] : 40
Antarctica	54 [pOG681] : 30
Dublin	72 [pOG675]
Rostock	72 [pOG675] (2)*
Bovismorbificans	85 [pOG679]
Gallinarum	85 [pOG676]
Pullorum	85 [pOG677]
Wangata	90 [pOG646]
Typhimurium	95 [pOG660]

( )* number of strains examined

Figure 3.1

REFP's of plasmids of *Salmonella* serotypes Enteritidis (En), Moscow (Mw), Blegdam (Bg) and Antarctica (At) digested with *Pst*I (Lanes 2–7) and *Sma*I (Lanes 8–13).

Lane 1. λ *Pst*I 2. pOG674(En) 3. pOG674(Mw) 4. pOG680(Mw) 5. pOG704(Mw) 6. pOG704(Bg) 7. pOG681(At) 8. pOG674(En) 9. pOG674(Mw) 10. pOG680(Mw) 11. pOG704(Mw) 12. pOG704(Bg) 13. pOG681(At)

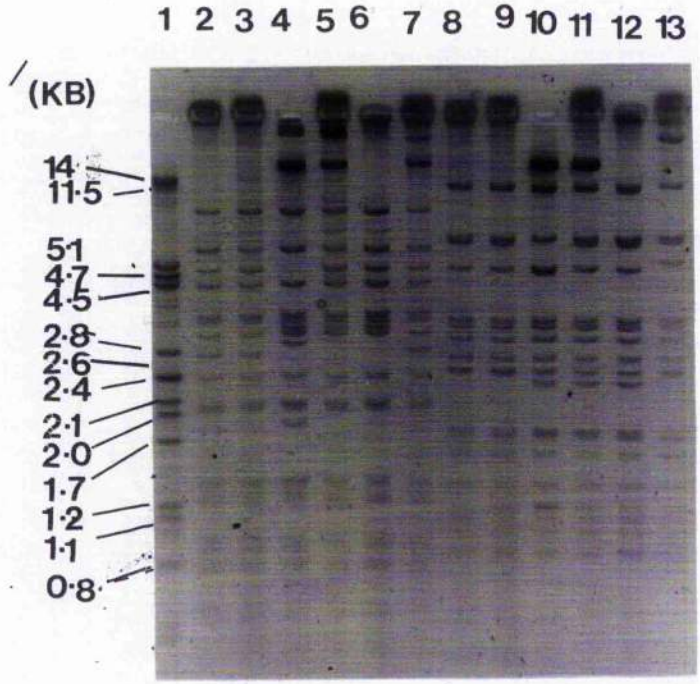


Figure 3.3

Computer generated REFP analysis of the plasmids of *Salmonella* serotypes Enteritidis(En), Moscow(Mw), Blegdam(Bg), Antarctica(At), Dublin(Du) and Rostock(Ro) digested with *Pst*I and *Sma*I

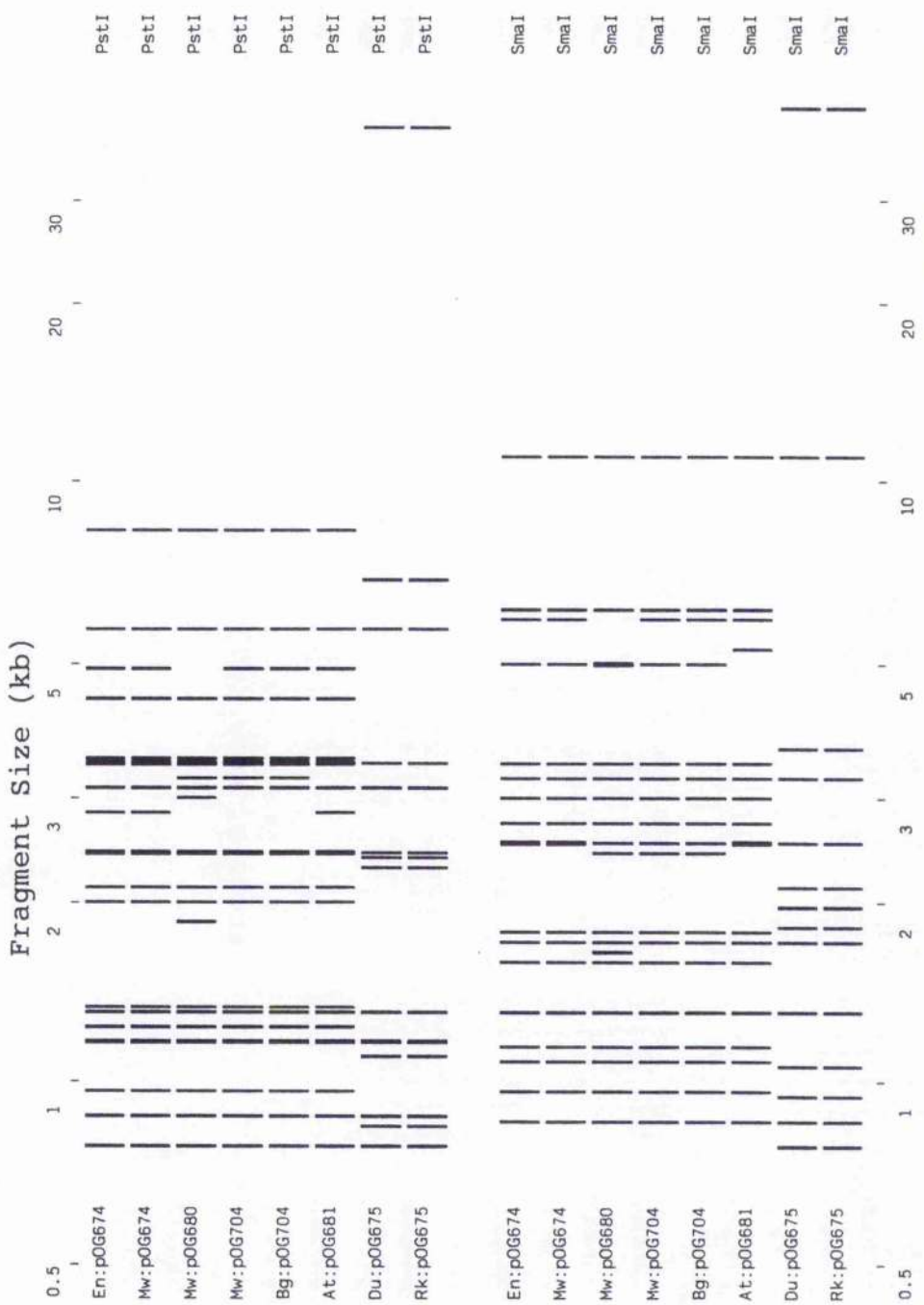


Table 3.2

Dice coefficients of similarity (%) between plasmids of Enteritidis (pOG674), Blegdam/Moscow (pOG704), Moscow (pOG680) and Antarctica (pOG681)

Plasmid	pOG674	pOG681	pOG704	pOG680
pOG674	—	**100**	**94**	**87**
pOG681	*94*	—	**94**	**87**
pOG704	*97*	*91*	—	**92**
pOG680	*91*	*86*	*94*	—

***Pst*I** values in bold

*Sma*I values in italic

plasmids, the smaller of which (40 kb) was not cleaved by the restriction enzymes used. The 54 kb plasmid present in one of these strains was indistinguishable from pOG704. The remaining three strains harboured an identical 54 kb plasmid which differed slightly from pOG704 designated pOG680. *Pst*I digestion revealed the 4.9 and 2.8 kb SAP fragments to be missing with additional fragments of 3.2, 3.0 and 1.85 kb generated. *Sma*I digestion revealed the 5.9 kb SAP fragment to be missing with additional 5.0, 2.4 and 1.65 kb fragments generated.

***Salmonella* Antarctica**

The strain of Antarctica examined harboured two plasmids the smaller one of which did not contribute any fragments to the REFP with the enzymes used. The 54 kb plasmid was indistinguishable from pOG674 after digestion with *Pst*I. However *Sma*I digestion showed the loss of the 5.0 kb fragment with an additional 5.3 kb fragment generated and was subsequently designated pOG646. Minor variations were also seen using other enzymes : *Ava*II and *Eco*RV generated additional fragments of 1.1 kb and 4 kb respectively.

REFP analysis of the serotype associated plasmids of the salmonellae

REFP analysis showed a wide range of similarity in the plasmids of the nine serotypes that possessed SAP's (Abortusovis, Bovismorbificans, Choleraesuis, Dublin, Enteritidis, Gallinarum, Pullorum, Typhimurium and Wangata). Results from digestion with *Pst*I and *Sma*I are shown in Figures 3.4 and 3.5 respectively and the resultant fragment sizes detailed in Table 3.3 (*Pst*I) and Table 3.4 (*Sma*I). The computer generated output from the REFP's of *Pst*I and *Sma*I digestion are presented in Figure 3.6. *Hin*dIII digestion of the plasmids is shown in Figure 3.7.

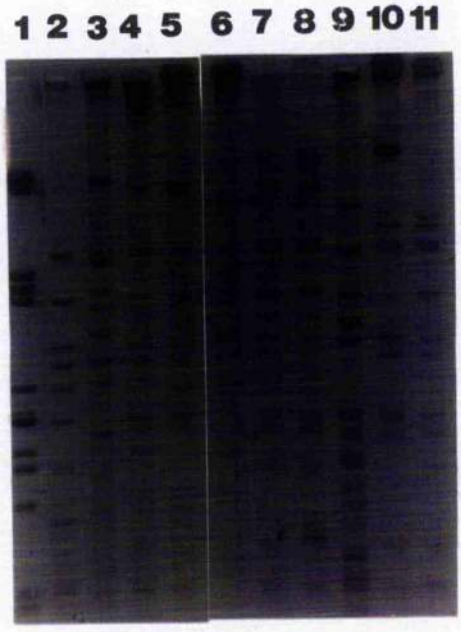
REFP analysis of the SAP of Abortusovis

The fragment sizes after digestion with *Pst*I and *Sma*I are detailed in Tables 3.3 and 3.4 respectively. The computer generated output of the REFP's are shown in Figure 3.6 and the comparison to other plasmids with Dice coefficients of similarity in Table 3.5.

Figure 3.4

***Pst*I digestion of the plasmids of *Salmonella* serotypes Abortusovis(Ab), Choleraesuis(Cs), Enteritidis(En), Typhimurium(Tm), Wangata(Wa), Gallinarum(Ga), Pullorum(Pu), Bovismorbificans(Bm) and Dublin(Du).**

Lane 1. λ *Pst*I 2. pOG678(Cs) 3. pOG645(Ab) 4. pOG660(Tm)
5. pOG646(Wa) 6. pOG674(En) 7. pOG676(Ga) 8. pOG677(Pu)
9. pOG679(Bm) 10. pOG675(Du) 11. pOG683(DuVar)*

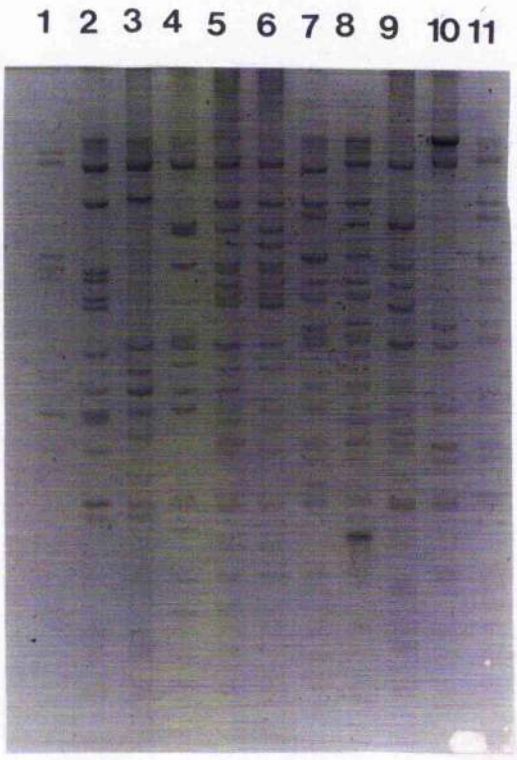


*Molecular variant of pOG675(see Chapter 5 Molecular variants of the SAP's)

Figure 3.5

***Sma*I digestion of the plasmids of *Salmonella* serotypes Abortusovis(Ab), Choleraesuis(Cs), Enteritidis(En), Typhimurium(Tm), Wangata(Wa), Gallinarum(Ga), Pullorum(Pu), Bovismorbificans(Bm) and Dublin(Du).**

Lane 1. λ *Pst*I 2. pOG645(Ab) 3. pOG678(Cs) 4. pOG674(En)
5. pOG660(Tm) 6. pOG646(Wa) 7. pOG676(Ga) 8. pOG677(Pu)
9. pOG679(Bm) 10. pOG675(Du) 11. pOG683(DuVar)*



*Molecular variant of pOG675 (see Chapter 5 Molecular variants of the SAP's)

Table 3.3 Fragment sizes (kb) of SAP's after digestion with *Pst*I

pOG645	pOG678	pOG674	pOG660	pOG646	pOG642	pOG677	pOG679	pOG675
								39.7
14.5			10.1	10.1				
					9.8			
						9.5		
		8.3					8.3	
								6.8
			5.8	5.8	5.8	5.8		
5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7
		4.9		4.9				
5.5								
4.6								
	4.4	4.4	4.4	4.4	4.4	4.4	4.4	
					4.3			
4.2								
			4.1					
			3.9	3.9	3.9	3.9	3.9	
3.85							3.85	
		3.5						
	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4
3.1	3.1	3.1	3.1	3.1	3.1	3.1		3.1
2.9								
	2.8	2.8						
						2.7		
				2.6				
	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
					2.35	2.35		2.35
			2.3	2.3	2.3	2.3	2.3	2.3
2.2							2.2	
		2.1	2.1				2.1	
		2.0					2.0	
	1.95				1.95	1.95		
1.9						1.9		
							1.8	
	1.7			1.7			1.7	
1.6			1.6	1.6				
			1.55					
1.50	1.50		1.50	1.50	1.50	1.50	1.50	
	1.45							
	1.40		1.40	1.40	1.40	1.40	1.40	
		1.35	1.35	1.35	1.35	1.35	1.35	
	1.32	1.32	1.32					1.32
1.23	1.23	1.23	1.23	1.23				
1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16
					1.10			1.10
			1.03					
0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	
0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88
								0.84
0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

Table 3.4 Fragment sizes (kb) of SAP's after digestion with *Sma*I

pOG645	pOG678	pOG674	pOG660	pOG646	pOG642	pOG677	pOG679	pOG675
								42.9
11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
	7.7							
7.4			7.4	7.4	7.4	7.4		
					6.6			
						6.3	6.3	
		6.2	6.2	6.2			6.2	
		5.9						
				5.3				
						5.2		
					5.1			
		5.0	5.0	5.0			5.0	
4.7			4.7	4.7				
4.55								
			4.4	4.4	4.4	4.4	4.4	
4.1			4.1	4.1				
					4.05	4.05		
4.0			4.0	4.0				
							3.9	
						3.6		3.6
					3.5			
		3.4			3.4	3.4		
	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
3.1						3.1		
		3.0	3.0	3.0				
	2.9							
2.7	2.7	2.7	2.7		2.7	2.7	2.7	
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
2.45								
2.4			2.4					
						2.35	2.35	
	2.3			2.3				
			2.2					
2.1			2.1		2.1	2.1	2.1	2.1
			2.0	2.0			2.0	
					1.95			1.95
							1.90	
					1.87			
				1.80	1.80	1.80		1.80
		1.77						
1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73	1.73
1.70								
1.68	1.68							
		1.58					1.58	
			1.46	1.46		1.46	1.46	
						1.40		
	1.32	1.32	1.32	1.32	1.32	1.32	1.32	1.32
					1.28		1.28	
		1.15						
1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08
0.96	0.96	0.96	0.96	0.96		0.96	0.96	0.96
0.87		0.87	0.87	0.87	0.87	0.87	0.87	0.87
	0.83				0.80	0.80		0.80

Figure 3.6

Computer generated REFP analysis of the plasmids *Salmonella enterica* serotypes Abortusovis(Ab), Choleraesuis(Cs), Enteritidis(En), Typhiumrium(Tm), Wangata(Wa), Gallinarum(Ga), Pullorum(Pu), Bovismorbificans(Bm) and Dublin(Du) digested with *Pst*I and *Sma*I.

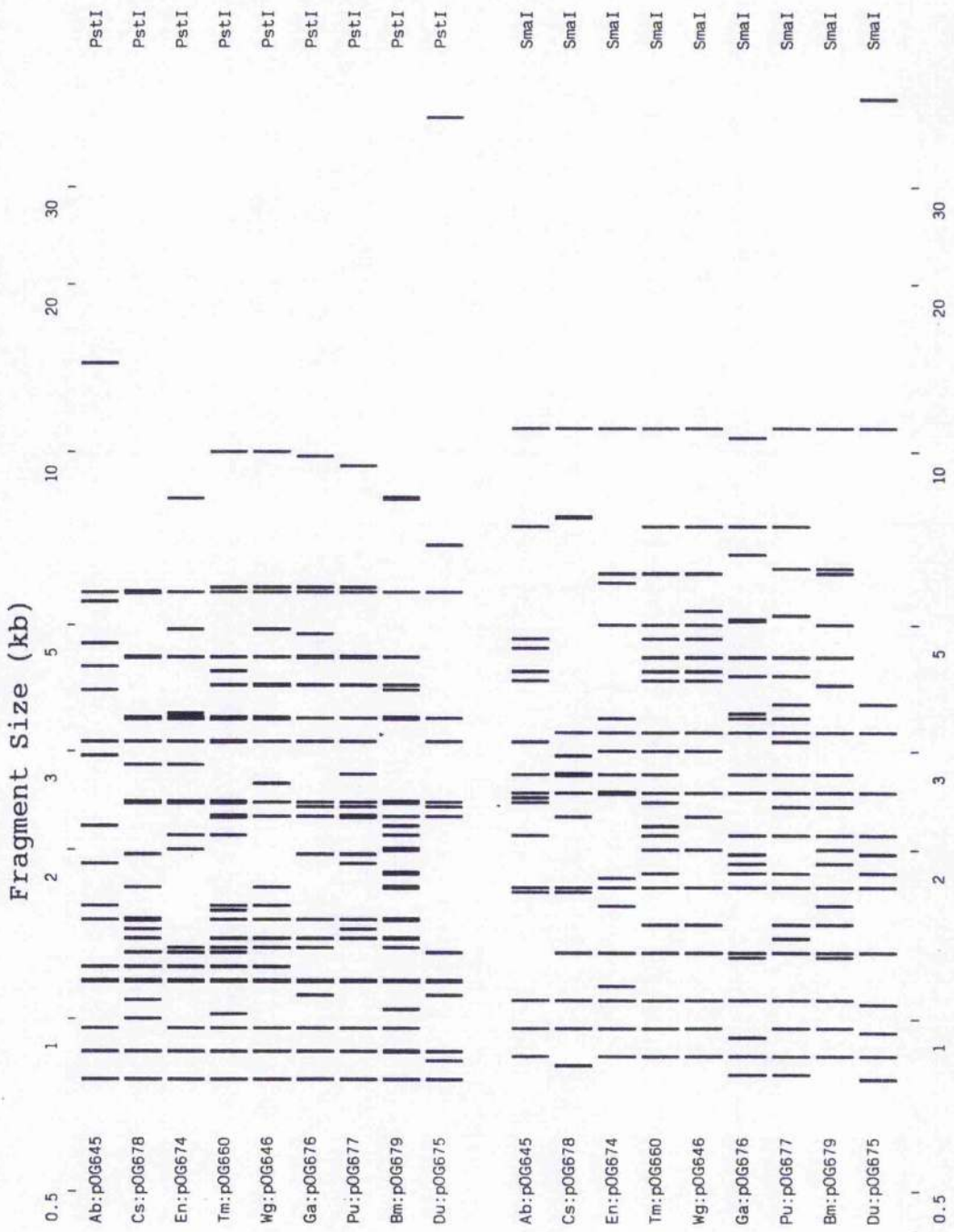
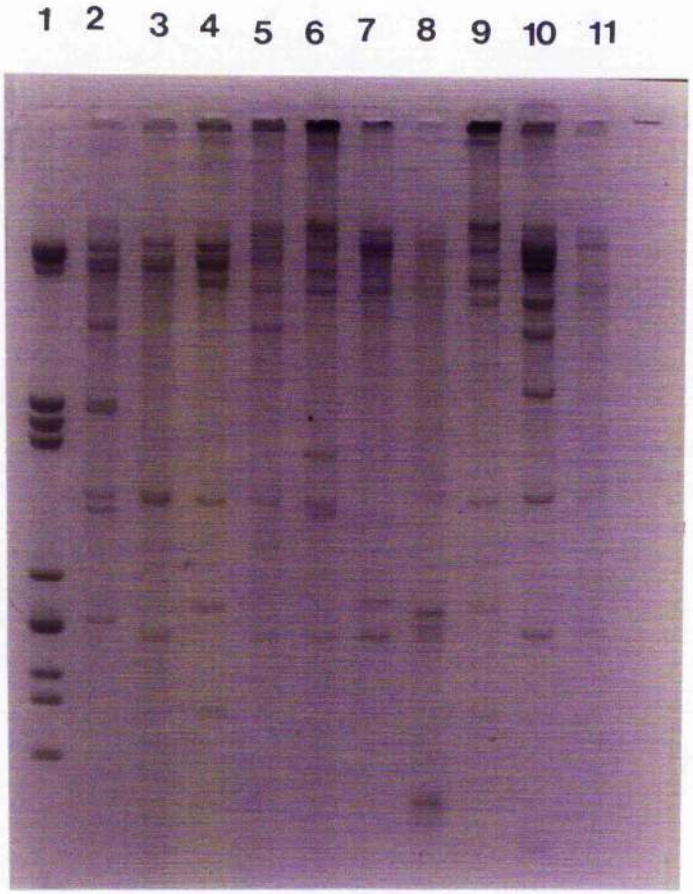


Figure 3.7

***Hin*dIII digestion of the plasmids of *Salmonella* serotypes Abortusovis(Ab), Choleraesuis(Cs), Enteritidis(En), Typhimurium(Tm), Wangata(Wa), Gallinarum(Ga), Pullorum(Pu), Bovismorbificans(Bm) and Dublin(Du).**

Lane 1. λ *Pst*I 2. pOG645(Ab) 3. pOG678(Cs) 4. pOG674(En)
5. pOG660(Tm) 6. pOG646(Wa) 7. pOG676(Ga) 8. pOG677(Pu)
9. pOG679(Bm) 10. pOG675(Du) 11. pOG683(DuVar)*



*Molecular variant of pOG675 (see Chapter 5 Molecular variants of the SAP's)

Table 3.5

Dice coefficients of similarity (%) between plasmids of Abortusovis (pOG645), Choleraesuis (pOG678), Enteritidis (pOG764), Typhimurium (pOG660), Wangata (pOG646), Gallinarum (pOG642), Pullorum (pOG677), Bovismorbificans (pOG679) and Dublin (pOG675)

Plasmid	pOG645	pOG678	pOG674	pOG660	pOG646	pOG642	pOG677	pOG679	pOG675
pOG645	—	*	*	**55**	**53**	**54**	*	*	*
pOG678	*	—	**68**	**63**	**68**	**68**	**63**	**67**	**56**
pOG674	*	*60*	—	**63**	**62**	**58**	**53**	**62**	**50**
pOG660	*63*	*	*65*	—	**82**	**70**	**65**	**68**	*
pOG646	*51*	*	*63*	*86*	—	**73**	**68**	**68**	**51**
pOG642	*	*	*56*	*67*	*61*	—	**80**	**68**	**65**
pOG677	*59*	*50*	*60*	*74*	*73*	*80*	—	**63**	**53**
pOG679	*51*	*53*	*63*	*77*	*71*	*65*	*64*	—	**57**
pOG675	*	*52*	*58*	*54*	*51*	*72*	*65*	*57*	—

*** Similarity coefficient < 50%**
PstI values in bold
***SmaI* values in italic**

The plasmid harboured by serotype Abortusovis, pOG645, showed more REFP similarity to pOG660 the plasmid of Typhimurium when digested with *Sma*I than *Pst*I. With *Sma*I, fragments of 11.0, 7.4, 4.7, 4.1, 4.0, 2.7, 2.5, 2.1 and various fragments < 1 kb were common. However with *Pst*I fragments of 5.7, 3.1, 1.6, 1.5, 1.23, 1.16 and various fragments < 1 kb were common to both plasmids. Dice coefficients of similarity showed the plasmids pOG645 and pOG660 to be 55 and 63 % similar with *Pst*I and *Sma*I respectively. Of all the plasmids examined, pOG645 was the least related to any of the other SAP's – with values < 50 % when compared to most other plasmids (Table 3.5).

REFP analysis of the SAP of Choleraesuis

The plasmid pOG678 showed REFP similarity with the plasmids of Enteritidis and Typhimurium with both *Pst*I and *Sma*I. *Pst*I fragments of 5.7, 4.4, 3.4, 3.1, 2.4 as well as fragments < 1 kb were common to pOG678, pOG674 and pOG660; a fragment of 2.8 kb was common to pOG678 and pOG674; fragments of 1.5 and 1.4 kb were common to pOG678 and pOG660. With SmaI fragments of 11.0, 3.2, 2.7, 2.5, 1.32 and fragments < 1 kb were common to pOG678, pOG674 and pOG660. Dice coefficients of similarity showed the plasmids of Choleraesuis and Enteritidis to be 68 and 60% similar with *Pst*I and *Sma*I respectively; Choleraesuis and Typhimurium plasmids were 63 and < 50% similar with *Pst*I and *Sma*I respectively.

REFP analysis of the SAP of Wangata

A study of 61 Scottish isolates in 1992 found 72% to be plasmid-free and 15 plasmid profile types to exist within the 28% of plasmid harbouring strains. The plasmids involved were diverse and showed geographical clustering of isolates (results not shown). However, one strain isolated from parrot faeces in 1986 was shown to harbour a plasmid indistinguishable from that of the NCTC strain which was also remarkably similar to other SAP's, notably pOG660 and was chosen as the representative SAP. The plasmid harboured by serotype Wangata (pOG646)

differed considerably from those harboured by other Group D1 serotypes. It was more closely related to pOG660 the reference plasmid of Typhimurium NCTC73 than to pOG674 (Enteritidis). Digestion with *Pst*I revealed four of the pOG660 fragments to be missing with four additional fragments generated. Similarly *Sma*I digestion showed four SAP fragments to be absent with two additional fragments generated. Dice coefficients of similarity showed pOG646 to be 82% and 86% related to pOG660 using *Pst*I and *Sma*I respectively whereas comparison with Enteritidis showed 65% and 53% similarity.

REFP analysis of *Salmonella* Bovismorbificans

The REFP of pOG679 showed 68% and 77% similarity with *Pst*I and *Sma*I respectively to pOG660. It was least similar to pOG645 < 50%, 51% with *Pst*I and *Sma*I respectively.

REFP analysis of *Salmonella* Gallinarum and Pullorum

The plasmids of Gallinarum and Pullorum showed a high degree of REFP similarity (Figures 3.4–3.6, Table 3.5). As mentioned previously, the SAP of Gallinarum is pOG642. Although this will be dealt with in the next chapter, it seems appropriate to mention here (since Figures 3.4 and 3.5 contain pOG676 – the NCTC reference strain plasmid) the minor differences between pOG642 and pOG676. With *Sma*I, the 11.0 kb fragment in pOG676 is replaced by one of 10.7 kb; with *Pst*I the 4.8 kb fragment is replaced by one of 4.3 kb.

pOG642 showed the highest degree of REFP similarity to pOG677 (80% with both *Pst*I and *Sma*I).

REFP analysis of the SAP of Dublin

The plasmid pOG675 showed the least amount of REFP similarity (after pOG645) to any of the other SAP's examined. This plasmid was unique in both *Pst*I and *Sma*I fingerprints with not only the fewest fragments generated (SmaI digestion produced no fragments in the 3.6–11 kb region) but also in the generation of a large ~40 kb fragment. In comparison to other plasmids, the REFP

of pOG675 was most similar to pOG642 (Gallinarum) – 65 & 72% with *Pst*I and *Sma*I respectively. Similarity to the other plasmids ranged from 50–58%.

HindIII analysis of the SAP's

The REFP's of the SAP's after digestion with *Hin*dIII is shown in Figure 3.7 and revealed all the plasmids except pOG676 to have a fragment of 3.9kb. This fragment has been shown to encompass the virulence region and further demonstrates that the plasmid of Gallinarum pOG676 was a variant plasmid.

Hybridization with probe generated from the 2.3 kb PstI fragment of pOG660

The transformation efficiency of the competent cells was ~5% i.e. 5 white colonies per 100 blue. Quantification of DIG-labeled probe showed the optimalprobe concentration to be 1/1000. A control hybridization revealed no reaction with the pUC19 labelled DNA therefore any hybridization resulted from the plasmid DNA. The hybridization of the probe to a *Pst*I Southern blot of the SAP's is shown in Figure 3.8. and reveals the 2.3 kb fragment to hybridize with the 2.3 kb fragments of the plasmids of serotypes Wangata, Gallinarum, Pullorum, Bovismorbificans, Dublin (and the Dublin variant plasmid pOG683 – see Chapter 5). This fragment also hybridized with the 1.95 kb fragment of the Choleraesuis plasmid and the 1.6 kb fragment of the plamid of Abortusovis. This fragment did not hybridize with the plasmid of Enteritidis.

Discussion

The interpretation of Dice coeffiicients of similarity must be undertaken with considerable caution and with several considerations in mind. The comparison of fragment numbers does not take into account the proportion of DNA in common either in relation to overall plasmid size (e.g. the comparison of a 90 kb plasmid with a 50 kb plasmid) or the fragment size itself (e.g. a match of 1.2 kb is given the same 'weight' as a match of 10 kb of DNA). Computer generated analysis of REFP's on a logarithmic scale tends to emphasize fragment variation however with a permissible level of variation of 5% the potential for mis-matched fragments was minimised.

Figure 3.8

Hybridization of a DIG-labeled probe generated from the 2.3 kb *Pst*I fragment of the Typhimurium plasmid pOG660 with a *Pst*I REFP Southern blot of the plasmids of *Salmonella* serotypes Abortusovis(Ab), Choleraesuis(Cs), Enteritidis(En), Typhimurium(Tm), Wangata(Wa), Gallinarum(Ga), Pullorum(Pu), Bovismorbificans(Bm) and Dublin(Du).

Lane 1. pOG683(DuVar)* 2. pOG675(Du) 3. pOG679(Bm) 4. pOG677(Pu) 5. pOG676(Ga) 6. pOG646(Wa) 7. pOG660(Tm) 8. pOG674(En) 9. pOG678(Cs) 10. pOG645(Ab)

The lowest degree of similarity was seen between the plasmid in Abortusovis and the other SAP's, most of which were <50% similar with both *Pst*I and *Sma*I. An interesting feature of this plasmid was that although one of the smallest at 50 kb it showed remarkable REFP similarity to pOG660 the largest of the SAP's (95 kb) and suggests that the plasmid of Abortusovis could have arisen from Typhimurium by deletion of DNA.

pOG675 also shows a fairly low degree of similarity to other SAP's, except with pOG676 (Gallinarum) with which it shares 65% and 68% similarity with *Pst*I and *Sma*I respectively.

An early study by Brunner (1952) demonstrated that changes in H antigens could be induced in certain serotypes of serogroup D1 and included the unidirectional conversion of serotype Blegdam (gmq) into both Enteritidis (gm) and Moscow (gq), all of which contained the g-complex of flagellar antigens. He concluded that the many serological types within this group may have originated from a single ancestral strain or from a few primitive strains. REFP analysis of these plasmids is consistent with clonality such that plasmids from different serotypes are the same or closely related to Enteritidis, Dublin or Typhimurium. Molecular variation among the plasmids within and between Enteritidis and Dublin has been demonstrated previously and possible mechanisms discussed to explain the marked REFP and incompatibility difference with Dublin (Platt *et al* 1988). Such variation is also is evident between further uncommon serotypes within a serogroup. It is therefore evident that although limited sequence homology was demonstrated previously (by hybridization to an *spv* probe) between serotypes Moscow, Blegdam, Antarctica, Rostock and Wangata these serotypes in fact harboured plasmids that were either identical to or were molecular variants of the established SAP's of Enteritidis and Typhimurium. The non-conjugative nature of the SAP's suggests that the surface structures of these serotypes have evolved independently of the plasmid. It is also evident that high levels of similarity between plasmids is not restricted within a particular serogroup. The observation that the plasmid harboured by Wangata, serogroup D, is more closely related to that of Typhimurium which belongs to serogroup B is perhaps not entirely surprising and parallels the recent report (Rankin *et al* 1995) that described a variant of the

archetypal Enteritidis plasmid which was more related to the reference plasmid of Typhimurium than to that of Enteritidis.

The identification of a plasmid pOG704 that is present in a strain of Enteritidis (Rankin and Platt 1995) and a strain of Moscow suggests that not only do these plasmids evolve within a serotype but the conservation of the plasmids is such that they can remain stable whilst the serotype evolves. The demonstration that minor changes in plasmid REFP occurs both within and between serotypes shows that the plasmids have evolved with considerable subtlety.

The relatedness of the virulence plasmids of the salmonellae has until now been based on the conservation of an 8 kb region between plasmids. Any attempt to compare these plasmids by REFP analysis has relied on the use of infrequent base cutter enzymes such as *Eco*RI and *Hind*III which generate only about 6–10 fragments.

The use of enzymes which produce 20–25 fragments increased the sensitivity of the analysis. In this study the degree of relatedness between the plasmids is paralleled by both *Pst*I and *Sma*I analysis and is further corroborated by *Eco*RV results and strengthens the arguement that these data are valid for providing a window on evolutionary processes, particularly where genotypic data further correlates (Platt *et al* 1995).

These results suggest firstly that the salmonellae have undergone evolutionary changes of considerable subtlety, secondly that traditional phenotypic approaches may not provide an optimal baseline for the development of a phylogenetic framework for the genus and thirdly where extrachromosomal DNA is studied a more detailed analysis is required than an estimate of the approximate sizes of plasmids harboured. The results presented here confirm a family of related plamids associated with but not restricted by serotype. Not only do these plasmids evolve within serotypes but the conservation of these plasmids is such that they can remain stable whilst the serotype evolves. Moreover, the overall conservation of the plasmids suggests that detailed analysis can offer insight into the evolutionary process the serotypes that harbour them have undergone.

archetypal Enteritidis plasmid which was more related to the reference plasmid of Typhimurium than to that of Enteritidis.

The identification of a plasmid pOG704 that is present in a strain of Enteritidis (Rankin and Platt 1995) and a strain of Moscow suggests that not only do these plasmids evolve within a serotype but the conservation of the plasmids is such that they can remain stable whilst the serotype evolves. The demonstration that minor changes in plasmid REFP occurs both within and between serotypes shows that the plasmids have evolved with considerable subtelty.

The relatedness of the virulence plasmids of the salmonellae has until now been based on the conservation of an 8 kb region between plasmids. Any attempt to compare these plasmids by REFP analysis has relied on the use of infrequent base cutter enzymes such as *Eco*RI and *Hin*dIII which generate only about 6–10 fragments.

The use of enzymes which produce 20–25 fragments increased the sensitivity of the analysis. In this study the degree of relatedness between the plasmids is paralleled by both *Pst*I and *Sma*I analysis and is further corroborated by *Eco*RV results and strengthens the arguement that these data are valid for providing a window on evolutionary processes, particularly where genotypic data further correlates these findings (Platt *et al* 1995).

These results suggest firstly that the salmonellae have undergone evolutionary changes of considerable subtlety, secondly that traditional phenotypic approaches may not provide an optimal baseline for the development of a phylogenetic framework for the genus and thirdly where extrachromosomal DNA is studied a more detailed analysis is required than an estimate of the approximate sizes of plasmids harboured. The results presented here confirm a family of related plamids associated with but not restricted by serotype. Not only do these plasmids evolve within serotypes but the conservation of these plasmids is such that they can remain stable whilst the serotype evolves. Moreover, the overall conservation of the plasmids suggests that detailed analysis can offer insight into the evolutionary process the serotypes that harbour them have undergone.

CHAPTER 4

Incompatibility properties of the serotype associated plasmids of the salmonellae

Introduction

Plasmid incompatibility is defined by the inability of two plasmids to co-exist stably in the same cell line in the absence of continued selection pressure for both plasmid types (Timmis 1979). The phenomenon of plasmid incompatibility was first recognised by Maas and Maas (1962) and was shown by Dubnau and Maas (1968) to involve the failure of one of the plasmids to replicate.

In its simplest form, incompatibility occurs when the introduction of a second plasmid into a plasmid-bearing host results in the elimination of the resident plasmid (Datta 1979). Compatibility between plasmids is indicated by the retention of both resident and incoming plasmids. Various mechanisms were put forward to account for plasmid incompatibility (Timmis 1979). One hypothesis, based on the Replicon Model postulated that the replication of plasmids occurred at specific attachment sites on the cell surface andas such compatible plasmids utilized different sites. The Inhibitor Dilution Hypothesis (Pritchard 1978) suggested that DNA replication was controlled by the presence of inhibitor substances that limits the frequency of replication events i.e cell growth decreases the concentration of the inhibitor and eventually results in the initiation of plasmid replication. This in turn generates an increase in the inhibitor which suppresses plasmid replication until sufficient cell growth has taken place. Plasmid incompatibility was concluded to result from the mutual inhibition of two related plasmids that produce cross-reacting repressor molecules : disproportionate concentrations of both plasmid populations became amplified during subsequent replication events led to the production of a population of bacteria that lacked one of the original plasmids. Another model for plasmid incompatibility suggested that random spatial distribution of copies of a single plasmid occurs prior to cell division and results in a random population of DNA molecules being partitioned to each daughter cell. As a result two compatible plasmids in a single bacterial clone would be partitioned as distinct plasmid populations and be inherantly stable.

This led to the conclusion that co-existing plasmids operated different replication systems and vice versa : the replication systems of incompatible plasmids were the same and as a result only one plasmid type could be maintained

in the cell. Plasmids were therefore assigned to incompatibility groups and further classification was based on DNA–DNA homology. Plasmids of the same incompatibility group have been shown to be of roughly the same size and share at least 80% of their DNA sequences whilst plasmids of different incompatibility groups showed less than 15% sequence similarity (Chabbert *et al* 1979). The demonstration that two plasmids are incompatible with each other reveals them to share either replication control or partition function – functions which both plasmids would otherwise compete for. Further classification was based on DNA–DNA homology. Plasmid incompatibility thus indicates the relatedness of plasmids.

More recently the incompatibility functions of the plasmid of Typhimurium have been located at a 4.3 kb region (Cerin and Hackett, 1989). Tinge and Curtiss (1990b) identified three replicons on the Typhimurium plasmid *rep*A, B and C involved with replication functions and a *par* region shown to increase the segregation stability of the *rep*C replicon. The exact mechanism involved in the partitioning of this plasmid have not been defined.

The serotype-associated plasmids of the salmonellae have not been assigned to an incompatibility group although various studies have attempted to resolve the situation. Ou *et al* (1990) found the virulence plasmids of Gallinarum and Pullorum belonged to a different incompatibility group to those of Typhimurium, Enteritidis, Choleraesuis and Dublin. However it has already been established that the plasmid of Dublin exhibits IncX properties (Platt *et al* 1988) and thus has different properties to the plasmids of Enteritidis and Typhimurium. Michiels *et al* (1987), Cerin and Hackett (1989) and Tinge and Curtiss (1990) identified two distinct replicons on the Typhimurium virulence plasmid – although the exact location and incompatibility group of the replicons differed in each study.

The naturally occuring cointegrate plasmid pOG669 is composed of a conjugative IncX plasmid (pOG670) that mediates ampicillin and kanamycin resistance and pOG660 the Typhimurium virulence plasmid (Platt *et al* 1988). The introduction of pOG669 into each of the SAP's was performed to assess their incompatibility properties. Any plasmid that showed incompatibility to pOG669 was then tested against pOG670 (the IncX component) to determine whether the

incompatibility was a result of ther Typhimurium or the IncX component of the cointegrate plasmid.

Materials and Methods

Bacterial strains and their plasmid designations are detailed in Table1. Methods are as detailed in Chapter 2.

Results

Incompatibility analysis of the SAP's of the salmonellae

The introduction of pOG669 into SAP--bearing strains resulted in the elimination of the SAP. Introduction of pOG670 resulted in the retention of both plasmid types except in the case of Dublin where pOG675 was eliminated. Plasmid incompatibility results are detailed in Table 4.1.

Incompatibility results of SAP's or variant plasmids harboured by atypical serotypes (Blegdam, Moscow, Antarctica and Rostock)

The introduction of pOG669 into Blegdam resulted in the elimination of the 54 kb plasmid. The introduction of pOG670 resulted in the retention of both plasmids.

The introduction of pOG669 into both types of Moscow resulted in the elimination of both resident plasmids (54 and 40 kb). The introduction of pOG670 resulted in the retention of the 54 kb plasmid but the elimination of the 40 kb plasmids.

The introduction of pOG669 into Antarctica resulted in the elimination of both resident plasmids. The introduction of pOG670 resulted in the retention of the 54 kb plasmid and the elimination of the 30 kb plasmid.

The introduction of pOG669 into Rostock resulted in the elimination of the 72 kb plasmid. The introduction of pOG670 also resulted in the elimination of the 72 kb plasmid. These results are summarised in Table 4.2

Table 4.1

Incompatibility analysis of the SAP's of the salmonellae with pOG669 and pOG670.

SEROTYPE	PP [Designation] kb	INCOMPATIBLE WITH	
		pOG669	pOG670
Abortusovis	50 [pOG645]	+	ND
Choleraesuis	50 [pOG678]	+	–
Enteritidis	54 [pOG674]	+	–
Typhimurium	95 [pOG660]	+	–
Wangata	90 [pOG646]	+	–
Gallinarum	90 [pOG676]	+	–
Pullorum	90 [pOG677]	+	–
Bovismorbificans	90 [pOG679]	+	–
Dublin	72 [pOG675]	+	+

+ plasmid incompatible

– plasmid compatible

ND = Not Done

Table 4.2

Incompatibility analysis of SAP's or variant plasmids harboured by atypical serotypes

SEROTYPE	PP [Designation] kb	INCOMPATIBLE WITH	
		pOG669	pOG670
Blegdam	54 [pOG704]	+	–
Moscow	54[pOG680]	+	–
	40	+	+
Moscow	54 [pOG704]	+	–
	40	+	+
Antarctica	54 [pOG681]	+	–
	30	–	+
Rostock	72 [pOG675]	+	+

+ incompatible

– compatible

Discussion

The demonstration that the plasmid of Rostock exhibited the same incompatibility results with pOG669 and pOG670 as that of Dublin confirms the REFP data – that these plasmids are the same.

The 40 kb co-resident plasmids in Blegdam and Moscow strains were incompatible with both pOG669 and pOG670 and thus suggests that these plasmids exhibit IncX properties.

The 30 kb co-resident plasmid of Antarctica was compatible with pOG669 and compatible with pOG670. This result was surprising since IncX properties should result in the plasmids elimination with the introduction of pOG669 also. Since this plasmid was co-resident with the virulence associated plasmid its properties were not examined further. Possible explanations as to the anomalous inc results could be the plasmids instability or other host restriction factors acquired during plasmid transfer. For the purposes of this study though the 54 kb plasmid of Antarctica showed incompatibility to the plasmid of Typhimurium; the 30 kb co-resident plasmid merits future analysis.

Plasmid incompatibility analysis of the SAP's of the salmonellae and related plasmids from other serotypes revealed that the SAP's of Enteritidis, Choleraesuis, Abortusovis, Wangata, Pullorum, Gallinarum and Bovismorbificans were related to the SAP of Typhimurium. The plasmid of serotype Dublin IncX incompatibility properties and suggests a different mechanism of evolution to the other SAP's which will be dealt with later. The plasmids of serotypes Blegdam, Moscow, Rostock and Antarctica were shown by REFP analysis to be the same as existing SAP's in other serotypes or molecular variants thereof. Therefore the incompatibility results of these plasmids is not entirely surprising and strengthens the arguement that these plasmids are serotype-associated rather than serotype-specific. The 40 kb plasmids co-resident in the strains of Moscow and Dublin were incompatible with both pOG669 and pOG670. It is almost certain that these are IncX plasmids – for three reasons. Firstly, their size; IncX plasmids are usually around 40 kb in size. Secondly, the REFP of the plasmids are similar. Although the co-resident plasmids of the salmonellae did not generate any fragments after digestion with *Pst*I or *Sma*I, pOG670 itself only produces 5 fragments. Digestion

of pOG670 with *Eco*RV generated more fragments as did the 40 kb plasmids of the salmonellae (results not shown). Thirdly, if these plasmids like that of Dublin, exhibited dual incompatibility properties then they would not be able to co-exist stably with the virulence plasmid

Previous work by Ou *et al* (1990) stated that the virulence plasmids of serotypes Typhimurium, Choleraesuis, Dublin and Enteritidis belonged to a different incompatibility group to those of Gallinarum and Pullorum. However, various anomalies were apparent from this work–firstly, the supposed same incompatibility group of the plasmids of Dublin and Typhimurium. Previous work by Platt *et al* (1988) established that the plasmid of Dublin exhibited IncX properties – results which were successfully repeated in this study. Secondly, the apparent different incompatibility group of Gallinarum and Pullorum plasmids from the others. The results presented here are at variance with those of Ou *et al* (1990) as regards the incompatibility group of Gallinarum, Pullorum and Dublin. However, previous work by McConnell *et al* (1979) found that the *Salmonella* plasmids of the F_1me group were incompatible with the F factor and MP10 plasmids (found in Typhimurium) but did not show any DNA homology. Therefore the inability of these plasmids to coexist must be a result of unexplained host factors rather than incompatibility properties. These results may explain the anomalous incompatibility results obtained by Ou *et al* (1990). The REFP data presented here (i.e. the high degree of similarity of plasmids of Pullorum and Gallinarum to Typhimurium and the low degree of similarity of Dublin to all the other SAP's) strengthens the arguement that the SAP's of Abortusovis, Chloeraesuis, Enteritidis, Wangata, Gallinarum, Pullorum and Bovismorbificans are related to Typhimurium whilst that of Dublin is related but at a much lower level.

CHAPTER 5

Molecular variants of the serotype associated plasmids of the salmonellae

Introduction

Chapter 3 dealt with the the serotype–associated plasmids of the salmonellae and their restriction endonuclease fragmentation patterns. However, molecular variation among such plasmids has been demonstrated previously in Enteritidis (Brown *et al*, 1993) and Typhimurium (Platt *et al* 1988) at a level of 5%. Molecular variation was detected in Dublin plasmids at a level of 23% (Browning *et al* 1995). The definition of molecular variant plasmids has previously been described (Platt *et al* 1988, Browning *et al* 1995). Briefly, it requires that the plasmid be identified initially in a serotype known to contain an SAP and although the majority of fragments are common, at least one SAP fragment be absent in strains that harbour additional plasmids or are of a different size.

As part of a survey of *Salmonella* plasmids in 1985 strains of Dublin were all found to harbour the SAP alone. This contrasted the situation in both Typhimurium and Enteritidis where a small but significant proportion of strains were plasmid free. The combined data from both plasmid profile and REFP analysis was then used to address the question of clonality in Dublin.

Most of the serotypes that harboured SAP's were found to harbour variant plasmids. These results are discussed.

Materials and Methods

Plasmid profile and REFP analyses were performed as described previously in Chapter 2.

Bacterial strains

Five strains of Choleraesuis were analysed from the UK and the USA and their plasmid REFP compared to the NCTC strain. Fifty strains of Gallinarum were analysed from an international collection that included isolates from Europe, USA, Africa and Asia and their plasmid REFP's compared to that of the NCTC reference strain. Thirty one strains of Pullorum were analysed that comprised a UK collection and their plasmid REFP's compared to the NCTC reference strain. A total of 65 distinct isolates of Dublin were examined. They were geographically

diverse; UK (26), USA (15), Holland (8), Canada (4), Denmark (4), France (4) and Germany (4). The strains studied comprised part of international collection held by Central Veterinary Laboratory (Weybridge) and a wide range of Scottish isolates. Additional strains were kindly provided by Dr P Jones (Animal health Institute; Compton). GR34285, pOG675 was used throughout as a reference strain of Dublin for the comparison of REFPs. Where epidemiological information indicated that multiple isolates were from a single outbreak or incident and molecular data was consistent with this conclusion a single isolate was included. However, all isolates epidemiologically defined as sporadic were included. 104 isolates of Bovismorbificans were examined that comprised a collection of Scottish strains and their plasmid REFP's compared to that of the NCTC reference strain.

Results

Molecular variation among plasmids of Choleraesuis

Six strains of *Salmonella* Choleraesuis were available for plasmid analysis, one of which was acquired from NCTC and to which the others were compared. Plasmid details are given in Table 5.1, computer generated output of the plasmids in Figure 5.1. Both pOG633 and pOG634 shared the same REFP when digested with *Pst*I which differed from pOG678 in the absence of the 1.7 kb SAP fragment and the addition of one of 1.6 kb. *Sma*I digestion however revealed pOG633 to contain all the SAP fragments with additional fragments of 1.25 and 1.2 kb generated. pOG634 contained one fragment of 1.25 kb in addition to the SAP fragments when digested with *Sma*I.

These results indicate minor differences in these plasmids which could have arisen by :

a) a deletion which resulted in the loss of a single *Pst*I cleavage site and the addition of a *Sma*I restriction site

or b) a rearrangement which generated an additional restriction site which resulted in the production of two smaller fragments one of which was below the level of detection of the system

Table 5.1.

Molecular characterization of *Salmonella* choleraesuis plasmids

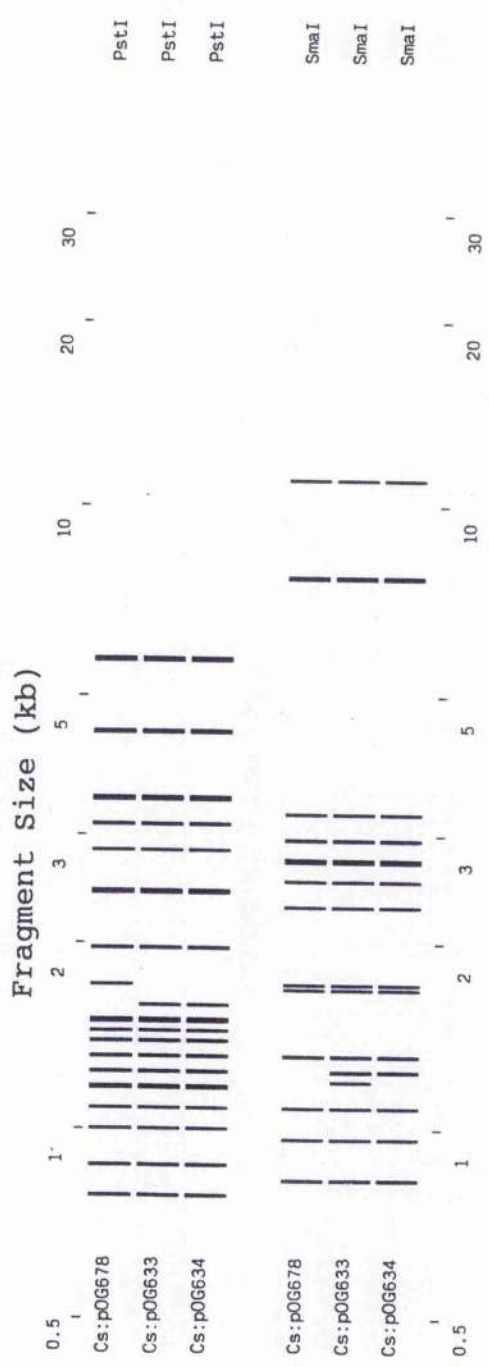
Strain Designation	Origin	PPA (designation)	Source
GR6489	UK	50 (pOG678)	NCTC 5735 isolated in 1939
GR6889	USA	50 (pOG678)	Univ. of Pennsylvania[#]
GR6989	USA	50 (pOG678)	Univ. of Pennsylvania[#]
GR7695	Not Known	50 (pOG633)	IAH, Compton*
GR7795	Not Known	50 (pOG634)	IAH, Compton*
GR7895	Not Known	50 (pOG634)	IAH, Compton*

[#] Strains received as a gift from Dr C Benson, University of Pennsylvania

* Strains recieved as a gift from Dr Tim Wallace, Institute of Animal Health, Compton, Berkshire.

Figure 5.1

Computer generated REFP analysis of the plasmids of *Salmonella* Choleraesuis



or c) the insertion of a piece of DNA which disrupted a *Pst*I restriction site and included or generated a *Sma*I site.

Molecular variation among plasmids of Gallinarum

Fifty one strains of Gallinarum were examined the results of which are given in Table 5.2, the computer–generated graphical printout in Figure 5.2 and Dice coefficients of similarity in Table 5.3.

Of the 51 strains examined 2 (4%) were plasmid free.

One strain GR7294 harboured a plasmid indistinguishable from pOG676 the NCTC strain.

pOG641 differed from pOG676 when digested with *Pst*I in that the 4.4 kb doublet was only a single fragment. *Sma*I digestion revealed the 11.0 kb and 2.5 kb fragments to be absent with an additional fragment of 11.9 kb generated.

pOG642, when digested with *Pst*I, revealed the 4.8 kb fragment to be absent with an additional fragment of 4.30 kb generated. *Sma*I digestion showed the 10.7 kb fragment was replaced by one of 11.0 kb.

Due to the rarity of this serotype the NCTC strain was initially used as the basis for the comparison of other strains and its plasmid was considered to be the SAP of this serotype. However, further analysis of an international collection revealed the majority of strains (90%) harboured an identical plasmid, the REFP of which was a molecular variant of the NCTC strain. The widespread geographical origins of these strains suggested that this plasmid is the predominant type and as a result pOG642 was thereafter designated as the SAP for Gallinarum. Dice coefficients of similarity between the plasmid variants show them to be > 95% related.

Molecular variation among plasmids of Pullorum

Thirty two strains of Pullorum were analysed. Strain details and plasmid designations are given in Table 5.4, the computer–generated analysis of REFP's after digestion with *Pst*I and *Sma*I in Figure 5.3 and Dice coefficients of similarity in Table 5.5. Of the 32 strains of Pullorum examined, one was plasmid free. The

Table 5.2

Molecular characterization of plasmids from *Salmonella* Gallinarum

Strain designation	Origin	PP [designation] kb
GR6589	NCTC 9240	85[pOG676] : 8
GR4590 (7)	Tanzania	85[pOG642]
GR5090 (4)	Saudi Arabia	85[pOG642]
GR5490	Mombasa	85[pOG642]
GR5590	Nairobi	85[pOG642]
GR5690	Lebanon	85[pOG642]
GR5790 (8)	Greece	85[pOG642]
GR5890 (10)	Jordan	85[pOG642]
GR4594	Greece	85[pOG642] : 8
GR4894	Yemen	85[pOG642]
GR5294 (3)	Kenya	85[pOG642]
GR5394 (2)	UK	ND
GR5494 (8)	UK	85[pOG642]
GR6694	Not Known	85[pOG642]
GR6794	Saudi Arabia	85[pOG641]
GR7294	Kenya	85[pOG676]

( ) number of strains examined

ND = None Detected

Figure 5.2

Computer generated REFP analysis of the plasmids of *Salmonella* Gallinarum after digestion with *Pst*I and *Sma*I

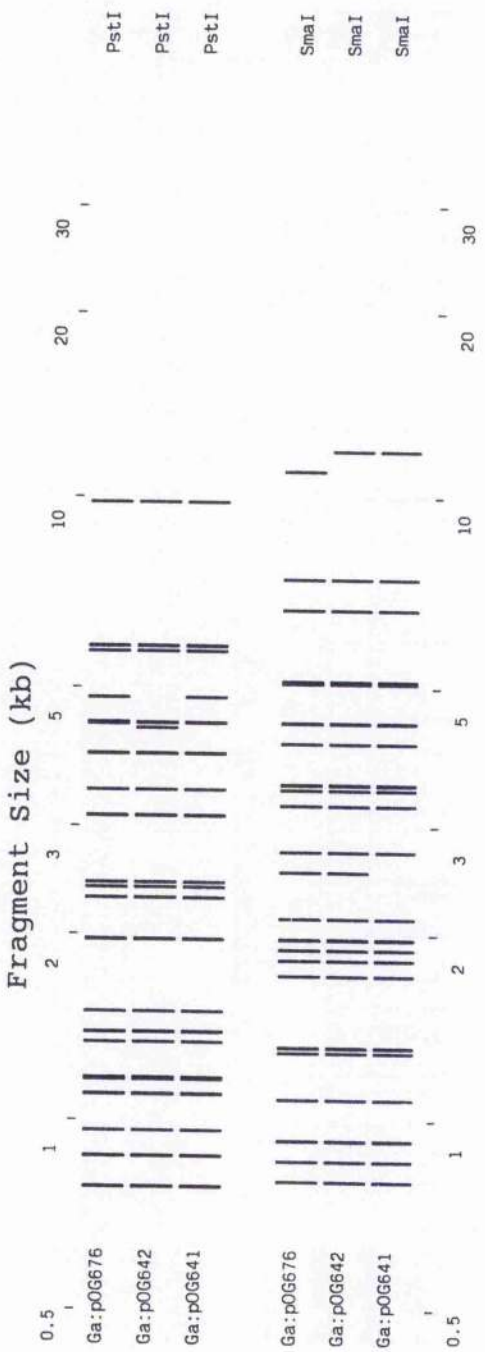


Table 5.3

Dice coefficients of similarity (%) of variant plasmids of Gallinarum.

Plasmid	pOG642	pOG676	pOG641
pOG642	—	**95**	**95**
pOG676	*100*	—	**100**
pOG641	*98*	*98*	—

PstI values in bold
SmaI values in italic

Table 5.4

Strain designation, source and plasmid profile of *Salmonella* Pullorum strains analysed

Strain designation	Origin	PP [designation]kb
GR6689	NCTC 10706	85[pOG677] : 8
GR1494	NCTC 5667	85[pOG677]
GR1594	England	85[pOG677] : 8
GR1694 (6)	UK	85[pOG677] : 8
GR1794 (4)	UK	85[pOG636] : 8
GR1894	UK	85[pOG640]
GR2094	UK	85[pOG639] : 8
GR2194	UK	85[pOG638] : 8
GR2494	UK	ND
GR2794	UK	85[pOG677]
GR3194	UK	85[pOG635]
GR3294 (3)	England	85[pOG677] : 8
GR3494 (4)	Scotland	85[pOG636] : 8
GR3694	England	85[pOG638]
GR3794 (2)	England	85[pOG677]
GR4194	England	85[pOG638] : 8
GR4294	England	85[pOG677] : 8
GR4394	NCTC 8044	85[pOG637] : 8
GR4494	Scotland	85[pOG677] : 8

( ) Number of strains examined

ND = None detected

Figure 5.3

Computer generated REFP's of the plasmids of *Salmonella* Pullorum after digestion with *Pst*I and *Sma*I.

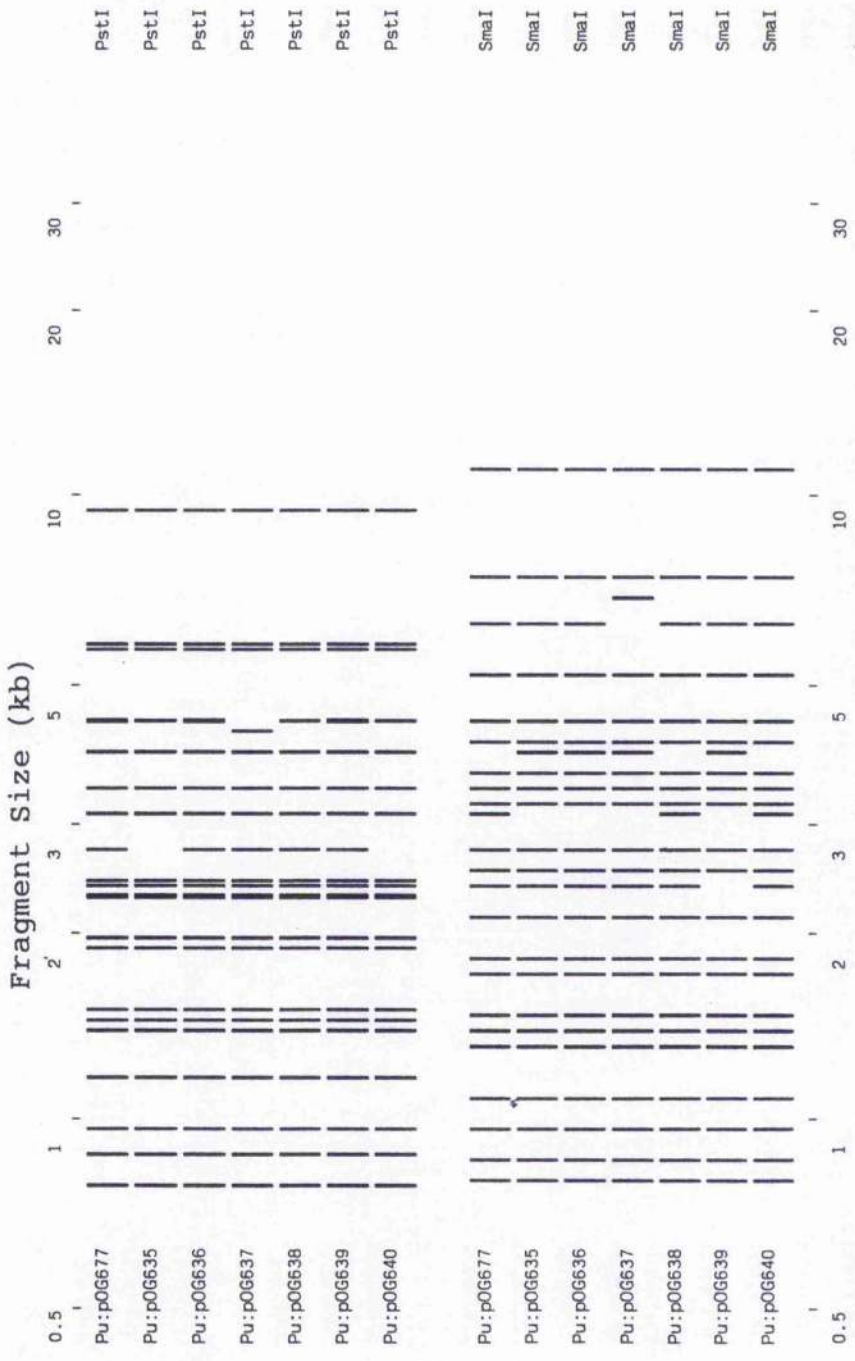


Table 5.5

Dice coefficients of similarity (%) between the variant plasmids of Pullorum

Plasmid	pOG677	pOG640	pOG639	pOG638	pOG637	pOG636	pOG635
pOG677	—	**95**	**100**	**98**	**95**	**100**	**97**
pOG640	*100*	—	**95**	**98**	**90**	**95**	**97**
pOG639	*93*	*93*	—	**98**	**95**	**100**	**97**
pOG638	*100*	*100*	*93*	—	**93**	**98**	**95**
pOG637	*91*	*91*	*93*	*91*	—	**95**	**92**
pOG636	*96*	*96*	*98*	*96*	*96*	—	**97**
pOG635	*96*	*96*	*98*	*96*	*96*	*100*	—

***Pst*I values in bold**

SmaI values in italic

other 31 strains all harboured an 85 kb plasmid. 16 (50%) were indentical to pOG677, the SAP. The other 15 strains harboured variant plasmids which comprised 6 patterns. 8 strains harboured the plasmid designated pOG636 which differed from pOG677 in the *Sma*I REFP only : the 3.1 kb fragment was replaced by one of 3.9 kb.One strain harboured a plasmid designated pOG640 which was evident as a variant only after digestion with *Pst*I – the 4.4 kb doublet fragment was replaced by single fragments of 4.4 kb and 4.35 kb and the 2.7 kb fragment was absent.

Conversely, pOG639 was a variant plasmid only after digestion with *Sma*I where two fragments of 3.1 and 2.35 kb were absent, and one additional 3.9 kb fragment seen. This plasmid was present a single : isolate.

Three strains harboured the plasmid, designated pOG638, which showed variation from pOG677 only when digested with *Pst*I – the 4.4 kb doublet was replaced by fragments of 4.4 and 4.35 kb.

Two strains harboured individual plasmids which were variants with both *Pst*I and *Sma*I. The plasmid of GR4394, when digested with *Pst*I, revealed the absence of the 4.4 kb doublet fragments and the addition of a 4.2 kb fragment. *Sma*I digestion revealed two fragments to be missing (6.3 and 3.1 kb) which were replaced by fragments of 6.9 and 3.9 kb. This plasmid was designated pOG637. The other plasmid that showed variation with both enzymes was found in strain GR3194. *Pst*I digestion revealed this plasmid to have the same REFP as pOG640. *Sma*I digestion however showed this plasmid to be the same as pOG636. This plasmid was designated pOG635.

Overall, the six distinct variant plasmids were closely related to each other and the archetype; dice coefficients of similarity between the plasmids ranged from 91–99% (mean values calculated from *Pst*I and *Sma*I results).

Molecular variation among plasmids of Bovismorbificans

Direct statistical analysis of the plasmids of *Salmonella* Bovismorbificans was not possible due to the lack of detailed epidemiological information in connection with these strains although their distribution suggests that some were epidemiologically related. As a result the actual incidence of plasmid carriage is

probably higher than indicated here. Of the 104 isolates examined, 30 harboured one or more plasmids (Table 5.6). The REFP results of the three 90 kb plasmid types can be seen in Fig 5.4 and the Dice coefficients of similarity in Table 5.7. The majority of strains of Bovismorbificans isolated in Scotland that harboured plasmids fell into two categories : the first plasmid pattern being the same as that of pOG679, the second differed from pOG679 in the *Sma*I digest pattern only in which it generated a single fragment of 1.2 kb in addition to all the SAP fragments and was designated pOG644. The REFP of pOG679 and pOG644 were indistinguishable after digestion with *Eco*RV (results not shown). These two plasmid types are seen with approximately the same frequency as each other although the majority of human isolates (9/12) harboured pOG679 whilst all the non-human isolates harboured pOG644.

pOG643 was identified in one human strain isolated in 1993 and differs from the SAP when digested with both *Pst*I and *Sma*I. After digestion with *Pst*I three fragments (3.85, 2.2 and 2.0 kb) were absent with three additional fragments generated (5.0, 3.0 and 2.8 kb). *Sma*I digestion showed three fragments to be absent (6.3, 2.35 and 1.9 kb) with two additional fragments generated (6.1 and 3.3 kb). This plasmid may have arisen as a result of the insertion of DNA which interupted a restriction site. The plasmid harboured by S/910475 remained uncut after digestion with *Pst*I and *Sma*I. *Eco*RV digestion revealed a plasmid unrelated to the other plasmids of Bovismorbificans.

Molecular variation among plasmids in Dublin

Strain designation, antimicrobial resistance pattern and plasmid profile are shown in Table 5.8. *Sma*I digestion of some of the variant plasmids is shown in Figure 5.5 and the computer generated REFP's with *Pst*I, *Sma*I and *Eco*RV in Figure 5.6. Dice coefficients of similarity of the REFP's with *Pst*I, *Sma*I and *Eco*RV are given in Table 5.9.

None of the isolates was plasmid free; all except 4 harboured a 72kb plasmid and 31 (48%) possessed one or more additional plasmids. Fifteen different strains were distinguished on the basis of PPA : 82 kb (1), 72 kb (30), 72:65 kb (5), 72:60 kb (1), 72:45 kb (2), 72:40 kb (4), 72:30 kb (1), 72:60:6 kb (4), 72:30:3.3 kb (1), 72:3.8 kb (1), 72:3.3 kb (9), 72:9 kb (1), 72:4.3:3.3 kb (1), 72:12

Table 5.6

Strain designation, source and plasmid details of Bovismorbificans strains

Strain designation	Source (Year isolated)	PP [designation] kb
GR6389	NCTC 5754 (1939)	90(pOG679)
S/890132	HUMAN (1989)	90(pOG679) : 7
S/890281	BOVINE (1989)	90(pOG644)
S/890337	BOVINE (1989)	90(pOG644)
S/890382	BOVINE (1989)	90(pOG944)
S/890796	BOVINE (1989)	90(pOG644)
S/890976	BOVINE (1989)	90(pOG644)
S/890411	OVINE (1989)	90(pOG644)
S/890463	CANINE (1989)	90(pOG644)
S/890608	ENVIRONMENTAL (1989)	90(pOG644)
S/890648	OVINE (1989)	90(pOG644)
S/892850	HUMAN (1989)	90(pOG679)
S/894174	SEAL (1989)	90(pOG644)
S/901430	AVIAN (1990)	3
S/902090	QC (1990)	90(pOG679)
S/903191	HUMAN (1990)	90(pOG679)
S/904040	SEAL (1990)	90(pOG644)
S/910475	BOVINE (1991)	60
S/915148	SEAL (1991)	90(pOG644)
S/922633	HUMAN (1992)	90(pOG679) : 6
S/922814	HUMAN (1992)	90(pOG679)
S/922898	HUMAN (1992)	90(pOG679)
S/923879	HUMAN (1992)	90(pOG644)
S/930803	OTTER (1993)	90(pOG644)
S/930869	HUMAN (1993)	90(pOG644)
S/931030	HUMAN (1993)	90(pOG643)
S/931354	ENVIRONMENTAL (1993)	90(pOG644)
S/934107	HUMAN (1993)	90(pOG679)
S/935530	HUMAN (1993)	90(pOG679)
S/935538	HUMAN (1993)	90(pOG679)
S/940709	AVIAN (1994)	90(pOG644)

Figure 5.4

Computer generated REFP analysis of the plasmids of *Salmonella* Bovismorbificans after digestion with *Pst*I and *Sma*I

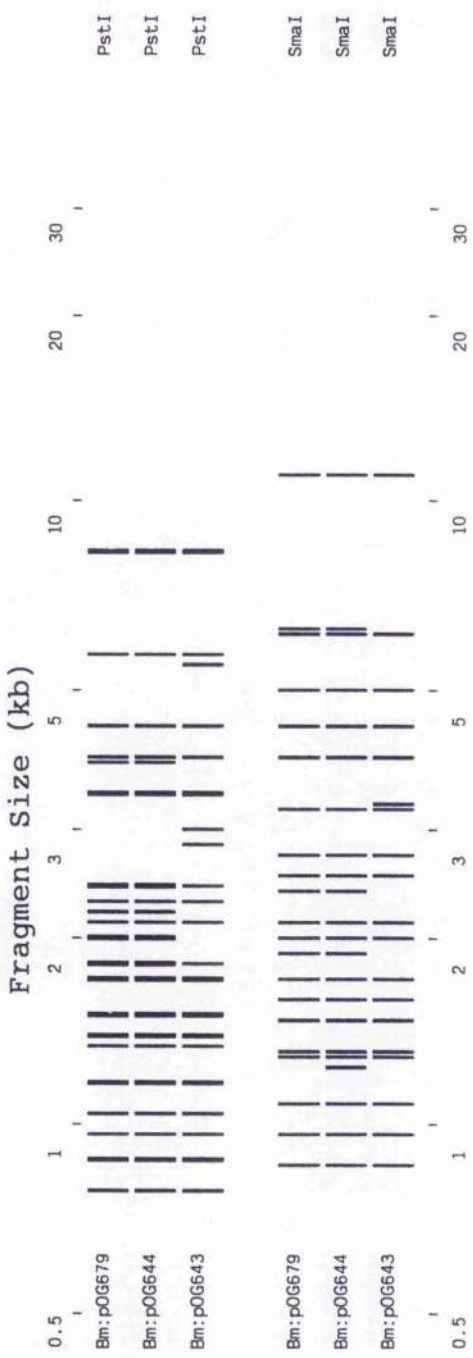


Table 5.7

Dice coefficients of similarity (%) of the variant plasmids of Bovismorbificans

Plasmid	pOG679	pOG644	pOG643
pOG679	—	**100**	**86**
pOG644	*98*	—	**86**
pOG643	*93*	*91*	—

PstI values in bold
SmaI values in italic

Table 5.8 Characteristics of *Salmonella* Dublin strains studies

Origin	Strain Designation	PP (designation) kb	Antibiotic resistance
UK	GR34285	72(pOG675)	ND
	GR10290 (12)	72(pOG675)	ND
	S/923907	72*(pOG688) : 40	ND
	S/920682	72*(pOG689)	ND
	GR10190 (2)	72*(pOG682) :45	ND
	S/921207 (3)	72 : 3.3	ND
	S/920386 (5)	[72 : 70(pOG647)]#	ND
	S/922378	[72 : 70]# : 3.3	ND
	S/921941	70(pOG647) : 30	ND
	S/922442	70(pOG647) : 40	ND
USA	GR9890	72	ND
	GR9690	72	Ap
	GR9990 (2)	72	Ap,Tc,Km,Gm, Tc
	GR1590 (2)	72*(pOG683)	ND
	GR2290	72*(pOG640) : 65	Ap, Km
	GR9490	72 : 65	Ap, Km
	GR9590	82*(pOG685)	Ap, Km
	GR9790	72*(pOG686) : 60	Ap,Km,Su
	GR1890	72 : 65	Ap,Tc,Km
	GR1990	72 : 65	Ap,Tc,Km
	GR2090	72 : 65	Ap,Tc,Km,Sm
	GR1790	125*(pOG650)	Ap
	GR2190	125*(pOG649)	Ap
Canada	GR8290	72 : 60 : 6	Ap,Cm
	GR8390	72 : 60 : 6	Ap,Tc,Su,Cm
	GR8490	72 : 60 : 6	Ap,Sm,Su
	GR8590	72 : 60 : 6	Ap,Tc,Cm,Km,Sm
Denmark	GR9090 (2)	72	ND
	GR9290	72 : 3.8	ND
	GR9390	72 : 30 : 3.3	ND
France	GR8790	72	Tc,Su,Cm
	GR8690 (2)	72*(pOG687) : 40	ND
	GR8990	72 : 4.3 : 3.3	ND
Germany	GR8090	72	Tc,Su,Cm
	GR7890	125*(pOG648)	Ap
	GR7990	72 : 3.3	Cm,Su
	GR8190	72 : 9	Ap,Cm,Su
Holland	GR2490	72	Tc,Su,Cm
	GR2690	72	Km,Su,Sm,Cm
	GR2590	72*(pOG684)	Ap,Tc,Km,Su,Cm
	GR2390	72 : 12	Ap,Tc,Su,Cm
	GR2790	72 : 3.3	Nal,Tc,Su,Cm
	GR2890	72 : 3.3	Tc,Su,Sm
	GR2990	72 : 3.3	Su,Sm
	GR3090	72 : 3.3	Tc,Su,Cm

( ) No. of strains examined

* indicates a variant plasmid on the basis of REFP analysis

#[72 : 70] indicates comigration of plasmids of similar size; distinguished only after REFP analysis.

Figure 5.5 REFP analysis of plasmids of *Salmonella* Dublin digested with *Sma*I.

Lane 1. λ *Kpn*I 2. pOG675 3. pOG689 4. pOG688 5. pOG685 6. pOG687 7. pOG684 8. pOG683 9. pOG675 10. λ *Pst*I

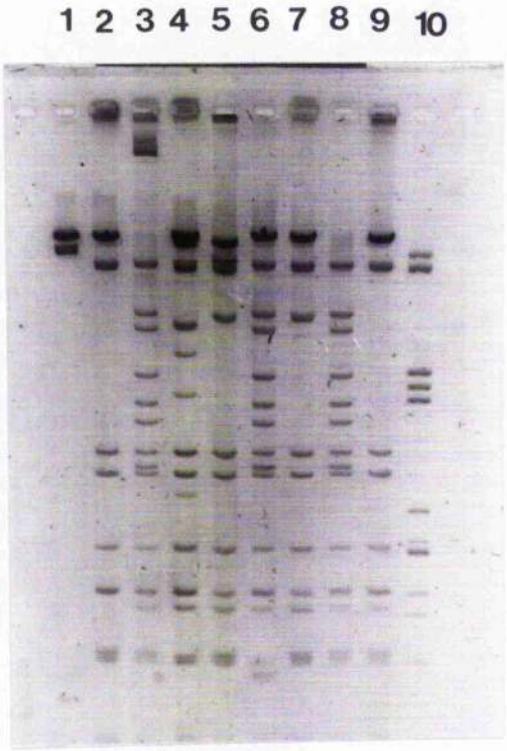
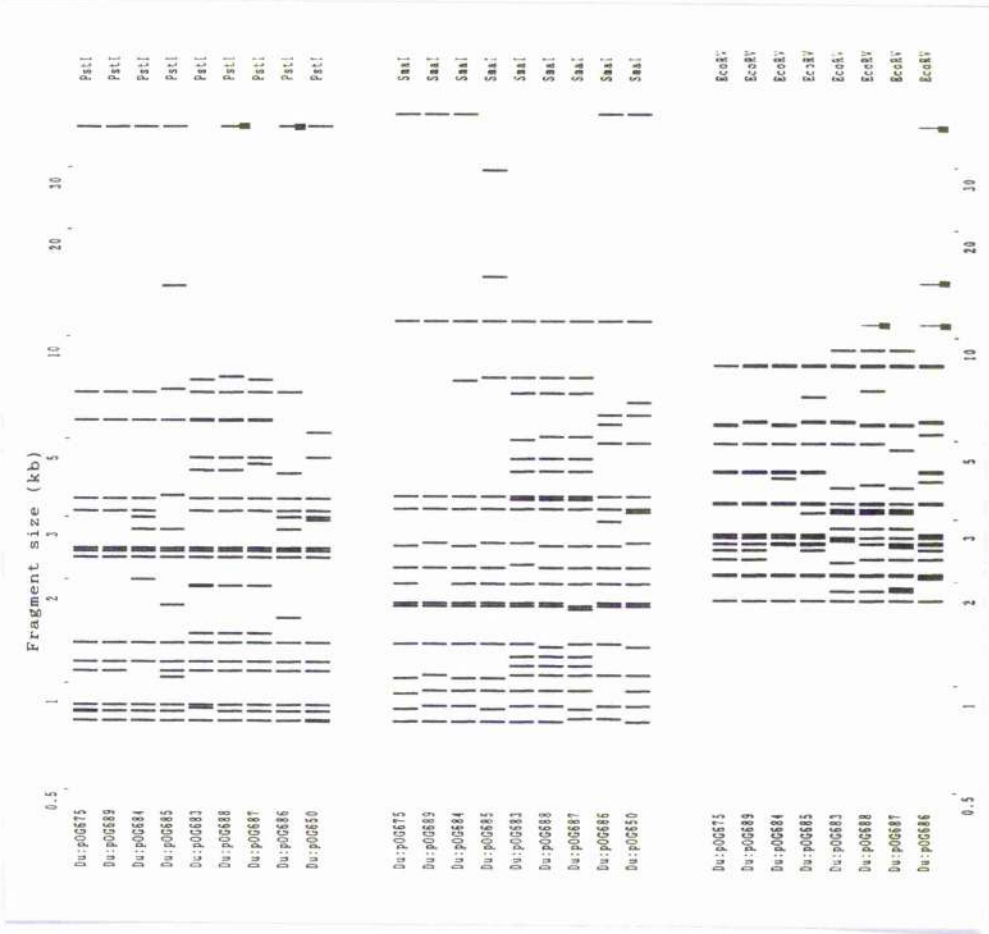


Figure 5.6

Computer generated REFP analysis of the plasmids of Dublin digested with *Pst*I, *Sma*I and *Eco*RV. ■ indicates fragments derived from a co-resident plasmid.



Plasmid	pOG675	pOG683	pOG640	pOG684	pOG685	pOG686	pOG687	pOG688	pOG689	pOG682
pOG675	–	74	84	90	86	83	72	74	99	75
pOG683	81 *74* 67	–	69	70	66	62	94	97	73	96
pOG640	80 *88* ND	71 *67* ND	–	80	72	80	70	71	82	71
pOG684	87 *97* 87	71 *78* 62	75 *85* ND	–	84	80	66	70	89	71
pOG685	84 *87* 88	69 *65* 64	*67* *77* ND	79 *90* 83	–	73	68	69	85	71
pOG686	84 *77* 87	69 *63* 55	79 *80* ND	85 *75* 80	77 *67* 74	–	63	63	81	62
pOG687	81 *74* 62	94 *100* 88	71 *68* ND	71 *78* 50	69 *76* 60	69 *63* 58	–	91	71	93
pOG698	85 *74* 63	97 *100* 94	74 *68* ND	74 *78* 57	72 76 60	72 *58* 60	92 *100* 83	–	73	95
pOG689	100 *96* 100	81 *71* 67	80 *84* ND	87 *93* 87	84 *83* 88	84 *73* 85	81 *71* 62	85 *71* 62	–	74
pOG682	85 *74* 67	97 *100* 91	74 *68* ND	74 *78* 62	72 *76* 65	72 *58* 56	92 *100* 86	100 *100* 86	85 *71* 67	–

Table 5.9 Dice coefficients of similarity between the variant plasmids of Dublin. Upper matrix shows mean values and lower matrix individual values for *Pst*I(bold), *Sma*I(Italic) and *Eco*RV(Standard).

kb (1), 125 kb (3). The 15 recognisable plasmid-profile types were further subdivided on the basis of REFP analysis into 28 strains.

Of 30 isolates which possessed a single 72 kb plasmid from PPA data, 5 were shown to harbour two comigrating plasmids after REFP analysis and the additional plasmid designated pOG647. The latter strain was found only among UK isolates and was associated with both outbreak and sporadic isolation.

The two human strains isolated in 1992 both possessed two plasmids (72:40 kb) and (72:30 kb). The 72 kb plasmid was identical in both strains. It was however unrelated to the SAP, and had the same fragmentation pattern as pOG647. However REFP analysis showed both the 40 kb and 30 kb plasmids to remain uncut with the enzymes used. The single ovine and the environmental isolates both contained the SAP alone.

One strain (GR2390), showed the presence of a single 72 kb plasmid. However, REFP analysis showed an additional 12 kb low copy-number plasmid which was obscured by chromosomal DNA and was undetected by PPA alone.

All the remaining strains harboured the SAP either alone, in addition to one or more plasmids or as a molecular variant.

Thirteen strains harboured plasmids which were molecular variants of the reference SAP pOG675 – and comprised 10 distinct patterns (Figure 5.6).

Plasmid pOG689 was recognised as a variant plasmid only after digestion with *Sma*1 (*Pst*I and *Eco*RV digestion both gave patterns identical to pOG675). *Sma*I digestion showed the 1.94 kb SAP fragment to be missing. No additional fragments were observed.

Plasmid pOG684 contained all the SAP fragments when digested with *Sma*I together with an additional fragment of 7.5 kb. However, this plasmid was classified as a variant SAP after digestion with *Pst*I. It lacked the 1.1 kb SAP fragment and three additional fragments were detected which totalled 7.8 kb. *Eco*RV digestion revealed loss of the 2.5 and 2.3 kb SAP fragments with one additional fragment of 4 kb generated. REFP analysis with these three enzymes indicates a net gain of 5 kb DNA. (This was not evident in a plasmid profile).

Plasmid pOG685, when digested with *Sma*I was shown to lack the 42.9 kb SAP fragment. Additional fragments of 29.4, 14.7 and 7.5 kb were present. This

resulted in an overall DNA gain of approximately 9 kb. *Pst*I digestion revealed the loss of the 3.1 kb SAP fragment with five additional fragments generated that totalled 19.4 kb. *Eco*RV digestion revealed the loss of the 2.3 kb SAP fragment with two additional fragments of 6.8 and 3.1 kb generated. The net result of these REFP data was an overall DNA gain of 10.8 kb DNA as indicated by PP and confirmed by REFP analyses.

The *Sma*I digest of plasmid pOG640, contained all the SAP fragments with four additional fragments that totalled 20.5 kb. *Pst*I digestion revealed the absence of both the 6.8 and 5.7 kb SAP fragments with four additional fragments generated that totalled 15.6 kb. This strain died before completion of the investigation. The presence of an additional 65 kb plasmid further complicated the analysis but the loss of two *Pst*I fragments unequivocally indicated the plasmid to be a variant.

Plasmid pOG686, when digested with *Sma*I, revealed the 0.95 kb SAP fragment to be absent with four additional fragments totalling 19.4 kb generated. *Pst*I digestion revealed the absence of the 5.7 kb SAP fragment with four additional fragments generated that totalled 11.3 kb DNA. EcoRV digestion revealed the loss of the 4.9 kb SAP fragment with three additional fragments of 5.6, 5.2 and 3.9 kb, together with three fainter fragments (40, 14.1 and 10.7 kb) which is consistent with their derivation from a lower copy number (60 kb) plasmid.

Plasmid pOG683 lacked the 42.9 kb SAP fragment after digestion with *Sma*I; eight additional fragments were generated which totalled approximately 34kb. *Pst*I digestion revealed loss of the 39.7 kb SAP fragment with five additional fragments generated which totalled 19.4kb DNA. *Eco*RV digestion revealed the 4.06, 2.71 and 2.46 kb SAP fragments to be absent with additional fragments of 9.1, 3.7, 3.2, 3.1 and 2.8 kb generated. Overall, the three enzymes indicate a DNA loss of 5.5 kb.

When plasmid pOG688 was digested with *Sma*I REFP was the same as that of pOG683 but for the additional 40 kb plasmid which lacked *Sma*I sites. After *Pst*I digestion the REFP of pOG688 was also identical to pOG683. However, the additional 40kb plasmid had a single *Pst*I restriction site and contributed a single 40 kb fragment. EcoRV digestion was the same as that of pOG683 with two

additional doublets of 10.7 and 6.98 kb which totalled 35 kb and probably comprised the co-resident plasmid.

The *Sma*I digest of pOG687 lacked both the 42.9 and 1.74 kb SAP fragments; additional fragments present matched those of pOG683. *Pst*I digestion produced the same REFP as that of pOG683 with the exception of the 4.2 kb fragment of pOG687 which was paralleled in pOG683 and pOG688 by an additional 4.1 kb fragment. *Eco*RV digestion matched that of pOG683 together with two additional fragments of 2.48 and 1.92 kb.

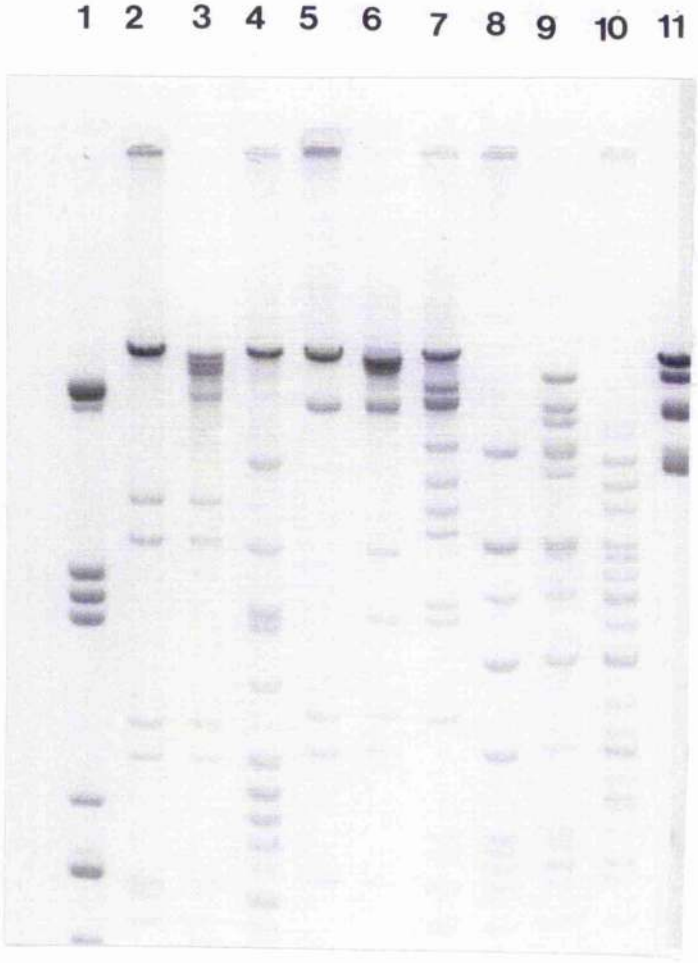
One strain GR8890 originally harboured 2 plasmids but after repeated sub-culture lost the 40kb plasmid. From the comparison of initial and derivative strains it was possible to determine from REFP data which fragments originated from the co-resident plasmid.

It is clear from *Pst*I and *Sma*I digest results that plasmids pOG683, pOG687 and pOG688 are almost identical. *Eco*RV digestion supported these findings: all three were shown to lack the 4.06 kb SAP fragment with the same additional fragments generated – with the exception of pOG687 which had two fragments of 2.02 and 2.57 kb not present in pOG683 and pOG688

The three 125 kb plasmids all contain the majority of SAP fragments. pOG648 lacked the 6.84, 3.41, 2.44 and 1.32 kb SAP fragments when digested with *Pst*I; *Sma*I digestion revealed the loss of the 3.2 kb fragment. pOG649 and pOG650 after *Pst*I digestion showed the loss of the 39.7 kb fragment. Figure 5.7 shows the plasmids pOG648 and pOG649 digested with *Pst*I, *Sma*I, *Eco*RV and compared topOG675. Eight common additional fragments were generated and pOG649 contained a further two additional fragments of 1.5 and 1.45 kb. The *Sma*I digest showed both plasmids to lack the 42.9 kb fragment; three common additional fragments were seen and pOG649 produced one unique fragment and pOG648 two unique fragments. Each of these plasmids was conjugative, incompatible with pOG675 and were thus co-integrate plasmids. pOG649 and pOG650 were closely related (S_D values >90% for both *Pst*I and *Sma*I). pOG648 was not closely related to either except in regard to the fragments derived from the SAP. This result is not surprising since pOG648 originated in Germany and pOG649 and pOG650 were both from American strains of Dublin and indicates

Figure 5.7 REFP analysis of the cointegrate plasmids of *Salmonella* Dublin after digestion with *Pst*I (Lanes 2–4), *Sma*I (Lanes 5–7) and *Eco*RV (Lanes 8–10).

Lane 1. λ *Pst*I 2,5&8. pOG675 3,6&9 pOG649 4,7&10 pOG648 11. λ *Kpn*I



unrelated cointegration events.

Incompatibility analysis of Variant-SAP and other plasmids harboured by Dublin

The introduction of pOG669 into GR1590 resulted in the elimination of the 72 kb variant plasmid. The introduction of pOG670 resulted in the retention of both plasmids.

The introduction of pOG669 into GR8890 resulted in the elimination of both parental plasmids whilst the introduction of pOG670 resulted in the elimination of the 40kb plasmid only.

The introduction of pOG669 into S/923907 resulted in the elimination of both resident plasmids whilst pOG670 resulted in the elimination of the 40kb plasmid only.

The introduction of pOG669 into S/921941 and S/922442 resulted in the elimination of both resident plasmids whilst pOG670 resulted in the elimination of the 30 and 40 kb respective plasmids only. The introduction of pOG669 into GR14792 resulted in the elimination of both co-migrating plasmids whilst the introduction of pOG670 resulted in the elimination of the SAP (72 kb) only. These results are summarised in Table 5.10.

The incidence of molecular variation in the serotype associated plasmids of the salmonellae is shown in Table 5.11.

Antimicrobial Resistance

None of the UK strains were resistant to any of the antimicrobials tested. Excluding UK isolates 77% of "rest of the world" strains were resistant to between one and five antimicrobial agents.

There was no overall correlation between antimicrobial resistance and plasmid presence as some strains which possessed the typical SAP alone were also resistant to 2–5 antimicrobials and indicates a chromosomal location for the resistance determinants.

Table 5.10

Plasmid incompatibility analysis of plasmids of *Salmonella* Dublin

Strain Designation	PP[designation]kb	Incompatible with	
		pOG669	pOG670
GR 1590	72 [pOG683]	+	–
S/921941	70 [pOG647]	+	–
	30	+	+
S/922442	70 [pOG647]	+	–
	40	+	+
GR 8890	72 [pOG687]	+	–
	40	+	+
S/923907	72 [pOG688]	+	–
	40	+	+
GR 14792	72 [pOG675]	+	+
	70 [pOG647]	+	–

+ incompatible

– compatible

Table 5.11

The incidence of molecular variation in the SAP's

Serotype	No strains	%P –ve	% variants	Range S_D to SAP*
Bovismorbificans	**104**	**61**	**3**	**86 - 99**
Gallinarum	**51**	**4**	**6**	**95 - 98**
Pullorum	**32**	**3**	**47**	**91 - 99**
Dublin	**65**	**0**	**23**	**62 - 94**
Typhimurium	#	**5**	#	#
Enteritidis	#	**5**	#	#

* Mean Dice coefficients of similarity of variant plasmids compared to the SAP

Variant plasmids previously demonstrated at 5% in these serotypes (Platt *et al* 1988, Brown *et al* 1993)

Discussion

The majority of serotypes of *Salmonella* that harbour SAP's also possess variant plasmids. Plasmid variation has been demonstrated at a level of 5% in serotypes Typhimurium and Enteritidis. This study showed Dublin to exhibit plasmid variants at a level of 23%, Gallinarum at around 6% and Pullorum at 47%. The plasmid of Bovismorbificans appears to be present in the population in two forms albeit this variation was only detectable with one enzyme. These plasmids were present at the same frequency as each other, and variation to them was at a level of around 3%. In the cases of Dublin, Gallinarum, Pullorum and Bovismorbificans there is evidence of the distribution of the plasmid world-wide as well as the existance of sub-clones within a localized geographical area. The presence of a plasmid in Gallinarum that is widespread and differs from that seen in the NCTC strain reveals this plasmid to have the 11.0 kb *Sma*I fragment in common with Typhimurium (and all other SAP's) whereas in pOG676 this fragment is replaced by one of 10.7 kb. The existance of a plasmid variant in Dublin (pOG683) which shows a high degree of similarity to Gallinarum (pOG676) indicates that these plasmids are more closely related to each other and may in fact provide an link in the evolutionary development of plasmids.

Although the incidence of plasmid variation in Dublin seems to be much higher than that of Enteritidis and Typhimurium (23% compared to 5%), the strains of Dublin involved were obtained from world–wide sources and thus not be truly representative of the population. For example the plasmids from two out of the four French isolates were designated molecular variants yet this probably does not reflect the overall situation in Dublin plasmids isolated in France. Similarly, 4% of Dublin isolated in Scotland in 1995 possessed molecular variants of the SAP. In these instances the epidemiology of the strains is important in determining the overall picture of plasmid identification.

The combination of plasmid profile and REFP analysis has confirmed the international dispersion of common clones of Dublin and also demonstrated distinct strains to be common within a locality and restricted to it. Strains that harbour the 72 kb plasmid alone are particularly common and their demonstration confers no epidemiological specificity unless REFP analysis indicates molecular variation.

Population genetic studies of Dublin (Selander *et al* 1992) based on MLEE distinguished four clones, three of which were closely related (Du1, Du3 and Du4); Du1 had a world-wide distribution, Du3 was restricted to the UK and France and Du4 was unique to the USA. The distribution of plasmid within Du1 was limited to either the SAP alone or ther SAP together with a single small (3 kb) plasmid whereas Du3 harboured no small plasmids and either the SAP alone, the SAP together with a plasmid of intermediate size (40-50 kb) or an intermediate sized plasmid alone. A survey of Danish isolates of Dublin (Olsen *et al* 1990) showed nine plasmid profiles, four of which corresponded to profiles typical of Du1 and Du3 and thus extends the range of Du3 to Denmark.

The more detailed analysis of plasmids presented here indicates considerably greater diversity in which there is no direct correspondence between the plasmid profiles of many strains and Du1 and Du3. Nevertheless, by inference both clones were represented among UK, Danish or French strains. The 3.3 kb plasmid found among isolates from all European countries sampled was identical on the basis of REFP analysis with four restriction enzymes although no individual enzyme generated a fingerprint with an optimal information content. Thus strains with the profile 72:3.3 kb are widely distributed and equate with Du1 and indicate the stability of the small plasmid. One strain from France harboured an additional 4.3 kb plasmid but is presumably clonally related. Individual strains from Denmark (PPA 72:30:3.3) and the UK (72:70:3.3), neither of which had acquired drug resistance may belong to Du1 but the presence of additional plasmids of intermediale size also raises the possibility of association with Du3. Similarly the Canadian strain that harboured small (6 kb) and intermediate (60 kb) sized plasmids and the American isolates that possessed additional plasmids cannot be readily assimilated into the clonal framework proposed by Selander *et al* (1992).

The demonstration of identical REFP's among the 72 kb SAP's from all seven countries confirms the overall conservation of this plasmid. However molecular variation in the SAP was detected in 15 (23%) of the isolates studied of which three were co-integrate plasmids. On the basis of REFP similarity and dissociative behaviour in vitro two of these were associated with the strains of PP 72:65. A further seven of the molecular variants were detected in strains that

harboured additional plasmids of intermediate size. Five of these corresponded to clone Du3; pOG682 and 688 (UK) and pOG687 (France) were almost identical to each other and also to pOG688 (USA). The latter plasmid, present alone in two American isolates, provides circumstantial evidence that Du3 is also present in the USA. The three remaining molecular variants were each recognised in single strains from different countries and none had diverged greatly from the reference SAP pOG675. Together these data suggest the possibility that co-resident plasmids may have influenced the evolution of the SAP in Dublin and go some way to explain why the SAP's of Dublin and Enteritidis are more markedly divergent than the genomic DNA appears to be on the basis of MLEE and IS200 fingerprinting.

Overall this study has demonstrated considerable plasmid diversity within Dublin and evolutionary divergence of the Dublin SAP. These findings offer some scope for the application of plasmid analysis in epidemiological investigation but not where strains harbour the SAP alone. Further studies are required to clarify the association between plasmid variation and genotypic markers; comparative analysis of molecular variants of the SAP's from different serotpyes will contribute to a better understanding of salmonella phylogeny.

The result of the Dublin–Variant plasmid pOG683 (incompatible with pOG669, compatible with pOG670) suggests this plasmid is more related to Typhimurium than the Dublin SAP is. This plasmid may be an intermediate in the evolutionary development of *Salmonella* plasmids.

All the SAP's tested were incompatible with pOG669 and, with the exception of pOG675 (Dublin) compatible with pOG670. This indicated that the incompatibility with pOG669 was a result of the Typhimurium portion of the plasmid and not the IncX component.

The variant plasmids were all incompatible with pOG669. However pOG683 was compatible with pOG670 unlike the SAP.

The SAP of Dublin is known to exhibit IncX properties. It has been suggested that the plasmid of Dublin may have evolved by a different mechanism to that of other plasmids which may have involved cointegration of an IncX plasmid followed by a deletion event in which the IncX properties were retained by the Dublin plasmid. This plasmid may provide an insight into the reason behind the

different incompatibility and REFP properties of the Dublin SAP. pOG683 may be an evolutionay intermediate in the development of plasmids.

The similarily matrices (Table 5.9) show that each of the three enzymes corroborated the relationships between variant plasmids. In comparisons with pOG675 all variants were ≥80% similar (*Pst*I), ≥74% (*Sma*I) and ≥62% (*Eco*RV).

The highest degree of relatedness (>90%) between the variant plasmids was seen in comparisons of pOG682, 683, 687 and 688. In contrast each of these plasmids was <75% similar to pOG675. Thus these four strains represent either parallel or progressive divergence from a single variant.

One interesting feature of the study was the difference in the antimicrobial resistance seen in isolates from different countries : the most noticable observation being the lack of resistance markers in UK isolates, which markedly contrasts the situation in cattle with respect to Typhimurium DT204c which have progressively acquired resistance determinants since their initial detection in 1979 (Threlfall *et al* 1985). Plasmid analysis suggests much antibiotic resistance to be chromosomally determined as strains with the SAP alone possessed resistance markers and have been previously reported (Woodward *et al* 1989).

CHAPTER 6

The identification of restriction endonuclease sites on the Typhimurium SAP

Introduction

The identification of a limited number of restriction endonuclease sites on certain plasmids of the salmonellae has previously involved the analysis of Typhimurium (Norel *et al* 1989 a,b, Cerin and Hackett 1993, Gulig *et al* 1992, Gulig and Chiodo 1990, Friedrich *et al* 1993), Dublin (Williamson *et al* 1990) and to a lesser extent Enteritidis (Suzuki *et al* 1994). In all cases, plasmid examination involved either REFP analysis with enzymes that cleave infrequently e.g. *Bam*HI, *Bgl*II etc which resulted in the generation of a small number of fragments (Tinge and Curtiss 1990, Michiels *et al* 1987) or the sequence determination of a small section of the plasmid e.g the virulence region (Gulig *et al* 1992, Gulig and Chiodo 1990) or fimbrial biosynthetic genes (Friedrich *et al* 1993).

Although it was shown that an 8 kb *Sal*I–*Xho*I probe from the virulence region hybridised with plasmids of different serotypes (Williamson *et al* 1988b), it is not known to what extent other regions of homology exist between the different plasmids. The comparison of REFP's from a variety of SAP's has indicated a high degree of similarity exists between certain plasmids e.g. Pullorum/Gallinarum, Typhimurium/Pullorum, Choleraesuis/Enteritidis and also a low degree of relatedness between others e.g. Dublin/Choleraesuis, Dublin/Typhimurium.

The published details of the restriction map of the Typhimurium plasmid has relied on the results generated by the enzymes *Bam*HI, *Bgl*II, *Xho*I, *Hin*dIII, *Eco*RI and *Sal*I all of which (*Sal*I excluded) generate between 3–7 fragments, and which revealed a large section of the plasmid to contain none of these sites at all (*Sal*I excluded). However, although the exact size of the fragments were not detailed the position of each fragment with respect to each other was (Michiels *et al* 1987, Tinge and Curtiss 1990) e.g. H2, H8, H4, H1, H5, H7, H3, H6 (H1 = largest *Hin*dIII fragment). In addition another anomaly existed – the precise size of the plasmid itself. Friedrich *et al* (1993) published a map in which the plasmid was given as 90 kb whilst Korpela *et al* (1989) and Rhen *et al* (1989) both described a plasmid of 96 kb.

The usefulness of *Pst*I and *Sma*I REFP's in both epidemiology and the recognition of variation in SAP structure would be extended by relating the sites for these enzymes to the existing more limited restriction maps.

The identification of both *Pst*I and *Sma*I restriction sites on the plasmid of Typhimurium would greatly enhance our understanding of the SAP's. It has already been demonstrated that there exists a high degree of relatedness between the plasmids of different serotypes, however the extent of the relatedness of non-virulence regions remains unknown. Ultimately, the analysis of both common core regions of the plasmids and also regions that do not appear to be common throughout was intended. The construction of probes from both common and unique fragments and hybridization to Southern blots was intended. This would demonstrate the true extent of fragment homology and if the fragments were really common/unique and if they were present elsewhere on the plasmid. Of particular interest was pOG683, pOG688 and pOG687 – variant plasmids of the Dublin SAP which showed more overall REFP similarity to plasmids of other serotypes than to Dublin itself; they shared a common core group of fragments with both *Pst*I and *Sma*I with other SAP's.

From these results it was hoped to more precisely define the relationship between the various SAP's and from which the construction of an evolutionary framework of plasmid development might be possible.

Materials and Methods

DNA fragment extraction and redigestion and REFP analysis of plasmid DNA was carried out as described previously.

Digestion of DNA with two enzymes.

Where both enzymes required the same React buffer, the digestions were carried out simultaneously in a total volume of 50µl with an excess (3µl) of each enzyme to ensure complete digestion of the DNA.

Where both enzymes required different React buffers, the enzyme that required the buffer with the lowest salt concentration was used first in a total volume of 30µl (20µl DNA, 3µl buffer, 3µl enzyme and 4µl distilled water) and digestion carried out for 2 – 3 hours. The second digestion was carried out by the addition of 5µl buffer, 3µl enzyme and 12µl distilled water for a further 2–3 hours. For example a double digest that involved *Pst*I and *Bam*HI was carried out with *Pst*I first (React 2 – 50mM NaCl) followed by *Bam*HI (React 3 –100mM NaCl).

Anomalies within the literature of the size of the Typhimurium SAP

Published maps of the Typhimurium plasmid varied both with respect to plasmid size and as a result the size of restriction digest fragments. Initial mapping studies were designed to identify fragments which contained sequences of interest e.g the virulence region was identified on a 3.5kb *Hin*dIII fragment. Therefore, although the exact size of the fragments may differ between different workers, the limited number of fragments generated enabled each to identify the appropriate fragment.

Although the various published maps of the plasmid of Typhimurium only differed by 6 kb, both *Pst*I and *Sma*I generate fragments of <11 kb in size so this difference would be significant with these enzymes. Estimation of the *Bgl*II fragment sizes of these plasmids indicated the map of Friedrich *et al* (1993) to contain fragments of 61 : 12 : 10 : 3 : 3 : 1 whereas the map of Korpela *et al* (1989) contained fragments of 67 : 12 : 9 : 3.4 : 3 : 0.6.

pOG660 corresponded to the Korpela map. This was confirmed with a *Bgl*II–*Xho*I double digest. If the Friedrich map had been correct then this would have resulted in the generation of additional fragments of 8 and 4 kb; whereas the 96 kb plasmid of Korpela would have generated additional fragments of 10 and 8 kb. The latter was in fact the case and indicated that the 96 kb plasmids of Korpela was correct.

Results and discussion

Restriction endonuclease fragment sizes of the Typhimurium SAP.

pOG660 was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sal*I, *Xba*I and *Xho*I. The fragment sizes generated are given in Table 6.1. The exact calculation of the larger fragments (> 30 kb) was made difficult by the decreased sensitivity of control fragments by this method but was achieved after the subtraction of the other fragments.

The XbaI restriction site is at map position 0.

pOG660 contained a single *Xba*I restriction site and so this was placed at map position 0.

Map positions of *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sal*I and *Xho*I

The map positions, with respect to each other, of each restriction fragment is shown in Figures 6.1 and 6.2 and in Table 6.2 together with the fragment bisected by *Xba*I and the fragments generated from a double digest with *Xba*I and other enzymes.

The virulence region extends from 83–91 kb

The virulence region was previously demonstrated to be contained within an 8 kb *Sal*I–*Xho*I fragment (Williamson *et al* 1988) and to encompass a 3.7 kb *Hin*dIII fragment (Norel *et al* 1989c, Poppe *et al* 1991). This region extended from position 83–91 kb on this map.

XhoI restriction sites

A *Xho*I–*Xba*I double digest resulted in the loss of the 15 kb *Xho*I fragment and the generation of two fragments of 3 and 12 kb. It has already been established that there is a *Xho*I restriction site in the virulence region which extended from 83 to 91 kb, the correct orientation of these fragments with respect to *Xba*I was determined to be 3 and 83 kb. The other *Xho*I site was mapped at 36.8 kb. This was determined after double digest analysis of *Xho*I –*Bgl*II, *Xho*I–*Sal*I and *Xho*I – *Hin*dIII (see later).

Table 6.1 Restriction enzyme fragment sizes (kb) of the Typhimurium SAP digested with various enzymes

*Pst*I	*Sma*I	*Hin*dIII	*Eco*RI	*Bgl*II	*Bam*HI	*Sal*I	*Xho*I
		40		66	82		46
			30				34
						20.2	
		15.2				15.1	15
			14			14.5	
10.1	11	12.7	13_D	12			
		9.9	9.9	9		8.8	
	7.4	7.4			7.4	7.5	
	6.2					6.2	
5.8							
5.7							
	5					5	
	4.7						
4.4	4.4						
		4.3				4.3	
		4.2					
4.1	4.1						
	4.0						
3.9		3.9				3.7	
					3.6		
3.4							
	3.2	3.2			3.2	3.2	
3.1							
	3.0					3.0	
						2.8	
	2.7						
	2.5						
2.4	2.4	2.4					
2.3							
	2.2		2.2				
2.1	2.1						
	2.0						
	1.8						
	1.73						
1.6							
1.55							
1.5	1.46						
1.4						1.4	
1.35							
1.32	1.32					1.3	
1.23						1.2	
1.16							
1.03	1.08			1			
0.96	0.96				0.9_D		
0.88							
0.87	0.87	0.1					

D denotes fragment is present as a doublet

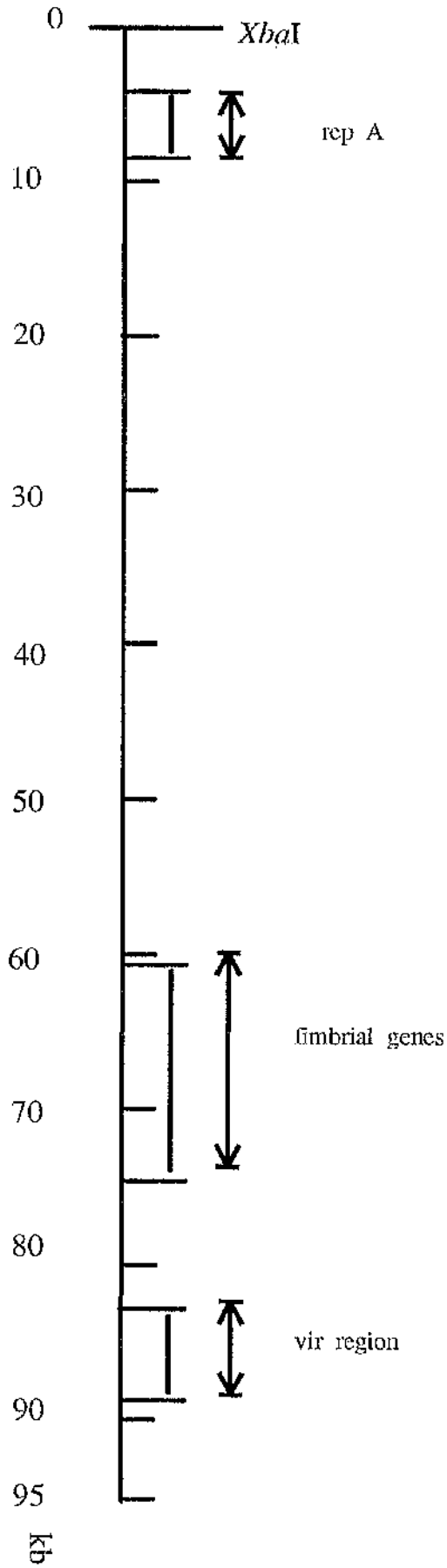


Figure 6.1 Sequenced regions of the plasmid of Typhimurium

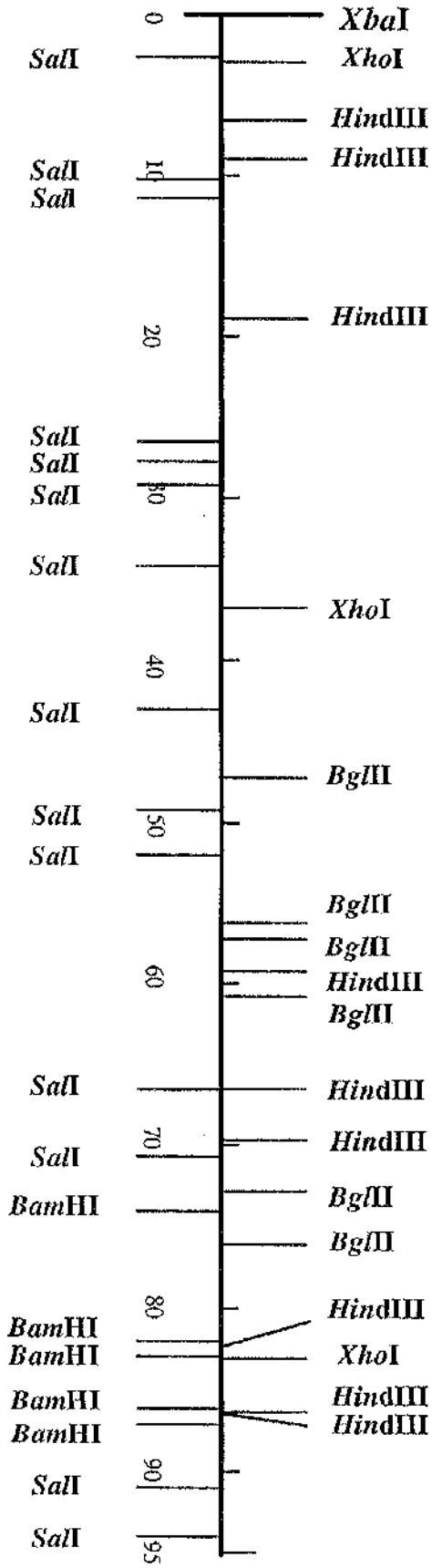


Figure 6.2

*Xba*I, *Xho*I, *Bam*HI, *Bgl*II, *Hin*dIII and *Sal*I restriction sites on the Typhimurium plasmid pOG660.

Table 6.2

Orientation of restriction endonuclease fragments with respect to each other of the Typhimurium plasmid pOG660 after digestion with various enzymes

ENZYME	MAP POSITION (kb)
*Xba*I	0
*Xho*I	15.0*(3,12), 33.8, 46.2
*Hin*dIII	15.2*(6.6,8.6), 2.4, 9.9, 40.2, 7.4, 3.2, 12.7, 3.9, 0.1
*Bgl*II	66.2*(47.2,19), 9.0, 1.0, 3.6, 12.0, 3.2
*Sal*I	3.7*(2.7,1), 7.5, 1.2, 15.1, 1.3, 1.4, 5.0, 8.8, 6.2, 2.8, 14.5, 4.3, 20.2
*Eco*RI	13.0*(6.8,6.2), 13.0, 1.7, 4.3, 1.7, 30.3, 0.9, 13.8, 2.3, 9.8, 4.2
*Bam*HI	82.6*(74.7,8), 7.4, 0.9, 3.2, 0.9

*Fragment bisected by *Xba*I (size of fragments generated)

BamHI restriction sites

A *Bam*HI–*Xba*I double digest generated an additional fragment of 8 kb. This indicated that the *Xba*I site was on the 82 kb *Bam*HI fragment; the resultant fragment of 74 kb remained indistinguishable from its original fragment. Again, because it has already been established that a *Bam*HI site is present in the virulence region, these *Bam*HI sites were at map positions 87 and 74.6 kb. Other *Bam*HI sites were positioned at 82, 82.9 and 86.1 kb.

BglII restriction sites

A *Bgl*II–*Xba*I double digest revealed the *Xba*I restriction site to be present on the 66 kb *Bgl*II fragment and generated two additional fragments, the smaller of which was ~20kb (Figure 6.3). Therefore the accurate map positions of *Bgl*II could not be achieved by this method alone.

A *Xho*I–*Bgl*II double digest generated additional fragments of 7 and 10 kb (Figure 6.3). The absence of *Bgl*II sites in the 15 kb *Xho*I fragment indicated no such sites were present in the region 83–95 kb. Therefore the *Bgl*II site must either be at map position 76 or 73 kb. The 7.4 kb *Bam*HI fragment which extends from 74.6–82 kb was eliminated upon simultaneous digestion with *Bam*HI and *Bgl*II (Figure 6.3) and thus indicated that a *Bgl*II site lay within this region. Therefore, a *Bgl*II restriction site must be present at map position 76 kb. This indicated that the *Xba*I–*Bgl*II double digest fragments were 19 and 47.2 kb. Therefore the *Bgl*II sites were positioned at 47.7, 56.2, 57.2, 60.8, 72.8 and 76 kb.

HindIII restriction sites

A *Hin*dIII–*Xba*I double digest generated additional fragments of 6.6 and 8.6 kb with the concomitant loss of the 15.2 kb *Hin*dIII fragment (Figure 6.3). Thus the map position of this *Hin*dIII fragment was established at 6.6 and 86.4 kb. This orientation was determined by the presence of the *Hin*dIII site within the virulence region (map position 86.4) which meant that the 6.6 kb fragment lay upstream from the *Xba*I site. Therafter *Hin*dIII sites were established at map positions 9, 18.9, 59.1, 66.5, 69.7, 82.4 and 86.3 kb.

Figure 6.3 Double digest REFP's of pOG660

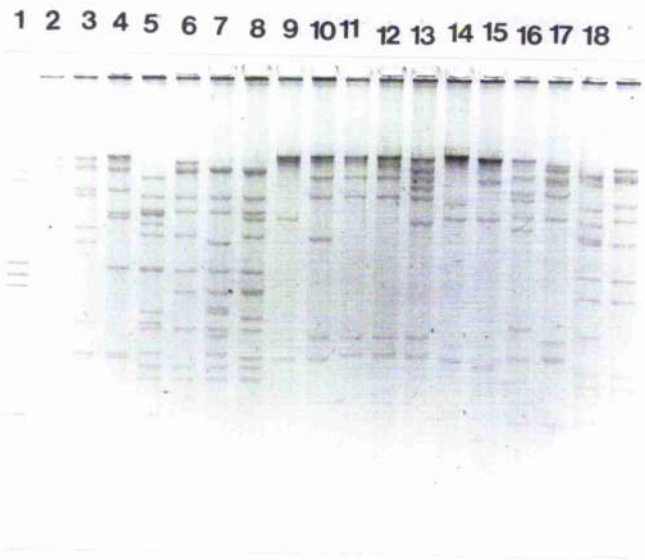
Lane 1. λ *Pst*I 2. *Hin*dIII 3. *Hin*dIII/*Bgl*II 4. *Hin*dIII/*Bam*HI

5. *Hin*dIII/*Sal*I 6. *Sal*I 7. *Bgl*II/*Sal*I 8. *Bam*HI/*Sal*I 9. *Bam*HI

10. *Bgl*II/*Bam*HI 11. *Bgl*II 12. *Xba*I/*Bgl*II 13. *Xho*I/*Bgl*II

14. *Xho*I/*Bam*HI 15. *Xba*I/*Hin*dIII 16. *Xho*I/*Hin*dIII 17. *Xho*I/*Sal*I

18. *Xba*I/*Sal*I



EcoRI restriction sites

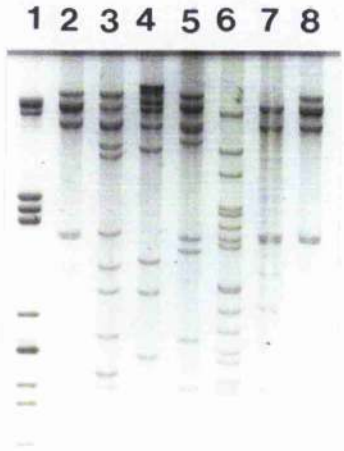
An *Xba*I–*Eco*RI double digest generated fragments of 6.8 and 6.2 kb (with the loss of a 13 kb *Eco*RI fragment). This meant that the fragment was either positioned at 6.2 & 88.2 kb or 6.8 & 88.8 kb. A *Hin*dIII–*Eco*RI double digest (Figure 6.4) resulted in the elimination of the 2.4 kb *Hin*dIII fragment (map position 6.6–9.0) therefore this fragment must contain an *Eco*RI site. Thus, the correct position of the 13 kb *Eco*RI fragment with respect to *Xba*I was 6.8 and 88.8 kb. Thereafter the *Eco*RI sites were established at map positions 19.8, 21.5, 25.8, 27.4, 57.8, 58.7, 74.8 and 84.6 kb.

SalI restriction sites

A *Sal*I – *Xba*I double digest (Figure 6.3) resulted in the loss of the 3.7 kb *Sal*I fragment and the generation of fragments of 2.7 and 1 kb. These were positioned at 2.7 and 94 kb and the other *Sal*I fragments were established at map positions 10.2, 11.5, 26.6, 27.8, 29.1, 34.1, 42.9, 49.1, 51.9, 66.4 and 70.7 kb. The positions of these fragments did not entirely correspond to those reported by Tinge and Curtiss 1990, who indicated that immediately upstream fron the 3.7 kb *Sal*I fragment were fragments of 1.2 and 7.5. However, the 7.5 kb *Sal*I fragment contained a *Xho*I site and therefore must lie adjacent to the 3.7 kb fragment (in order to encompass the *Xho*I site at 3.0 kb). Similarly Tinge and Curtiss ordered the fragments between 27.8 and 42.9 kb as 8.8, 1.3, 5 kb however the 8.8 kb fragment also encompassed a *Xho*I site and therefore the order of the fragments should be 1.3, 5, 8.8 kb. Therefore the 8.8 kb fragment must be positioned from 34.1–42.9 kb and the 5 and 1.3 kb fragments downstream. It must be noted that the restriction fragment sizes in the map of Tinge and Curtiss (1990) were not actually given; they were estimated from the diagram therefore the fragment orientations may be subject to variation.

Figure 6.4 Double digest REFP's of pOG660

Lane 1. λ *Pst*I 2. *Eco*RI 3. *Hin*dIII/*Eco*RI 4. *Hin*dIII 5. *Xho*I/*Eco*RI 6. *Sma*I 7. *Sma*I/*Eco*RI 8. *Eco*RI



Once these restriction sites were identified, the positions of *Pst*I and *Sma*I sites were determined. Obviously, the large number of small fragments generated by these enzymes meant that the exact position of all fragments was not feasible (without sequence analysis of the plasmid). However, it was possible to identify regions which contained small fragments < 1 kb but not the precise location of such fragments.

The identification of *Sma*I sites on the Typhimurium plasmid pOG660

With the previously established positions of *Bam*HI, *Bgl*II, *Hin*dIII, *Xho*I and *Sal*I as a template (see previously), the first step in the determination of the *Sma*I restriction sites was to insert those already identified by sequence analysis, both by Friedrich *et al* (1993) and by Gulig *et al* (1990). The virulence region was contained within an 8 kb *Sal*I–*Xho*I fragment which had a map position of 83–91 kb. A 6.2 kb region within this region was notable for the absence of *Sma*I restriction sites. Hence the virulence region was shown to be contained on a larger *Sma*I fragment – either 11.0, 7.4 or 6.2 kb. A *Xho*I–*Sma*I double digest revealed the 11 kb fragment to be absent, replaced by a slightly smaller fragment (~10 kb) and also the 4 kb fragment to be absent, with two additional fragments generated (2.0 and 1.95 kb). Therefore because the virulence region encompasses a *Xho*I site it must also be contained on the 11 kb *Sma*I fragment.

Positioning of the 11 kb *Sma*I fragment

This fragment encompassed the entire virulence region. Its position in the plasmid map was achieved with the use of double digests.

A *Xho*I–*Sma*I double digest already revealed the *Xho*I site to intersect the 11 kb fragment such that it generated two fragments of 10.2 and 0.8 kb. Therefore the fragment must either be positioned from 82.8–93.8 or 73.8–83.8 in order that it encompass the *Xho*I site at position 83 kb).

A *Sma*I - *Bam*HIdouble digest revealed the 3.2 kb *Bam*HI (map position 82.9–86.1 kb) fragment remained intact (i.e. it contained no *Sma*I sites). This confirmed the 11 kb *Sma*I fragment to extend from map position 82.8- 93.8.

Two *Sma*I fragments were however eliminated : 11 and 2.4 kb with one visible additional fragment of 6.8 kb produced. The 2.4 kb *Sma*I fragment must therefore encompass the *Bam*HI site at 82 kb. The only other *Bam*HI site it could surround was the one at 74.6 however sequence data revealed the *Sma*I fragment at this site to be >3.2 kb. Therefore the 2.4 kb *Sma*I fragment extends from map position 80.4–82.8 kb.

The REFP's of both *Sma*I and *Bam*HI contained a fragment of 7.4 kb. A fragment this size was also present after a double digest with these enzymes which means that either the 7.4 kb *Bam*HI fragment contains no internal *Sma*I restriction sites and is itself contained within a larger *Sma*I fragment or conversely the 7.4 kb *Sma*I fragment is contained within a larger *Bam*HI fragment. It has already been established that there are *Sma*I sites at map positions 74.6 and 80.5 kb thereby disrupting the 7.4 kb *Bam*HI site. In addition the only *Sma*I fragment that could possibly contain the 7.4 kb *Bam*HI fragment is the 11kb which has already been demonstrated to encompass the virulence region. Therefore the 7.4 kb *Sma*I fragment must be postioned within the 74 kb *Bam*HI fragment.

A *Sma*I–*Bam*HI double digest allowed the position of *Sma*I restriction sites in and around *Bam*HI sites to be determined (excluding a section 74.6–80.4 which shall be dealt with later, but probably consists of a few small fragments).

The work of Friedrich *et al* (1993) concerned a 14 kb section of the Typhimurium plasmid that extended from the *Bam*HI site downstream to a *Bgl*II site (74.6 and 60.8 kb respectively on my map). This sequence contained *Sma*I sites at positions 71.4, 70.5, 67.3, 65.6, 64.5, 64.4 and 63.1 and indicated fragments of 0.9, 3.2, 1.7, 1.1, 0.1, and 1.3 kb were present. This region contained a fragment ≥3.2 kb upstream from 70.4 and ≥2.4 kb downstream from 63.1 kb. The 3.2 kb *Bgl*II fragment (72.8–76 kb) was climinated after double digestion with *Sma*I and therefore contained an internal *Sma*I restricton site. Therefore the *Sma*I fragment that extended from 71.4 kb was ≥ 3.2 kb but < 4.6 kb. There were four

possible fragments that could have applied to this situation 3.2, 4.0 kb, 4.1 kb and 4.4 kb. The 4.0 kb fragment was eliminated during a *Xho*I double digest therefore this fragment could not be at this position. In addition the *Sma*I fragment would remain intact after double digestions with both *Hin*dIII (contained within the 12.7 kb *Hin*dIII fragment) and *Sal*I (contained within the 20 kb *Sal*I fragment). The 4.4 kb *Sma*I fragment, although intact after *Hin*dIII digestion (Figure 6.5), was eliminated upon digestion with *Sal*I. Therefore the 4.4 kb *Sma*I fragment must be positioned elsewhere on the map. The 4.1 kb *Sma*I fragment was not eliminated after digestion with *Bgl*II. Therefore the only fragment that could be positioned upstream from 71.4 kb was 3.2 kb and indicated that this fragment was present as a triplet in a *Sma*I REFP. Thus, the 3.2 kb fragment extends from 71.4–74.6 kb.

The *Sma*I fragment that extended downstream from 63.1 kb was > 2.3 kb. The 3.6 kb *Bgl*II fragment (57.2–60.8) was eliminated after digestion with *Sma*I and therefore must contain a *Sma*I site. Thus, the fragment in question must also be < 5.4 kb. The fragments which fell into this category were 2.5, 2.7, 3.0, 4.4, 4.7 and 5.0 kb. This fragment was contained within the 7.4 kb *Hin*dIII fragment (59.1–66.5). *Sma*I sites have already been established for part of this region and correspond to *Sma*I fragments of 1.1, 0.1 and 1.2 kb. Extraction of the 7.4 kb *Hin*dIII fragment and subsequent redigestion with *Sma*I generated fragments of ~2.4, 1.7, 1.2, 1.2 and 0.8 kb (Figure 6.6). Therefore the fragment that extends downstream from 63.1 must be 2.5 kb and is positioned from 60.6–63.1 kb. Thereafter, the region 59.1–60.6 must contain *Sma*I fragments too small to detect by this method (e.g. < 0.5 kb).

Orientation of the *Sma*I fragment around the *Xba*I site

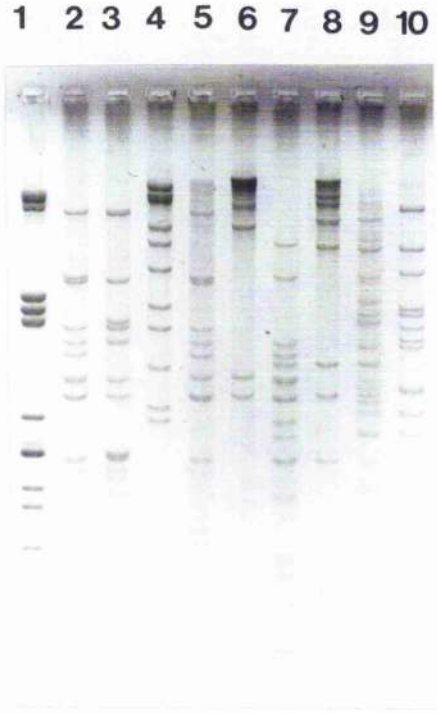
A *Sma*I–*Xba*I double digest resulted in the elimination of a 3.2 kb fragment (which was present as a doublet) and the generation of fragments of 2.0 and 1.2 kb. there were two possible orientations of this *Sma*I fragment

1) map positions 2.0 and 93.8

2) map positions 1.2 and 93.

Figure 6.5 Double digest analysis of pOG660

Lane 1. λ *Pst*I 2. *Pst*I 3. *Pst*I/*Sal*I 4. *Sal*I 5. *Pst*I/*Bgl*II 6. *Bgl*II 7. *Pst*I/*Hin*dIII 8. *Hin*dIII 9. *Sma*I/*Hin*dIII 10. *Sma*I



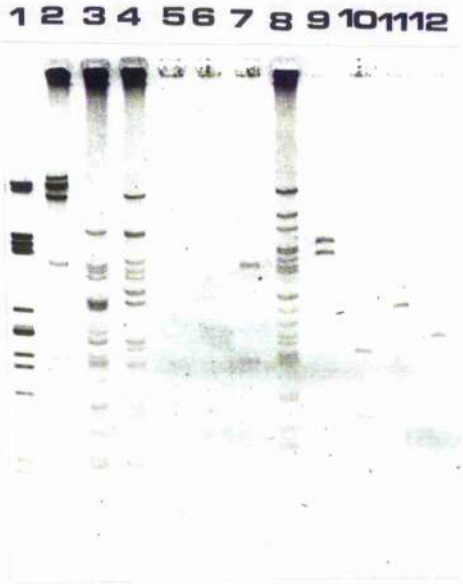
The 11 kb *Sma*I fragment has previously been demonstrated to extend to map position 93.8, therefore the 3.2 kb fragment must be situated at map positions 93.8 and 2.0.

Published work on the RepA (incompatibility) region of the plasmid (which on this map extended from 4.5–8.6 kb and incorporated the *Hin*dIII fragment situated at 6.9 kb) revealed *Sma*I fragments to be present at map positions 6.1 and 7.2 kb. This was confirmed by the extraction of the 2.4 kb *Hin*dIII fragment (6.6–9.0 kb) and redigestion with *Sma*I which generated two fragments of 0.2 and 2.2 kb (Figure 6.5). Extraction of the 9.9 kb *Hin*dIII fragment (9–18.9) and redigestion with *Sma*I generated two fragments of ~ 4.4 and 5.4 kb. Therefore both these fragments must have arisen from larger *Sma*I fragments; either 7.4, 6.2 or 5.0 kb. The 6.2 kb *Sma*I fragment was not possible at this position because a *Sma*I–*Sal*I double digest revealed a fragment of this size. Since both enzymes themselves generated a fragment of this size it meant that either the 6.2 kb *Sma*I fragment contained no *Sal*I sites or conversely the 6.2 kb *Sal*I fragment contained no *Sma*I sites. If the 6.2 kb *Sal*I fragment contained no *Sma*I sites then it would be part of a larger *Sma*I fragment i.e. either 11.0 or 7.4 kb. However both of these fragments were demonstrated to contain *Sal*I sites. Therefore, the 6.2 kb *Sma*I fragment did not contain any *Sal*I sites. The 7.4kb *Sma*I contained *Sal*I fragments and therefore could not be positioned upstream from 7.2 kb (because there were *Sal*I sites at 10.2 and 11.4 kb). Therefore, the 5.0 kb *Sma*I fragment must be positioned within the *Hin*dIII fragment situated at 9.0–18.0 kb and cross the *Sal*I sites at 10.2 and 11.4 kb. For this reason it must extend from 9.5–14.5 kb.

Similarly, the 6.2 kb *Sma*I fragment was positioned from 14.5–20.7 kb. This was the only option at this position; this fragment contained a *Hin*dIII site but no *Sal*I sites and extraction of the 9.9 kb *Hin*dIII fragment and redigestion with *Sma*I was shown to generate two fragments of approx 5.5 and 4.5 kb (Figure 6.6).

Figure 6.6 Double Digest REFP's of pOG660 and fragment extraction and redigestion analysis

Lane 1. λ *Pst*I 2. *Eco*RI 3. *Pst*I/*Eco*RI 4. *Pst*I 5. 15kb *Hin*dIII fragment redigested with *Pst*I 6. 12kb *Hin*dIII fragment redigested with *Pst*I 7. 7.4kb *Hin*dIII fragment redigested with *Pst*I 8. *Sma*I 9. 9kb *Hin*dIII fragment redigested with *Sma*I 10. 7.4kb *Hin*dIII fragment redigested with *Sma*I 11. 3.2 kb *Hin*dIII fragment redigested with *Sma*I 12. 2.4 kb *Hin*dIII fragment redigested with *Sma*I



4.0 kb *Sma*I fragment

A *Xho*I–*Sma*I double digest resulted in the elimination of the 4.0 kb *Sma*I fragment and the generation of two 2.0 kb fragments. This meant that the fragment encompassed either the *Xho*I site at position 3.0 or 36.8 kb. Therefore the fragment would either be positioned at 1–5 kb or 34.8–38.8 kb. Since a *Sma*I site has already been established at map position 1.2 kb the 4.0 kb *Sma*I fragment must extend from 34.8–38.8 kb.

4.7 kb *Sma*I fragment

The 4.7 kb *Sma*I fragment was eliminated by double digestions with both *Bgl*II and *Sal*I. The only site at which this could occur was the *Bgl*II site at 47.2 kb and *Sal*I site at 52 kb. A *Sma*I–*Bgl*II double digest generated two additional fragments of 2.4 and 2.3 kb. Therefore the exact orientation of the 4.7 kb *Sma*I fragment around the *Bgl*II site in unconfirmed however it was either 44.8–49.5 or 44.9–49.6 kb.

Of the available regions left in the map :

20.7 - 34.8

38.8 - 44.8

49.5 - 54.9

63.2 - 67.3

the only position the 7.4 kb *Sma*I fragment could be was somewhere in the region 20.7–34.2. This was the only appropriately sized region that resulted in the elimination of the 7.4 kb *Sma*I fragment by both *Hin*dIII and *Sal*I in their respective double digests, and also result in the elimination of the 5.0 kb SalI fragment (29.2–34.2). The elimination of the 7.4, 5.0, 4.7 and 4.4 kb *Sma*I fragments after digestion with *Sal*I resulted in the generation of fragments of 4.6, 4.2, 3.8 and 3.1 kb. The 3.1 fragment was generated as a result of digestion of the 5.0 kb *Sma*I fragment (9.5–14.5). Similarly the 4.6 kb fragment was a product of 4.7 kb *Sma*I fragment (44.8–49.5). The 3.8 kb fragment originated from the 4.4 kb *Sma*I fragment (30.4 - 34.8). Therefore the 4.2 kb fragmentwas generated from the 7.4 *Sma*I fragment. This meant that the 7.4 kb *Sma*I fragment was positioned either

from 26–33.4 kb or 22.6–30 kb. As will be demonstrated later, the actual position of this fragment was 22.6–30 kb.

The 4.1 kb *Sma*I fragment was contaitned within the region 38.3–43 as it was neither eliminated by double digestion with *Sal*I or *Hin*dIII. It extended from 38.8–42.9.

This indicated that there were only two possible positions at which the 4.4 and 3.0 kb *Sma*I fragments could occur. In order for them to be eliminated upon digestion with *Sal*I, these fragments must cross the *Sal*I sites at positions 34.2 and 52 kb. The presence of a *Hin*dIII site at map position 66.5 as well as *Sal*I ruled this site out. As mentioned previously, *Sal*I intersected the 4.4 kb *Sma*I fragment to produce a fragment of 3.8 kb. This meant that this S*ma*I fragment extended from 30.4–34.8. (If the 3.0 kb *Sma*I fragment had been at this position there would have been an additional 2.4 kb fragment generated after a *Hin*dIII–*Sal*I double digest. This was not the case).

Therefore the 3.0 kb *Sma*I fragment was positioned from 50.5–53.5 kb (A *Sma*I–*Sal*I double digest generated two fragments of ~1.5 kb)

The 2.7 kb *Sma*I fragment was undisturbed by *Sal*I or *Hin*dIII and therefore lay in the region 63.2–66.5 or 66.5–67.3.

The 1 kb *Bgl*II fragment (map position 56.2–57.2) was eliminated by double digestion with *Sma*I and generated at least two fragments below 0.7 kb. Therefore there must be a *Sma*I restriction site in this region. Due to the presence of a *Sma*I site at 57.3 this region must contain small *Sma*I fragments < 0.8kb. The two remaining *Bgl*II sites at map positions 56.2 and 76 kb must be surrounded by *Sma*I fragments of 2.1 and 2.2 kb. Both fragments must have been cut into roughly equal sizes; no extra fragments >1 kb were detected after double digestion. Therefore it was not known which *Sma*I fragment was at which *Bgl*II site and as a result their postions may be interchanged. For the purposes of mapping the 2.2 kb fragment has been positioned from 74.9 - 77.1 and the 2.1 kb from 54.9 - 57kb.

The 2.0 and 1.8 kb *Sma*I fragments were eliminated by *Hin*dIII digestion (Figure 6.5). There were only two possible *Hin*dIII sites at which they could occur : 9.0 and 59.1 kb. A *Hin*dIII–*Sma*I double digest generated additional fragments of

1.8 and 1.5 kb and implied that the 2.0 kb *Sma*I fragment was cut by *Hin*dIII and produced fragments of 1.8 and 0.2 kb; the 1.8 kb *Sma*I fragment generated fragments of 1.5 and 0.3 kb when digested with *Hin*dIII. The only orientation for these sites was for the 2.0 kb *Sma*I to extend from 7.2–9.2 kb and the 1.8 kb *Sma*I from either 57.6–59.4 or 58.8–60.6.

The *Sma*I restriction site of the Typhimurium plasmid pOG660 are shown in Figure 6.7 and Tables 6.1 and 6.2 .

Identification of *Pst*I restriction sites on the Typhimurium plasmid pOG660

Sequence data of the virulence region (Gulig *et al* 1990), which was situated at map position 83–91 kb, revealed *Pst*I restriction sites at map position 87.9 and 88.7 kb and demonstrated the virulence region to be located on a *Pst*I fragment >5.6 kb downstream from 87.9 kb. Three potential fragments were therefore possible at this position : 10.1, 5.8, 5.7 kb. This fragment also incorporated the 3.2 and 0.9 kb *Bam*HI fragments (82.9–86.1, 86.1–87 kb). A *Bam*HI–*Pst*I double digest revealed the 10.1 and 5.8 kb *Pst*I fragments to remain intact. Therefore, the virulence region was contained on the 5.7 kb *Pst*I fragment which extended from 82.2–87.9 kb.

The published sequence data of Friedrich *et al* (1993) demonstrated *Pst*I restriction sites at map positions 71.3, 70.9, 69.8, 69.0, 68.3, 66.7 and 62.6 kb and revealed fragments of 0.4, 1.1, 0.8, 0.7, 1.6 and 4.1 kb to be present. Therafter, downstream from 62.2 lay a fragment >1.8 kb and upstream from 71.3 was a fragment >3.3 kb.

A *Pst*I–*Bgl*II double digest resulted in the elimination of three *Pst*I fragments : 3.4, 2.1 and 1.6 kb (Figure 6.5). These fragments each encompassed a *Bgl*II site. Therefore, the 3.4 kb *Pst*I fragments extended upstream from 71.3 - 74.7 kb and thus incorporated the *Bgl*II site at 72.8kb.

Downstream from 62.6 therefore continued with the 2.1 kb *Pst*I fragment (60.5–62.6 kb) and incorporated the *Bgl*II site at map position 60.8 kb. This was

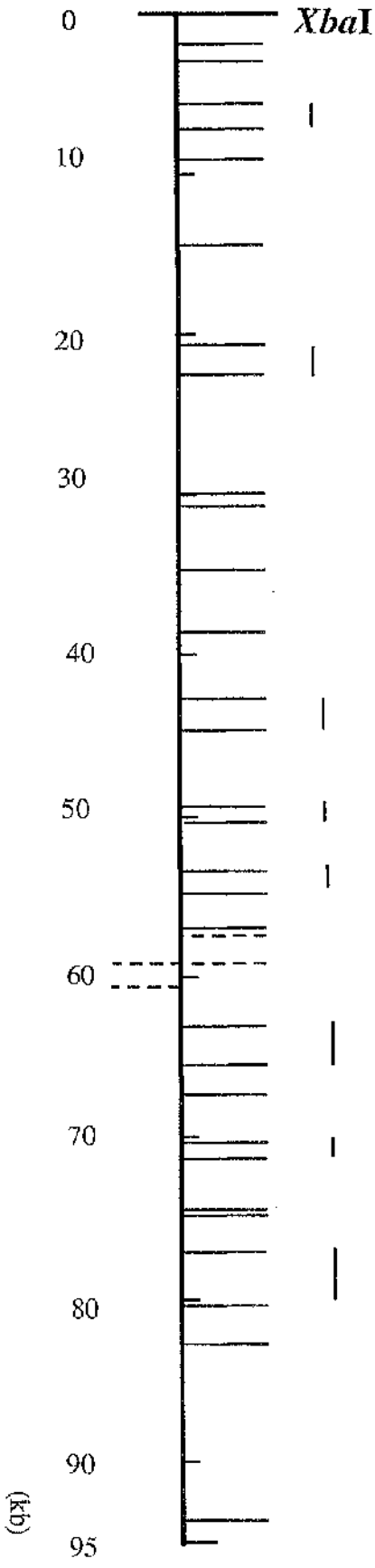


Figure 6.7 Identification of *Sma*I restriction sites on the Typhimurium SAP

——— Exact fragments unconfirmed at these regions

– – – Restriction sites at either of these positions

Table 6.3 ***Sma*I restriction sites on the Typhimurium SAP**

Map position (kb)
2
3.1
5.8
⇓
7.2
9.2
9.5
14.5
20.7
⇓
22.6
30
30.4
34.8
38.8
42.9
⇓
44.8
49.5
⇓
50.5
53.5
⇓
54.9
57
57.6 or 59.4
59.4 or 60.6
60.6
63.1
⇓
65.6
67.3
70.5
⇓
71.4
74.6
74.9
77.1
⇓
80.4
82.8
93.8

⇓ : *Sma*I fragments at these intervening regions remain unconfirmed although are <1.8kb.

Table 6.4 Map positions of *Sma*I Fragments of the Typhimurium SAP

*Sma*I fragment (kb)	Map position
11	82.8 – 93.8
7.4	22.6 – 30
6.2	14.5 – 20.7
5.0	9.5 – 14.5
4.7	44.8 – 49.5
4.4	30.4 – 34.8
4.1	38.8 – 42.9
4.0	34.8 – 38.8
3.2	67.3 – 70.5
3.2	71.4 – 74.6
3.2	93.8 – 2.0
3.0	50.5 – 53.5
2.7	within 63.2 – 67.3
2.5	60.6 – 63.1
2.4	80.4 – 82.8
2.2	74.9 – 77.1
2.1	54.9 – 57
2.0	7.2 – 9.2
1.8	within 57.6 – 59.4 or 58.8 – 60.6
1.73	65.6 – 67.3
1.46	*
1.32	*
1.08	*
0.96	*
0.87	70.5 – 71.4

* Exact fragment position unconfirmed.

confirmed by extraction of the 7.4 kb *Hin*dIII fragment (59.1–66.5 kb) and redigestion with *Pst*I. This generated fragments of 4.1, 2.1 and 1.2 kb and thus also demonstrated a 1.2 kb fragment extended from 59.3–60.5 kb.

Extraction of the 15 kb *Xho*I fragment (83–3 kb) followed by redigestion with *Pst*I generated fragments of ~5.8, 4 and 2.4 kb. Because of the *Pst*I sites already established at map position 82.2 and 87.9, this 5.8 kb fragment was not part of a larger (i.e 10.1 kb) fragment and indicated the 5.8 kb *Pst*I fragment extend upstream from the virulence region from 88.7 - 94.5 kb. The *Xba*I site bisected the 2.4 kb *Pst*I fragment in such a way that it generated two fragments of 2.3 and 0.1 kb after double digestion. therefore, the 0.1 kb portion of this fragment must extend from 94.9 - 95 kb (the 2.3 kb fragment could not be positioned on this side of the *Xba*I site because of the *Pst*I site at 94.5 kb). Therefore the 2.4 kb *Pst*I fragment extended from 94.9 - 2.3 kb.

Sequence data of the RepA gene (map position 4.5–8.8 kb) revealed *Pst*I sites at 8.0 and 8.6 kb. In addition this region was shown to be contained on a *Pst*I fragment >3.9 kb which extended downstream form 8.0 kb. this corresponded to the 4.4 kb *Pst*I fragment which was therefore positioned at 3.6 - 8.0 kb.

The 3.4 kb *Pst*I fragment was actually present as a doublet. Both fragments were eliminated after double digestion with *Bgl*II and *Sal*I respectively (Figure 6.5). One of these fragments has already been identified at map position 71.3–74.7 kb. The only other position at which the second 3.4 kb *Pst*I fragment could be situated was at the *Bgl*II site 47.2, *Sal*I site 49.2 kb. The fragment must encompass both these sites.

Extraction of the 6.2 kb *Sal*I fragment (43–49.2) and redigestion with *Pst*I generated fragments of 2.4, 1.4, 1.4 kb. The 2.4 kb double digest product must have arisen from the 3.4 kb *Pst*I fragment present in this region and must be positioned from 46.8–49.2 in order that the 3.4 kb *Pst*I fragment be situated from 46.8 - 50.2 kb. The region 43–46.8 consisted of fragments <1.4 kb.

The 2.3 kb *Pst*I was eliminated after digestion *Hin*dIII (Figure 6.5) and must therefore encompass a *Hin*dIII site. The only sites at which this fragment could occur were at 9.0 or 59.1 kb. A *Pst*I–*Eco*RI double digest resulted in the elimination of the 2.3 kb *Pst*I fragment. No *Eco*RI sites were present around the 9.0 kb region such that a 2.3 kb fragment would encompass it. Therefore the 2.3 kb *Pst*I fragment must extend from 58.2–60.5 kb.

The 3.1 kb *Pst*I fragment contained no *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sal*I or *Xho*I restriction sites (Figures 6.5, 6.6, 6.8). Extraction of the 5.0 kb *Sal*I fragment (29.2–34.2 kb) and redigestion with *Pst*I generated fragments of 2.0 and 1.0. Therefore since the 3.1 kb fragment must be contained within this region it cannot be situated here. It therefore could be situated in the following regions :

21.6(*Pst*I)–25.8(*Eco*RI)

36.8(*Xho*I)–43(*Sal*I)

52(*Sal*I)–56.2(*Bgl*II)

76(*Bgl*II)–80.9(*Pst*I)

The 1.50 kb *Pst*I fragment contained a *Bam*HI site. The only position this fragment could occur at was 80.7–82.2 kb which incorporated the *Bam*HI site at 82 kb.

The 3.9 kb *Pst*I fragment did not contain any *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII or *Xho*I sites. It did however contain *Sal*I site(s) (Figure 6.5). Extraction of the 9 kb *Bgl*II fragment (47.2–56.2) and redigestion with *Pst*I generated fragments of ~3.2, 1.7 and 1.1 kb (Figure 6.9). A *Pst*I site has already been demonstrated at map position 50.2 and so the 3.2 kb double digestion product must have resulted from this. Therefore the 3.9 kb *Pst*I fragment cannot lie in the region 47.2–56.2. This region must contain, in addition to the 3.4 kb fragment, fragment <1.7 kb.

Therefore, the 3.9 kb *Pst*I fragment could be situated in the following regions 27.4(*Eco*RI)–36.8(*Xho*I) and must cross the *Sal*I sites at 27.8, 29.2, 34.2 kb. The 1.4 kb *Sal*I fragment remained intact after double digestion and therefore did not contain an internal *Pst*I site

or 36.8(*Xho*I) - 46.8(*Pst*I) and must cross the *Sal*I site at 43 kb.

Figure 6.8 Double digest REFP's of pOG660

Lane 1. λ *Pst*I 2. *Pst*I 3. *Pst*I/*Hin*dIII 4. *Hin*dIII 5. *Xho*I/*Pst*I/*Hin*dIII 6. *Xho*I 7. *Bgl*II 8. *Pst*I/*Bgl*II 9. *Xho*I/*Pst*I/*Bgl*II 10. *Xho*I/*Pst*I 11. *Sal*I 12. *Pst*I/*Sal*I 13. *Xho*I/*Pst*I/*Sal*I

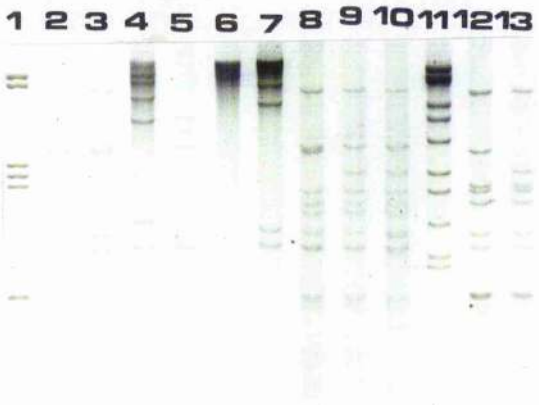


Figure 6.9

Extracted *Bgl*II fragments of pOG660 redigested with *Pst*I and *Sma*I.

Lane 1. λ PstI 2. pOG660/BglII 3. pOG660/PstI 4. 66kb/PstI 5. 12kb/PstI 6. 9kb/PstI 7. pOG660/SmaI 8. 66kb/SmaI 9. 12kb/SmaI 10. 9kb/SmaI

It was not possible to determine the position of the fragments smaller than 1.5 kb. However, the 1.55 and the 1.35 fragments both contained *Sal*I sites and so must encompass one of these sites.

The 2.4 kb *Pst*I fragment appeared to be present as a doublet. One of the fragments was shown to be positioned from 94.9–2.3 kb. The other fragment did not contain any internal *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sal*I or *Xho*I restriction

sites. Therefore its position on the plasmid map was subject to the same criteria as the 3.1 kb fragment.

Thus, although it was not feasible to map the restriction sites of the smaller *Pst*I fragments, the positions of the larger (>1.6 kb) fragments was determined.

Extraction of the 11 kb *Sma*I fragment and redigestion with *Pst*I confirmed the presence of fragments of around 5.8 kb (Figure 6.10). These consisted of the 5.7 kb fragment in its entirity and part of the adjacent 5.8 kb fragment.

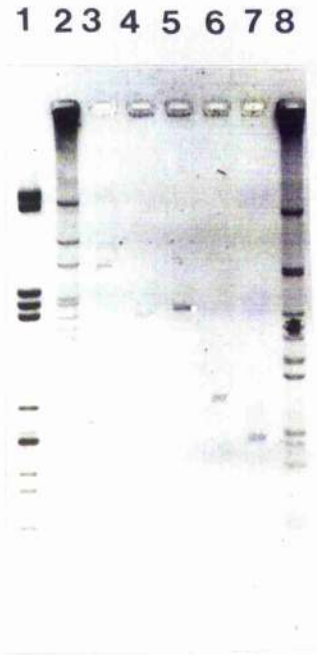
The *Pst*I restriction sites of the Typhimurium plasmid pOG660 are shown in Figure 6.11 and Tables 6.5 and 6.6.

One of the main reasons that the plasmid of Typhimurium was chosen, over the other SAP's, to map *Pst*I and *Sma*I restriction sites was its size. At 95 kb it is the largest of the SAP's.

The question of plasmid evolution has never been properly addressed – in particular the question as to whether the plasmid evolves by DNA loss (from Typhimurium to Enteritidis) or DNA gain (from Enteritidis to Typhimurium). The fact that one small section of the plasmid remains conserved between SAP's prompts the question as to how many other regions are conserved and where do they occur on the plasmid. This study was designed to identify *Pst*I and *Sma*I restriction endonuclease sites on the Typhimurium SAP with a view to further analysis of certain fragments by the construction of probes and hybridization to Southern blots.

Figure 6.10 ***Sma*I fragments extracted and redigested with *Pst*I**

Lane 1. λ PstI 2. pOG660/SmaI 3. 11kb/PstI 4. 7.4kb/PstI 5. 6.2kb/PstI 6. 3.2kb/PstI 7. 3.0kb/PstI 8. pOG660/PstI



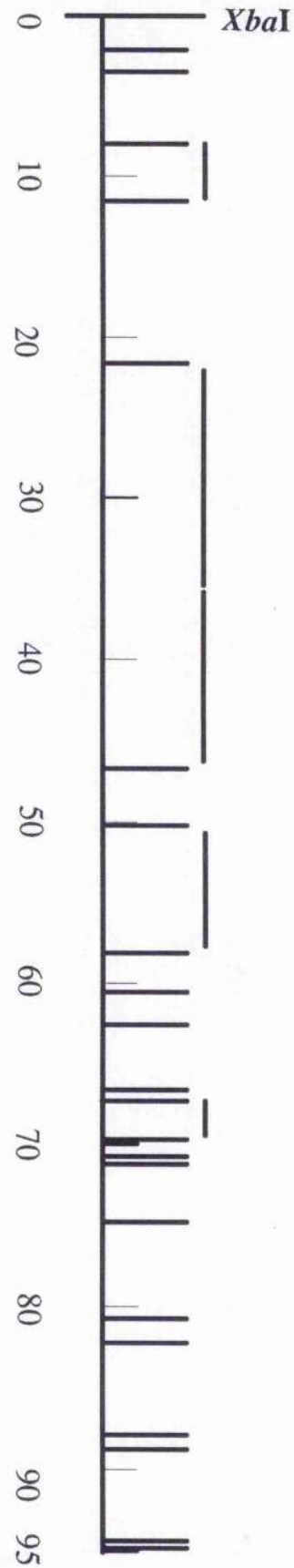


Figure 6.11

Identification of *Pst*I restriction sites on the Typhimurium plasmid

| Exact position of fragments in these regions unconfirmed

Table 6.5 ***Pst*I restriction sites on the Typhimurium plasmid pOG660**

*Pst*I fragment (kb)	Map position
10.1	11.5 – 21.6
5.8	88.7 – 94.5
5.7	82.2 – 87.9
4.4	3.6 – 8.0
4.1	62.6 – 66.7
3.9	within 27.4 – 36.8 or 36.8 – 46.8
3.4	71.3 – 74.7
3.4	46.8 – 50.2
3.1	within 21.6 – 25 36.8 – 43 or 52 – 56.2 76 – 80.9
2.4	94.9 – 2.3
2.4	As for 3.1 kb
2.3	58.2 – 60.5
2.1	60.5 – 62.6
1.6	66.7 – 68.3
1.55	*
1.4	*
1.35	*
1.32	*
1.23	*
1.16	*
1.03	69.8 – 70.9
0.96	*
0.88	*
0.87	*

*exact position not determined

Table 6.6 Map positions of the *PstI* restriction sites on the Typhimurium plasmid pOG660

Map position (kb)
2.6
3.6
8.0
⇓
21.6
25.8
⇓
⇓
46.8
50.2
58.2
60.5
62.6
66.7
68.3
69.8
70.9
71.3
74.7
⇓
80.7
82.2
87.9
88.7
94.5
94.9

⇓ Fragments unconfirmed in these regions

***Sma*I restriction fragments common to other SAP's**

The use of double digest analysis and also fragment redigestion enabled the map positions of the majority of *Sma*I sites to be determined. Obviously the exact position of the small fragments (<1.5 kb) was not possible.

An interesting feature of the *Sma*I map was the occurance of fragments 4.4, 4.0, 4.1 and 4.7 kb which lay adjacent to each other. These fragments occur in a region of the plasmid previously uncharacterized – in as much as it lies within the 40 kb *Hin*dIII, 82 kb *Bam*HI, 66 kb *Bgl*II and the 30 kb *Eco*RI fragments.

Fragments of 4.0, 4.1, 4.4 and 4.7 kb were also found in serotypes :

4.7 Abortusovis, Wangata, Bovismorbificans

4.4 Wangata, Gallinarum, Pullorum, Bovismorbificans, Dublin(variant)

4.1 Abortusovis, Wangata, Gallinarum, Pullorum, Dublin(variant)

4.0 Abortusovis, Wangata

A *Sma*I fragment of 4.7 kb (which was positioned immediately downstream from the *rep*B region on the Typhimurium plasmid) was present in the plasmids of Abortusovis, Wangata and Bovismorbificans. The plasmid of Abortusovis is one of the smallest SAP's (along with Choleraesuis) at 50 kb. If this fragment is indeed present in Abortusovis as well as Wangata and Bovismorbificans, then it would suggest that these plasmids may have arisen via the same process – be it deletion, rearrangement or insertion of DNA.

A 2.9 kb *Sma*I fragment was present in the REFP of the plasmid of Choleraesius but no other SAP's. The plasmid of Choleraesuis shows a most similarity to the plasmd of Enteritidis (68%–*Sma*I, 60%–*Pst*I) which would suggest that these plasmids evolved similarly. Their size supports this 50 and 54 kb respectively. The 3.0 kb *Sma*I fragment was not present in the plasmid of Enteritidis (in this form) therefore either the fragments are not the same or the plasmid of Choleraeraesuis did not evolve from that of Enteritidis.

A 5.0 kb *Sma*I fragment (which was demonstrated to be situated immediately upstream from the *rep*A gene in Typhimurium) was found to be present in the SAP's of Wangata and Enteritidis. If the plasmid is evolving by the

loss of DNA (as we would expect–from the point of view of genetic burden etc) then either the plasmid of Enteritidis has evolved from a separate lineage than the other SAP's (It would be extremely unlikely for a plasmid to lose a piece of DNA only to regain it again later) or this 5.0 kb fragment is not in fact the same.

*Pst*I restriction fragments common to other SAP's

The majority of *Pst*I restriction fragments were successfully mapped. The position of three fragments remain unconfirmed; the 3.9 kb fragment was possible at two separate sites whilst the 3.1 and the 2.4 kb fragments were possible at four sites. These undetermined sites all lay within the region previously only identified as part of larger fragments e.g. 40 kb *Hin*dIII, 66 kb *Bgl*II, 30 kb *Eco*RI.

The *rep*A gene was demonstrated to be present on the 4.4 kb *Pst*I fragment. A fragment of this size was present in all the SAP's except those of Abortusovis and Dublin. pOG683 (Dublin-Variant plasmid) possessed a fragment of this size.

The 4.1 kb fragment was unique to the Typhimurium plasmid. This fragment was also present in pOG690, an Enteritidis plasmid that shows more similarity to Typhimurium and is thought to be an evolutionary intermediate. This fragment was also of interest to probe against other SAP's, to see if it is truly unique or if it is present in the plasmids in another form.

A fragment of 2.3 kb was present in the plasmids of Typhimurium, Wangata, Pullorum, Gallinarum and Bovismorbificans. This fragment appears to be present in plasmids of around 90 kb in size but not in those plasmids smaller and indicates that they may have arisen via the same molecular event.

There was not as much fragment similarity with *Pst*I between pOG683 and othe SAP's as there was with *Sma*I. However, with the combined results of both enzymes the molecular development of the serotype-associated plasmids of the salmonellae can be investigated further.

The results of *Pst*I and *Sma*I restriction site analysis has demonstrated more potential similarity between the plasmids of different serotypes. The identification

of fragments of similar size within different plasmids and within non-virulence associated regions corroborates previous suggestions, that a family of related plasmids exist, rather than the suggestion that the plasmids evolved by the transposition of a virulence determinant as suggested by Williamson *et al* (1988a).

These results emphasise the need for hybridization analysis of these fragments. Of particular interest are the common fragments in the pOG683. Further analysis of these fragments may provide insight into the development of these plasmids and may explain the differences seen in the plasmid of *Salmonella* Dublin which has possibly arisen via an unstable intermediate which resulted from a cointegration event.

Chapter 7

Discussion

The identification of *Salmonella* with regard to clinical epidemiology has relied primarily on phenotypic typing methods such as serotyping (either with or without phage typing), biochemical typing or antimicrobial resistance typing. These methods have proved invaluable both diagnostically and commercially with the rapid identification of an outbreak situation or prevention of spread of disease in the clinical, animal husbandry and food industries. Although such typing methods provide little or no information as to the genotypic relationships that exist within the salmonellae, most clinicians and veterinarians are only concerned with solving the problem of infection at the local level i.e. assigning a "name" to the problem and subsequently how to eliminate it. Thus, although much debate surrounds the correct nomenclature of the salmonellae, especially in this day and age of advanced molecular techniques and bacterial systematics, the practice of identifying salmonellae by name will continue.

The advent of molecular biology greatly enhanced our understanding of the salmonellae. The study of population genetics revealed the salmonellae to be basically clonal. Selander and colleagues (1990) used multilocus enzyme electrophoresis studies to show that the majority of serotypes analysed belonged to a single world-wide clone from which a small number of subclones have arisen by mutation (Beltran *et al* 1988) or recombination (Smith *et al* 1990). Helmuth *et al* (1988) came to the same conclusion based on outer membrane protein patterns and plasmid profile analysis.

The introduction of plasmid analysis enabled a greater understanding of the epidemiology of the salmonellae. Plasmid analysis has provided some solutions to the lack of diversity within serotypes in epidemiological investigations (Platt and Smith 1991). The concept of clonality plays an important, although not always recognised, part in the epidemiology of *Salmonella*. Accordingly, it is important to know the plasmid pool of an organism before conclusions can be made concerning its epidemiology.

The association of virulence plasmids with certain serotypes led to their increased analysis. Different aspects of virulence from outer membrane proteins, pilin proteins, flagellar involvement to resistance to host attack mechanisms have been investigated but only served to emphasise that virulence is a multifactorial

process. The contribution of the *Salmonella* plasmid was undeniable, even if the exact mechanism remained unexplained.

However, many questions remained unanswered. Did the plasmid population reflect the overall situation in the salmonellae? Were the plasmids clonal? Did they evolve at a different rate to the genome? Did the SAP of a host adapted serotype such as Dublin behave any differently than non adapted serotypes? What was the extent of molecular variation among SAP's and lastly what was the extent of molecular relatedness between SAP's?

One of the first discoveries during this study was that the term serotype specific plasmid as introduced by Helmuth in 1985 was a misnomer. The plasmid associated with serotypes Dublin and Enteritidis were identified in strains of . *Salmonella* Rostock and Moscow respectively. Plasmids previously designated as molecular variants of Enteritidis were identified in Blegdam and Moscow. Although these serotypes only vary in the structure of their H–antigens they had previously been shown to exhibit limited sequence homology (Williamson *et al* 1988, Stanley *et al* 1994). It was also demonstrated that these similarities between plasmids were not restricted to Group D serotypes. The plasmid of *Salmonella* Wangata shows a high degree of relatedness to that of Typhimurium both in size (90 kb compared to 54 kb of other Group D serotypes studied) and REFP (~84% similarity). These observations not only prompted the proposal of the term "serotype–associated plasmid" but demonstrated that the levels of similarity between plasmids was not restricted to a particular serogroup. The observation that the plasmid of Wangata (serogroup D) is more closely related to Typhimurium (group B) parallels a recent report (Rankin *et al* 1995) that described a molecular variant of the archetypal Enteritidis plasmid which was more closely related to the reference plasmid of Typhimurium than to Enteritidis itself. Together these results indicated a family of related plasmids associated with but not restricted to serotype.

Restriction analysis of the SAP's has progressed in two different ways dependant on purpose. Early mapping approaches emphasized the spatial relationships between small numbers of fragments whereas approaches designed with epidemiology in mind require a greater information content and employ

enzymes that cleave frequently but with few data available with regard to the spatial relationship between fragments.

REFP analysis of the SAP's of Abortusovis, Choleraesuis, Enteritidis, Typhimurium, Wangata, Gallinarum, Pullorum, Bovismorbificans and Dublin suggested that these plasmids shared more homology than just the 7.8 kb virulence region and confirms the heteroduplex results of Montenegro *et al* (1991).

The serotype associated plasmids have been previously demonstrated to be stable and highly conserved with respect to REFP in each serotype. This prompted the question – were the evolutionary relationships demonstrated between different serotypes at the level of the genome paralleled by the plasmids associated with certain serotypes? The size of the plasmids enabled their direct sequence analysis, however this has remained limited to particular areas associated with plasmid gene function such as virulence (Gulig *et al* 1993) and fimbrial biosynthesis (Friedrich *et al* 1993).

This study demonstrated the relationship between the plasmids of serotypes Abortusovis, Choleraesuis, Dublin, Enteritidis, Typhinurium, Wangata, Gallinarum, Pullorum, Bovismorbificans, Moscow, Blegdam Antarctica and Rostock by the comparison of the fragmentation patterns or "fingerprints" generated after digestion with various enzymes. Some plasmids showed more REFP similarity than others. For example, the REFP's of the plasmids of Typhimurium and Wangata were 84% similar, Gallinarum and Pullorum 80% similar, Gallinarum and Typhimurium 69% similar whereas the plasmids of Dublin and Abortusovis showed less than 50% REFP similarity. The analysis of a large number of strains from serotypes that contained SAP's revealed that molecular variants of the established SAP's existed. Obviously in a population of predominantly plasmid–free (Wangata) or of limited accessability (Abortusovis) this was not the case. However, if the sample population had been increased it is highly likely that these serotypes too will contain molecular variants of the SAP.

Molecular variation of the SAP of *Salmonella* Gallinarum was observed to be around 6% and paralleled the conclusions of Li *et al* (1993) who found the natural population of Gallinarum consisted of one predominant world–wide clone

which was present at a level of 93%. Plasmid analysis of Gallinarum by Christensen *et al* 1992 also observed three different restriction profiles.

Molecular variation of the SAP of *Salmonella* Pullorum was observed at a level of 47%. Although this figure is high compared to the incidence of variant plasmids in other serotypes, the actual population was different to the other strains. All the strains were isolated in the UK and the variant plasmids involved 15 strains and comprised 6 patterns. Most of these plasmids exhibited minor differences in one or two fragments and probably reflect local variations in the plasmid population. Whether the differences in the plasmid pool of Gallinarum and Pullorum is a reflection on geographical/regional variations or the mechanisms of the spread of disease is not known. *Salmonella* Pullorum has a predilection for the reproductive tissues of poultry and disease is commonly spread by ovarian transmission to eggs (Barrow 1994) whereas Gallinarum is usually acquired by the ingestion of food or water contaminated by the faeces of diseased birds (Li *et al*, 1993). It is possible that the selection pressures exerted on Pullorum results in the spontaneous mutation of the plasmid, albeit minor.

The evolution of some serotypes to specific habitats or niches is relatively common within the salmonellae. This ranges from the strictly host adapted e.g. Pullorum in poultry to the not so restricted e.g. Dublin which is occasionally isolated fron ovine and human sources. Selander has suggested that the clones of host adapted serotypes may have arisen more recently than those of broad host range serotypes. Host adapted serotypes encounter a narrow range of ecological conditions which implies a limited amount of genetic diversity (therefore fewer clones or subclones). Broad host range serotypes encounter a wide range of ecological niches and therefore scope for more genetic diversity.

The adaptation of some serotypes has resulted in changes in amino acid metabolism of the organism. Whether this is a direct result of the environmental pressures that are exerted has yet to be established, however the extent of auxotrophy exhibited by some serotypes tends to favour this theory. Dublin is auxotrophic for nicotinic acid – cattle do not require niacin in their diet therefore it is possible that *Salmonella* Dublin utilises the hosts niacin. Similarly, Pullorum is

auxotrophic for cystine, which is found in abundance in poultry where it is utilised to synthesise feathers.

The results of plasmid analysis of host adapted serotypes indicates a higher degree of diversity in some serotypes. Of the three host adapted serotypes widely studied (with respect to geography and numbers) namely Gallinarum, Pullorum and Dublin only Gallinarum showed limited plasmid diversity, with one world wide plasmid restriction profile. Pullorum and Dublin, on the other hand, showed a greater degree of plasmid divergence than indicated by chromosomal analysis.

Possible explanations as to the results obtained from the analysis of the plasmids of these serotypes include :

1) External physiological stimuli (predilection for reproductive tissue) may affect the Pullorum population, at least in the UK. This may account for the spontaneous production of minor plasmid variants. Whether these molecular variants remain stable in the population remains to be seen. This hypothesis could be analysed *in vivo* – by the experimental infection of poultry with strains of Pullorum that harbour variant plasmids, and *in vitro* by trying to mimic the conditions exerted by the chicken. The analysis of a wide range of strains from throughout the world will also enhance our understanding of the plasmid population within *Salmonella* Pullorum.

2) The widespread clonal expansion of Dublin refutes the idea of limited genetic diversity within a host adapted serotype – at least in the plasmid population. The occurance of subclones within a population however also exists in Dublin especially in isolates from the UK – the majority of which harbour the SAP alone and are fully sensitive to the antimicrobial agents tested.

Plasmid analysis extended the observations of Selander *et al* (1992) that clone Du1 was distributed worldwide and contained either the SAP alone or in combination with a small 3 kb plasmid whilst Du3 was restricted to the UK and France and was associated with either the SAP alone or in combination with another plasmid of intermediate size – no small plasmids were associated with this clone. Plasmid REFP analysis indicated considerably greater diversity in the plasmid population in which there was no direct correspondance between PP and clones Du1 and Du3. However, by inference both clones were represented and

Du3 extended to include Denmark (Platt *et al* 1995). The analysis of whole cell DNA REFP of these strains revealed that nine strains that could not be assigned to either Du1 or Du3 on the basis of PPA (because they possessed both an intermediate and a small plasmid in addition to the SAP) were identified as belonging to Du1 (Platt *et al* 1995) and generally paralleled MLEE analysis but also showed genomic variation in both Du1 and Du3.

One particular molecular variant of Dublin pOG683 proved particularly interesting. This plasmid was isolated from two strains from the USA; three strains isolated fron the UK and France showed REFP's nearly identical to pOG683 i.e. they only varied in one or so fragments. The wholecell REFP of these strains assigned them to Du3. These plasmids have diverged greatly from the Dublin SAP and their presence in the USA provides evidence that Du3 is present there too. pOG683 showed more REFP similarity to the SAP's of Gallinarum (79%) and Pullorum (77% – although one of the molecular variants of Pullorum showed 82% similarity) than to Dublin itself (74%) when digested with *Sma*I. Five fragments were noticable in both their absence from pOG675 (the Dublin plasmid itself) and their presence in the REFP's of other SAP's. These fragments were 7.4 kb (also present in the SAP's of Typhimurium, Wangata, Gallinarum, Pullorum, and Abortusovis), 6.6 kb (present in Gallinarum), 5.2 kb (present in Pullorum), 4.4 kb (present in Typhimurium, Wangata, Gallinarum, Pullorum and Bovismorbificans) and 4.05 kb (present in Gallinarum and Pullorum). In addition to the REFP similarity to other SAP's, pOG683 exhibited incompatibility properties different to Dublin but the same as the other SAP's. The SAP of Dublin was unique in having IncX properties. The other SAP's and pOG683 showed incompatibility to pOG660 only. This plasmid would appear to be an evolutionary intermediate in the development of the Dublin plasmid from a common ancestor. This would also explain the differences in both REFP and incompatibility. Dublin may at some point have formed a cointegrate with an IncX plasmid which subsequently degraded but left the DNA responsible for IncX function. Three cointegrate plasmids were identified in the study of the Dublin plasmids which were conjugative and unstable. This would also explain the differences in *Pst*I and *Sma*I REFP's – both of which generate fragments of ~40 kb. These fragments were not present in pOG683 and

suggests that the 40 kb fragment present in pOG675 after digestion with *Pst*I and *Sma*I are a derivative of an IncX plasmid. Du3 also contains IncX plasmids and is a geographically restricted clone (Selander *et al* 1992). Therefore it is less successful on the basis of the worldwide distrubution of Du1. This scenario may have arisen as a result of a primitive strain of Dublin (which possessed the SAP seen currently) under restricted conditions coexisting with a 40 kb IncX plasmid. Changes in the genome of the Dublin may have resulted in the cointegration and subsequent destabililisation of these plasmids. In this manner a different clone of Dublin with a greater 'fitness' and different incompatibility properties may have spread worldwide.

Whether these findings are a result of practises in animal husbandry remains speculative at the moment. Different farming methods in different countries may affect the population and the importation of livestock may serve to complicate the analysis of evolutionary genetics.

It is not impossible, however that changes in external stimuli are reflected in the molecular genetics of the cell. The bacterial chromosome is less likely to be affected in such a way as to be noticed by current methods of analysis e.g. MMLE or PFGE. Changes in plasmid function and structure are much easier to monitor.

The 4.4 kb *Pst*I fragment was shown to contain the repA functional genes – associated with incompatibility and partition functions. A fragment of this size was present in all SAP's except those of Abortusovis and Dublin (again this fragment was evident in pOG683). This suggests that the plasmids of Abortusovis and Dublin pOG675 are more distantly related to that of Typhimurium or have undergone recent evolutionary change.

The identification of the majority of *Pst*I and *Sma*I restriction sites on the Typhimurium serotype–associated plasmid was achieved with the use of double–digest data, fragment extraction and redigestion and the established orientation of other restriction endonuclease fragments with respect to each other. Although it was intended that this information would enable the construction of a library of DNA probes with which to hybridize Southern blots of the SAP's, both technical

difficulties and time constraints meant that this could not be completed. One successful fragment was cloned – the 2.3 kb *Pst*I fragment – which hybridized with a 2.3 kb fragment from the SAP's of Wangata, Gallinarum, Pullorum, Dublin, Bovismorbificans and the Dublin–Variant plasmid pOG683. This fragment also hybridized with the 1.95 kb fragment of Choleraesuis and the 1.6 kb fragment of Abortusovis. This fragment did not hybridize with Enteritidis. This avenue would form a usefulapproach for future investigation of the evolution of both the plasmids and by extension the salmonellae.

A recent report by Woodward *et al* (1996) identified *pef* A genes in the plasmids of Typhimurium, Bovismorbificans, Choleraesuis and a strain of Enteritidis that harboured a 90 kb plasmid, the lack of such genes in the plasmids of Abortusovis, Blegdam, Gallinarum and Dublin and weak hybridization with a strain of Enteritidis that harboured a the SAP. The *pef* region as sequenced by Friedrich *et al* (1993) was shown to contain the 4.1 kb *Pst*I fragment and a 3.2 kb *Sma*I fragment (this study). The plasmid of Typhimurium was the only SAP to have a 4.1 kb *Pst*I fragment. The fact that the *pef*A gene probe hybridized weakly with the Enteritidis SAP suggested that at least part of this gene was present. This is corroborated – at least in theory – by the REFP data which showed that *Pst*I fragments of 3.1 and 2.1 kb, present in the Typhimurium *pef* region were also present in the plasmid of Enteritidis; similarly 3.2 and 2.5 kb *Sma*I fragments were present in both plasmids. The identification of *Pst*I and *Sma*I restriction sites on the Typhimurium plasmid revealed that the 2.1 kb *Pst*I was contained within the 2.5 kb *Sma*I fragment. The fact that a fragment of 2.1 kb was only evident in plasmids of serotypes Typhimurium, Enteritidis and Bovismorbificans yet a 2.5 kb *Sma*I fragment was identified in all serotype–associated plasmids suggests that either these fragments are of the same size coincidentally or molecular rearrangement within the 2.5 kb fragment has occured such that the *Pst*I recognition site was disrupted.

The implication of plasmid evolution from a common ancestor was confirmed. Given that the *Sma*I REFP of Typhimurium revealed the presence of a fragment of 7.4 kb which was not present in the plasmids of Enteritidis or pOG690

(the suggested plasmid intermediate between those of Typhimurium and Enteritidis – Rankin, Benson and Platt 1995) it is more likely that these plasmids arose from Typhimurium by deletion of DNA; in order for these plasmids to have arisen by DNA acquisition, the event would have had to happen twice; once to generate pOG690 and again to generate pOG660. Similar arguements can be presented for the presence or absence of fragments. However the only satisfactory answer to the development of these plasmids will result from the further investigation either by direct sequence analysis or the use of probes.

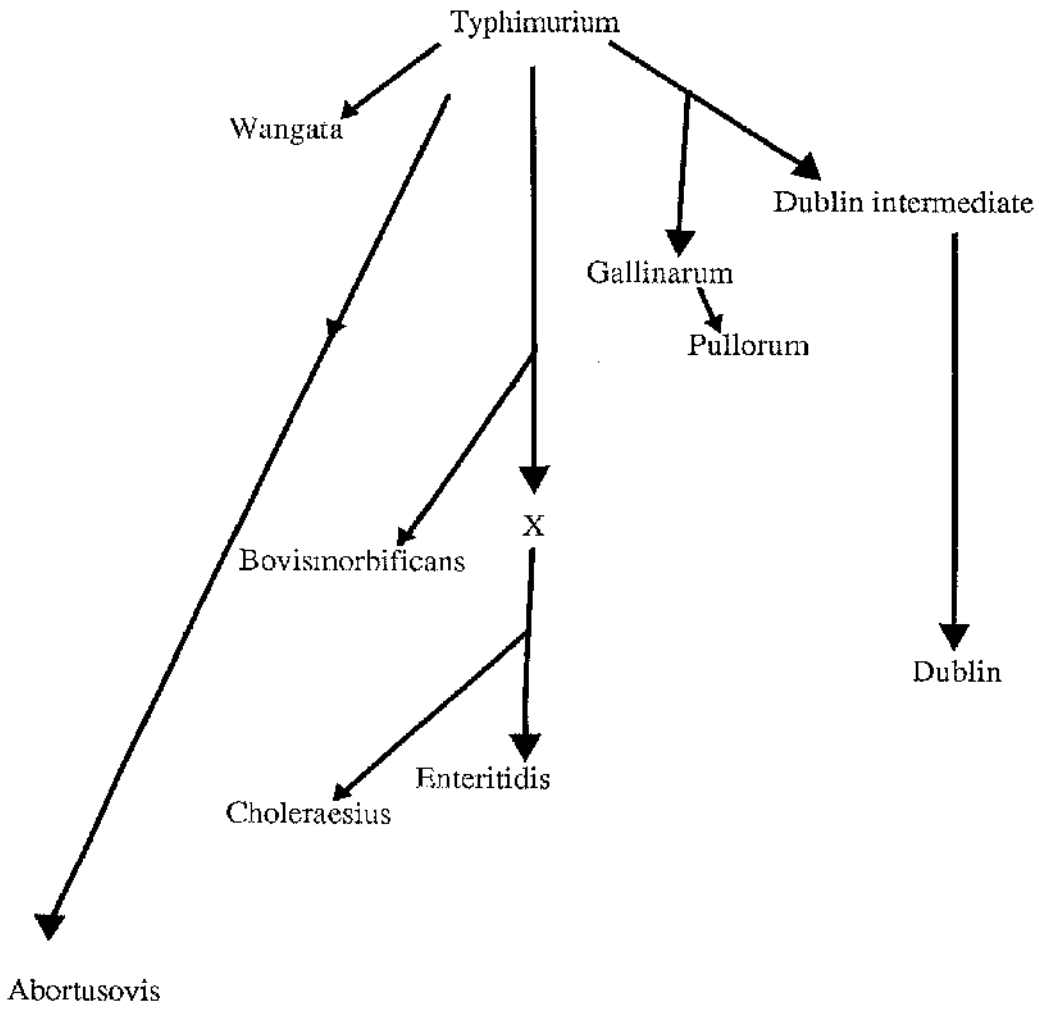
A possible framework for the evolution of the serotype associated plasmids of the salmonellae is given in Figure 7.1.

Overall conclusions

The analyses presented here confirm a family of related plasmids within the salmonellae. Molecular variation within the SAP's of host adapted serotypes appears to occur at a higher frequency than in ubiquitous serotypes. Whether this is a function of host environment e.g temperature, nutrient status remains to be seen. The serotype associated plasmid of *Salmonella* Dublin belongs to a separate evolutionary lineage than the other SAP's. An intermediate in the development of this plasmid exists which suggests that the incursion of an IncX plasmid has occured and the resultant unstable cointegrate plasmid gave rise to the plasmids we see today. The spread of infection throughout the world is also evident from this study. The spontaneous occurance of the same plasmid variants in both Europe and the USA implies the cross boundary transfer of strains. The possibility of an identical molecular variant arising in two separate incidents is remote if not impossible. One of the unanswered questions about the evolution of the serotype associated plasmids of the salmonellae was whether they developedfrom a common ancestor by deletion or incursion of DNA. The results presented here argue in favour of the former.

The stability and conservation of the serotype associated plasmids of the salmonellae suggest that they could be considered as quasi-genomic DNA for the study of *Salmonella* evolution.

Figure 7.1 Possible framework for the evolution of the serotype associated plasmids of the salmonellae



X = evolutionary intermediate

References

Aaij, C. and Borst, P. (1972) The gel electrophoresis of DNA. *Biochim-Biophys. Acta.* **269:** 192-200

Anderson, E.S., Ward, L.R., De Saxe, M.J. and de Sa, J.D.H. (1977) Bacteriophage-typing designations of *Salmonella typhimurium*. *J. Hyg. Camb.* **78:** 297-300.

Arbeit, R.D., Arthur, M., Dunn, R., Kim, C., Selander, R.K. and Goldstein, R. (1990) Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulse-field electrophoresis to molecular epidemiology. *J. Infect. Dis.* **161:** 230-235.

Barker, R.M., Kearney, G.M., Nicholson, P., Blair, A.L., Porter, R.C. and Crichton, P.B. (1988) Types of *Salmonella paratyphi* B and their phylogenetic significance. *J.Med.Microbiol.* **26:** 285-293.

Barker, R.M. and Old, D.C. (1989) The usefulness of biotyping in studying the epidemiology and phylogeny of salmonellae. *J. Med. Microbiol.* **29:** 81-88.

Barker, R.M., Old, D.C. and Tyc, Z. (1982) Differential typing of *Salmonella agona* : type divergence in a new serotype. *J. Hyg.Camb* **88:** 413-423.

Barrow, P.A. and Lovell, M.A. (1988) The association between a large molecular mass plasmid and virulence in a strain of *Salmonella pullorum*. *J. Gen. Microbiol.* **134:** 2307-2316.

Barrow, P.A., Simpson, J.M., Lovell, M.A. and Binns, M.M. (1987) Contribution of *Salmonella gallinarum* large plasmid toward virulence in fowl typhoid. *Infect. Immun.* **55:** 388-392.

Baumler, A., Tsolis, R., M., Bowe, F., A., Kusters, J., G., Hoffmann, S. and Heffron, F. (1996) The *pef* fimbrial operon of *Salmonella typhimurium* mediates

adhesion to murine small intestine and is neccessary for fluid accumulation in the infant mouse. *Infect. Immun.* **64:** 61-68.

Baumler, A.J. and Heffron, F. (1995) Identification and sequence analysis of *lpf*ABCDE, a putative fimbriae operon of *Salmonella typhimurium*.. *J. Bacteriol.* **177:** 2087-2097.

Beltran, P.J., Musser, J.M., Helmuth, R., Farmer III, J.J., Frerichs, W.M., Wachsmuth, I.K., Ferris, K., McWhorter, A.C., Wells, J.G., Cravioto, A. and Selander, R.K. (1988) Toward a population genetic-analysis of *Salmonella*: genetic diversity and relationship among strains of serotypes, *S. choleraesuis, S. derby, S. dublin, S. enteritidis, S. heidelberg, S. infantis, S. newport,* and *S. typhimurium. Proc. Natl. Acad. Sci. USA* **85:** 7753-7755.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7:** 1513-1523.

Brahmbhatt, H.N., Wyk, P., Quigley, N.B. and Reeves, P.R. (1988) Complete physical map of *rfb* gene cluster encoding biosynthetic enzymes for the O antigen of *S.typhimurium* LT2. *J. Bacteriol.* **170:** 98-102.

Brown, D.J., Munro, D.S. and Platt, D.J. (1986) Recognition of the cryptic plasmid pSLT, by restriction fingerprinting and a study of its incidence in Scottish Salmonella isolates. *J. Hyg.Camb.* **97:** 193-197.

Brown, D.J., Threlfall, E.J., Hampton, M.D. and Rowe B. (1993) Molecular characterization of plasmids in *Salmonella enteritidis* phage types. *Epidemiol. Infect.* **110:** 209-216.

Browning, L.M. and Platt, D.J. (1995) Restriction endonuclease fragmentation pattern analysis of plasmids from Salmonellae that belong to serogroups D1. *Med. Microbiol. Lett.* **4:** 132-139.

Browning, L.M., Wray, C. and Platt, D.J. (1995) Diversity and molecular variation among plasmids in *Salmonella enterica* serotype Dublin based on restriction enzyme fragmentation pattern analysis. *Epidemiol. Infect.* **114:** 237-248.

Brubaker, R.R. (1985) Mechanisms of bacterial virulence. *Ann Rev Microbiol* **39:** 21-50.

Brunner, D.W. (1952) Changes induced in the H antigens of *Salmonella bleydam. J. Bacteriol.* **64:** 138-139.

Brunner, F., Margadant, A., Peduzzi, R. and Piffaretti, J. (1983) The plasmid pattern as an epidemiological tool for *Salmonella typhimurium* epidemics: comparison with the lysotype. *J. Infect. Dis.* **148:** 7-11.

Carle, G.F., Frank, M. and Olson, M.V. (1986) Electrophoretic separation of large DNA molecules by periodic inversion of the electric field. *Science* **232:** (65):68

Carsiotis, M., Weinstein, D.L., Karch, H., Holder, L.A. and O'Brien, A.D. (1984) Flagella of *Salmonella typhimurium* are a virulence factor on infected c57BL/6J mice. *Infect. Immun.* **46:** 814-818.

Casalino, M., Commanducci, M., Nicoleti, M. and Maimone, F. (1984) Stability of plasmid content in *Salmonella wein* in late stages of epidemic history. *Antimicrob. Agent. Chemo.* **25:** 499-501.

Cerin, H. and Hackett, J. (1989) Molecular cloning and analysis of the incompatability and partition functions of the virulence plasmid of *S.typhimurium. Microb. Pathog.* **7:** 85-100.

Cerin, H. and Hackett, J. (1993) The *par*VP region of the *Salmonella typhimurium* virulence plasmid pSLT contains four loci required for incompatibility and partition. *Plasmid* **30:** 30-38.

Chabbert, Y.A., Roussel, A., Witchitz, J., L., Lepo, M.J. and Courvalin, P. (1979) Restriction endonuclease generated patterns of plasmids belonging to *inc* groups I_1, C, M and N; application to plasmid taxonomy and epidemiology. In: Timmis, K.N. and Puhler, A. (Eds.) *Plasmids of medical, environmental and commercial importance,* Amsterdam: Elseiver

Chambers, R.M., McAdam, P., de Sa, J.D.H., Ward, L.R. and Rowe, B. (1987) A phage-typing scheme for *Salmonella virchow*. *FEMS Microbiol. Lett.* **40:** 155

Chart, H., Rowe, B., Threlfall, E.J. and Ward, L.R. (1989) Conversion of *S.enteritidis* phage type 4 to phage type 7 involves loss of lipopolysacchride with concomitant loss of virulence. *FEMS Microbiol. Lett.* **60:** 37-40.

Christensen, J.P., Olsen, J.E., Hansen, H.C. and Bisgaard, M. (1992) Characterization of *Salmonella enterica* serovar *gallinarum* biovars *gallinarum* and *pullorum* by plasmid profiling and biochemical analysis. *Avian. Pathol.* **21:** 461-470.

Christie, A.B. (1974) *Infectious Diseases- Epidemiology and clinical practice*, 2nd edn. pp. 55-63. Edinburgh: Churchill Livingstone

Colombo, M.M., Leori, G., Rubino, S., Barbato, A. and Cappuccinelli, P. (1992) Phenotypic features and molecular characterization of plasmids in *Salmonella abortusovis*.. *J. Gen. Microbiol.* **138:** 725-731.

Cowden, J.M., O'Mahony, M., Bartlett, C.L.R., Rana, R., Smyth, B., Lynch, D. and Tillett, H. (1989) A national outbreak of *Salmonella typhimurium* DT124 caused by contaminated salami sticks. *Epidemiol. Infect.* **103:** 219-225.

Craigie, J. and Felix, A. (1947) Typing of typhoid bacilli with Vi bacteriophage. Lancet **i:** 823-827.

Crichton,P.B. and Old,D.C. (1990) Salmonellae of serotypes Gallinarum and Pullorum grouped by biotyping and fimbrial-gene probing. *J.Med.Microbiol.* **32:** 145-152.

Craven, P.C., Mackel, D.C., Baine, W.B., Barker, W.H., Gangarosa, E.J., Goldfield, M., Rosenfeld, H., Altman, R., Lachapelle, G., Davies, J.W. and Swanson, R.C. (1975) International outbreak of *Salmonella eastbourne* infection traced to contaminated chocolate. *Lancet* **1:** 788-792.

Crosa, J.H., Brenner, D.J., Ewing, W.H. and Falkow, S. (1973) Molecular relationships among the salmonellae. *J. Bacteriol.* **115:** 307-315.

Datta, N. (1979) Plasmid classification : incompatibility grouping. In: Timmis, K.N. and Puhler, A. (Eds.) *Plasmids of Medical, Environmental and Commercial importance*. pp. 3-12. New York: Elsevier

de Sa, J.D.H., Ward, L.R. and Rowe, B. (1980) A scheme for the phage typing of *Salmonella hadar*. *FEMS Microbiol. Lett.* **9:** 175

Dice, L.R. (1945) Measures of the amount of ecological association between species. *Ecology* **26:** 297-302.

Dorman, C.J., Chatfield, S., Higgins, C.F., Hayword, C. and Dougan, G. (1989) Characterization of porin and *omp*R mutants of a virulent strain of *Salmonella typhimurium* : *omp*R mutants are attenuated *in vivo*. *Infect. Immun.* **57:** 2136-2140.

Dubnau, E. and Maas, W.K. (1968) Inhibition of replication of an F'lac episome in Hfr cells of Escherichia coli. *J. Bacteriol.* **95:** 531-539.

Dugiud, J.P., Anderson, E.S., Anderson, G.A., Barker, R. and Old, D.C. (1975) A new biotyping scheme for *Salmonella typhimurium* and its phylogenic significance. *J. Med. Microbiol.* **8:** 149-166.

Duguid, J.P., Darekar, M.R. and Wheater, D.W.F. (1976) Fimbriae and infectivity in *Salmonella typhimurium*. *J. Med. Microbiol.* **9:** 459-473.

Esteban, E., Snipes, K., Hird, D., Kasten, R. and Kinde, H. (1993) Use of ribotyping for characterization of *Salmonella* serotypes. *J. Clin. Microbiol.* **31:** 233-237.

Ewing, W.H. (1972) The nomenclature of *Salmonella*, its usage and definitions for the three species. *Can. J. Microbiol.* **18:** 1629-1637.

Ewing, W.H. (1986) Edwards and Ewing's identification of *Enterobacteriaceae*. 4th Ed. Elsevier, New York.

Farrar, W.E. (1983) Molecular analysis of plasmids in epidemiologic investigation. *J. Infect. Dis.* **148** 1-6.

Feutrier, J., Kay, W.W. and Trust, T.J. (1986) Purification and characterization of fimbriae from *Salmonella enteritidis*. *J. Bacteriol.* **168:** 221-227.

Fields, P.I., Swanson, R.V., Maidaris, C.G. and Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad Sci. USA*. **83:** 5189-5193.

Finlay, B.B. and Falkow, S. (1988) Virulence factors associated with *Salmonella* species. *Microbiol. Sci.* **5:** 324-327.

Friedrich, M.J., Kinsey, N.E., Vila, J. and Kadner, R.J. (1993) Nucletide sequence of a 13.9kb segment of the 90kb virulence plasmid of *S.typhimurium* : the presence of fimbrial biosynthetic genes. *Mol. Microbiol.* **8:** 543-558.

Frost, J.A., Ward, L.R. and Rowe, B. (1989) Acquisition of a drug resistance plasmid converts *Salmonella enteritidis* phage type 4 to phage type 24. *Epidemiol. Infect.* **103:** 243-248.

Gaertner, E. (1886) Ueber die Fleischuergiftung in Frankenhausen a Fyffh und der Erreger derselben. *Korresp. Allg. Artzl. Ver. Thurigen* **17:** 573-600.

Galan, J.E. and Curtiss, I.R. (1989) Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proc. Natl. Acad. Sci. USA* **86:** 6383-6387.

Gianella, R.A., Formal, S.B., Dammin, G.J. and Collins, H. (1973) Pathogenesis of salmonellosis. Studies of fluid secretion, mucosal invasion and morphological reaction in the rabbit ileum. *J. Clin. Invest.* **52:** 441-453.

Ginocchio, C.C. and Galan, J.E. (1995) Functional conservation among members of the *Salmonella typhimurium* InvA family of proteins. *Infect. Imnun.* **63:** 729-732.

Grimont, F. and Grimont, P.A.D. (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann. Inst. Pasteur Microbiol.* **137 B:**165-175.

Groisman, E.A., Fields, P.I. and Heffron, F. (1990) Molecular biology of *Salmonella* pathogenesis. In: Iglewski, B. and Clark, V.L. (Eds.) *Molecular Basis of Bacterial Pathogenesis*, pp. 251-272. Academic Press

Gulig, P.A., Caldwell, A.L. and Chiodo, V.A. (1992) Identification genetic analysis and DNA sequence of a 7.8kb virulence region of the *Salmonella typhimurium* virulence plasmid. *Mol. Microbiol.* **6:** 1395-1411.

Gulig, P.A. (1990) Virulence plasmids of *Salmonella typhimurium* and other salmonellae. *Microb. Pathog.* **8:** 3-11.

Gulig, P.A. and Chiodo, V.A. (1990) Genetic and DNA sequence analysis of the *Salmonella typhimurium* virulence plasmid gene encoding the 28000-molecular weight protein. *Infect. Immun.* **58**: 2651-2658.

Gulig, P.A. and Curtiss, R. (1988) Cloning and transposon insertion mutagenesis of virulence genes of the 100kb plasmid of *Salmonella typhimurium*. *Infection and Immunity* **56:** 3262-3271.

Gulig, P.A., Danbara, H., Guiney, D.G., Lax, A.J., Norel, F. and Rhen, M. (1993) Molecular analysis of *spv* virulence genes of the salmonella virulence plasmids. *Mol. Microbiol.* **7:** 825-830.

Heery, D.M., Gannon, F. and Powell, R. (1990) A simple method for subcloning DNA fragments from gel slices. *Trends in Genetics* **6:**173

Heffernan, E.J., Harwood, J., Frierer, J. and Guiney, D. (1992) The *Salmonella typhimurium* virulence plasmid complement resistance gene *rck* is homologous to a family of virulence -related outer membrane protein genes, including *pag*C and *ail*. *J. Bacteriol.* **174:** 84-91.

Heiskanen, P., Tiara, S. and Rhen, M. (1994) Role of *rpo*S in the regulation of *Salmonella* plasmid virulence (*spv*) genes. *FEMS Microbiol. Lett.* **123:** 125-130.

Helmuth, R., Stephan, R., Bunge, C., Hoog, B., Steinbeck, A. and Bulling, E. (1985) Epidemiology of virulence - associated plasmids and outer membrane

protein patterns with seven common Salmonella serotypes. *Infect. Immun.* **48:** 175-182.

Holmberg, S.D., Wachsmuth, I.K., Hickman-Brenner, F.W. and Cohen, M.L. (1984) Comparison of plasmid profile analysis, phage typing and antimicrobial susceptibility testing in characterizing *Salmonella typhimurium* isolates from outbreaks. *J. Clin Microbiol.* **19:** 100-104.

Jones, G.W., Rabert, D.K., Svinarich, D.M. and Whitfield, S.H. (1982) Association of adhesive, invasive and virulent phenotypes of *Salmonella typhimurium* with autonomous 60-megadalton plasmids. *Infect. Immun.* **38:** 476-486.

Kasatiya, S., Caprioli, T. and Champoux, S. (1978) Bacteriophage typing scheme for *Salmonella infantis*. *J. Clin. Microbiol.* **10:** 637-640.

Kauffmann, F. (1954) Enterobacteriaceae. 2nd Ed. Munksgaard,Copenhagen. pp 19-146.

Kauffmann, F. (1960) Two biochemical subdivisions of the genus *Salmonella*. *Acta Pathol Microbiol Scand* **49:** 393-396.

Kauffmann, F. (1966) Anonymous *The Bacteriology of the Enterobacteriaceae*, Copenhagen: Munksgaard

Kauffmann, F. and Orskov, F. (1956) Anonymous *Die Bakteriologie der Escherichia coli*-Enteritis, Stuttgart: Georg Thieme Verlag

Kawahara, K., Haraguchi, Y., Tsuchimoto, M., Terakado, N. and Danbara, H. (1988) Evidence of correlation between 50-kilobase plasmid of *Salmonella choleraesuis* and its virulence. *Microb. Pathog.* **4:** 155-163.

Koo, F.C.W. and Peterson, J.W. (1983) Cell free extracts of *Salmonella* inhibit protein synthesis and cause cytotoxicity in eukaryotoc cells. *Toxicon* **21:** 309-320.

Korpela, K., Ranki, M., Sukupolvi, S., Makela, P.H. and Rhen, M. (1989) Occurrence of *Salmonella typhimurium* virulence plasmid-specific sequences in different serovars of *Salmonella*. *FEMS Microbiol. Lett.* **58:** 49-54.

Krause, M., Fierer, J. and Guiney, D. (1990) Homologous DNA sequences on the virulence plasmids of pathogenic *Yersinia* and *S.dublin* Lane. *Mol Microbiol* **4:** 905-911.

Krause, M., Roudier, C., Fierer, J., Harwood, J. and Guiney, D. (1991) Molecular analysis of the virulence locus of the *Salmonella dublin* plasmid pSDL2. *Mol. Microbiol.* **5:** 307-316.

Lam, S. and Roth, J.R. (1983) IS200 : A Salmonella-specific insertion sequence. *Cell* **34:** 951-960.

Le Minor, L. (1984) In Kreig, N.R. and Holt, J.G. (Eds.) *Bergey's Manual of Systematic Bacteriology*, pp. 427-458. Baltimore: Williams and Wilkins

Le Minor, L. (1988) Typing of *Salmonella* species. *European Journal of Clinical Microbiology and Infectious Disease* **7:** 214-218.

Le Minor, L. and Popoff, M.Y. (1987) Designation of *Salmonella enterica* sp. nov., nom., rev., as the type and only species of the genus *Salmonella*. *Int. J. Syst. Bacteriol.* **37:** 465-468.

Le Minor, L. and Popoff, M.Y. (1988) Antigenic formulas of the *Salmonella* serovars, 5th rvn., Paris, WHO Collaborative Centre for Reference and Research on *Salmonella*. *Institut Pasteur France*. 1-146.

Le Minor, L., Veron, M. and Popoff, M.Y. (1982) Taxonomie des *Salmonella*. *Ann. Microbiol. (Paris)* **133B:** 222-243.

Le Minor, L., Veron, M. and Popoff, M.Y. (1982) Proposition pour une nomenclature des *Salmonella*. *Ann. Microbiol. (Paris)* **133B:** 245-254.

Li, J., Smith, N.H., Nelson, K., Crichton, P.B., Old, D.C., Whittam, T.S. and Selander, R.K. (1993) Evolutionary origin and radiation of the avian-adapted non-motile salmonellae. *J. Med. Microbiol.* **38:** 129-139.

Loeffler, F. (1892) Ueber Epidemieen unter den im hygienischen Institut zu Greifswald gehaltenen Masen und uber die Bekampfung der Feldmausplage. *Zentrabl. Bakteriol. Parasitenk Infectionskr. Hyg. Abt. I. Orig.* **11:** 129-141.

Luderitz, O., Galanos, C. and Rietschel, E.T. (1986) Endotoxins of Gram-negative bacteria. In: Dorner, F. and Drews, J. (Eds.) *Pharmacology of Bacterial Toxins.*

Maas, R. and Maas, W.K. (1962) Introduction of a gene from *Escherichia coli* B into Hfr and F- strains of *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA.* **48:** 1887-1893.

Mahon, J. and Lax, A.J. (1993) A quantitative polymerase chain reaction method for the detection in avian faeces of salmonellas carrying the *spv*R gene. *Epidemiol. Infect.* **111:** 455-464.

Maniatis,T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning, a Laboratory Manual (New York, Cold Spring Harbour Laboratory).

Manning, E.J., Baird, G.D. and Jones, P.W. (1986) The role of plasmid genes in the pathogenicity of *Salmonella dublin*. *J. Gen.Microbiol.* **21:** 239-243.

Martinetti, G. and Altwegg, M. (1990) rRNA gene restriction patterns and plasmid analysis as a tool for typing *Salmonella enteritidis*. *Res. Microbiol.* **141:** 1151-1162.

Matsui, H., Kawahara, K., Terakado, N. and Danbara, H. (1990) Nucleotide sequence of a gene encoding a 29kDa polypeptode in mba region of the virulence plasmid, pKDSC50, of *Salmonella choleraesuis*. *Nucleic Acids Res.* **18:** 1055

Mayer, L.W. (1988) Use of plasmid profiles in epidemiologic surveillance of disease outbreaks and in tracing the transmission of antibiotic resistance. *Clin. Microbiol. Rev.* **1:** 228-243.

McConnell, M.M., Smith, H.R., Leonardopoulos, J. and Anderson, E.S. (1979) The value of plasmid studies in the epidemiology of infections due to drug resistant *Salmonella wein. J.Infect.Dis.* 139: 178-190.

Michiels, T., Popoff, M.Y., Durviaux, S., Coynault, C. and Cornelis, G. (1987) A new method for the physical and genetic mapping of large plasmids: application to the localisation of the virulence determinants on the 90kb plasmid of *Salmonella typhimurium*. *Microb. Pathog.* **3:** 109-116.

Montenegro, M., Morelli, G. and Helmuth, R. (1991) Heteroduplex analysis of Salmonella virulence plasmids and their prevalence in isolates of defined sources. *Microb. Pathog.* **11:** 391-397.

Nakamura, M., Sato, S., Ohya, T., Suzuki, S. and Ikeda, S. (1985) Possible relationship of a 36-megadalton *Salmonella enteritidis* plasmid to virulence in mice. *Infect. Immun.* **47:** 831-833.

Nastasi, A., Mammina, C. and Villafrate, M.R. (1993) Epidemiology of *Salmonella typhi* : ribosomal DNA analysis of strains from human and animal sources. *Epidemiol. Infect.* **110:** 553-565.

Norel, F., Pisano, M.R., Nicoli, J. and Popoff, M.Y. (1989a) Nucleotide sequence of the plasmid-borne virulence gene *mkf*A encoding a 28kDa polypeptide from *Salmonella typhimurium*. *Res. Microbiol.* **140:** 263-265.

Norel, F., Pisano, M.R., Nicoli, J. and Popoff, M.Y. (1989b) Nucleotide sequence of the plasmid borne virulence gene *mkf*B from *Salmonella typhimurium*.. *Res. Microbiol.* **140:** 455-457.

Norel, F., Pisano, M.R., Nicoli, J. and Popoff, M.Y. (1989c) A plasmid-borne virulence region (2.8kb) from *Salmonella typhimurium* contains two open reading frames. *Res. Microbiol.* **140:** 627-630.

Old, D.C. (1990) In Topley and Wilsons principles of bacteriology, virology and immunity, 8th Edition pp 469-493. Parker, M.T. and Duerder (Eds), B.I. London.

Old, D.C. (1992) Nomenclature of *Salmonella*. *J. Med. Microbiol.* **37:** 361-363.

Old, D.C., Munro, D.M., Reilly, W.J. and Sharp, J.C.M. (1985) Biotype discrimination of *Salmonella montevideo*. *Lett. Appl. Microbiol.* **1:** 67-69.

Olsen, J., Skov, M.N., Threlfall, E.J. and Brown, D.J. (1994) Clonal lines of *Salmonella enterica* serotype Enteritidis documented by IS200-, ribo-, pulsed-field gel electrophoresis and RFLP typing. *J. Med. Microbiol.* **40:** 15-22.

Olsen, J.E., Baggesen, D.L., Nielsen, B.B. and Larsen, H.E. (1990) The prevalence of plasmids in Danish bovine and human isolates of *Salmonella dublin*. *APMIS* **98:** 735-740.

Olsen, J.E., Brown, D.J., Baggesen, D.L. and Bisgaard, M. (1992) Biochemical and molecular characterization of *Salmonella enterica* serovar *berta* and comparison of methods for typing. *Epidemiol. Infect.* **108:** 243-260.

Olsen, J.E. and Skov, M. (1994) Genomic lineage of *Salmonella enterica* serovar Dublin. *Vet. Microbiol.* **40:** 271-282.

Ou, J.T., Baron, L.S., Dai, X. and Life, C.A. (1990) The virulence plasmids of *Salmonella* serovars *typhimurium, choleraesuis, dublin* and *enteritidis* and the cryptic plasmids of Salmonella serovars *copenhagen* and *sendai* belong to the same incompatibility group, but not those of serovars *durban, gallinarum, give, infantis and pullorum.. Microb. Pathog.* **8:** 101-107.

Platt, D.J. (1983) Bacterial plasmids and their fingerprints. *Disease Markers* **1:** 107-115.

Platt, D.J., Brown, D.J., Old, D.C., Barker, R.M., Munro, D.S. and Taylor, J. (1987) Old and new techniques together resolve a problem of infection by *Salmonella typhimurium. Epidemiol. Infect.* **99:** 137-142.

Platt, D.J., Browning, L.M. and Candlish, D. (1995) Molecular analysis of *Salmonella enterica* serotype Dublin: building bridges between population genetic and molecular epidemiological studies. *Electrophoresis* **17:**1-5.

Platt, D.J., Chesham, J.S., Brown, D.J., Kraft, C.A. and Taggart, J. (1986) Restriction enzyme fingerprinting of enterobacterial plasmids: a simple strategy with wide application. *J. Hyg.Camb.* **97:** 205-210.

Platt, D.J. and Smith, I. (1991) Gentamicin-resistant *Salmonella typhimurium* phage type 204c: molecular studies and strain diversity in a putative bovine outbreak. *Epidemiol. Infect.* **107:** 213-223.

Platt, D.J. and Sullivan, L. (1992) Molecular epidemiology : Molmatch and beyond. *International Labmate* **110:** 657

Platt, D.J., Taggart, J. and Heraghty, K.A. (1988) Molecular divergance of the serotype-specific plasmid (pSLT) among strains of *Salmonella typhimurium* of human and veterinary origin and comparison of pSLT with the serotype-specific plasmids of *S.enteritidis* and *S.dublin.*. *Microbiol.* **27:** 277-284.

Plikaytis, B.D., Carlone, G.M., Edmonds, P. and Mayer, L.W. (1986) Robust estimation of standard curves for protein molecular weight and linear-duplex DNA base-pair number after gel electrophoresis. *Anal. Biochem.* **152:** 346-364.

Popoff, M.Y., Bockemuhl, J. and McWhorter-Murlin, A. (1994) Supplement 1993 (no.37) to the Kauffman-White scheme. *Res. Microbiol.* **145:** 711-716.

Popoff, M.Y., Miras, I., Coynault, C., Lasselin, C. and Pardon, P. (1984) Molecular relationships between virulence plasmids of *Salmonella* serotypes *typhimurium* and *dublin* and large plasmids of other *Salmonella* serotypes. *Annale. Microbiol. (Paris)* **135:** 389-398.

Poppe, C., Curtiss, R., III, Gulig, P.A. and Gyles, C.L. (1991) Hybridization studies with a DNA probe derived from the virulence region of the 60Mdal plasmid of *Salmonella typhimurium*.. *Can J Vet Research* **145:** 378-384.

Pritchard, R.H. (1978) DNA synthesis - present and future. In: Kohiyama, M. and Molineux, I. (Eds.) pp. 1-26. New York: Plenum Press

Pullinger, G.D., Baird, G.D., Williamson, C.M. and Lax, A.J. (1989) Nucleotide sequence of a plasmid gene involved in the virulence of salmonellas. *Nucleic Acids Res.* **17:** 7983

Purcell, B.K., Pruckler, J. and Clegg, S. (1987) Nucleotide sequences of the genes encoding Type1 Fimbrial subunits of *Klebsiella pneumoniae* and *Salmonella typhimurium*.. *J. Bacteriol.* **169:** 5831-5834.

Rankin, S. and Platt, D.J. (1995) Phage conversion in Salmonella enterica serotype Enteritidis: implications for epidemiology. *Epidemiol. Infect.* **114:** 227-236.

Rankin, S.C., Benson, C.E. and Platt, D.J. (1995) The distribution of serotype-specific plasmids among different subgroups of strains of *Salmonella enterica* serotype Enteritidis: characterisation of molecular variants by restriction enzyme fragmentation pattern. *Epidemiol. Infect.* **114:** 25-40.

Rexach, L., Dilasser, F. and Fach, P. (1994) Ploymerase chain reaction for salmonella virulence-associated plasmid genes detection: a new tool in salmonella epidemiology. *Epidemiol. Infect.* **112:** 33-43.

Rhen, M. and Sukupolvi, S. (1988) The role of the traT gene of the *S.typhimurium* plasmid for serum resistance and growth within the liver macrophages. *Microb. Pathog.* **5:** 275-285.

Rhen, M., Virtanen, M. and Makela, P.H. (1989) Localizatiom by insertion mutagenesis of a virulence-associated region on the *Salmonella typhimurium* 96 kilobase pair plasmid. *Microb. Pathog.* **6:** 153-158.

Riley, L.W., Ferdinando, D., De Melfi, T.M. and Cohen, M.L. (1983) Evaluation of isolated cases of salmonellosis by plasmid profile analysis: introduction and transmission of a bacterial clone by pre-cooked roast beef. *J. Infect. Dis.* **148:** 12-17.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Sharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487-491.

Salmon, D.E.and Smith, T. (1886) Investigations in swine plague. In *US Department of Agriculture Annual Report of the Bureau of Animal Industry 1885.* pp184. Washington Government Printing Office.

Selander, R.K., Beltran, P., Smith, N.H., Helmuth, R., Rubin, F.A., Kopecko, D.J., Ferris, K., Tall, B.D., Cravioto, A. and Musser, J.M. (1990) Evolutionary genetic relationships of clones of *Salmonella* serovars that cause human typhoid and other enteric fevers. *Infect. Immun.* **58:** 2262-2275.

Selander, R.K., Caugant, D.A., Ochman, H., Musser, J.M., Gilmour, M.N. and Whittam, T.S. (1986) Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **51:** 873-884.

Selander, R.K., Musser, J.M., Caugant, D.A., Gilmour, M.N. and Whittam, T.S. (1987) Population genetics of pathogenic bacteria. *Microb. Pathog.* **3:** 1-7.

Selander, R.K., Smith, N.H., Li, J., Beltran, P., Ferris, K.E., Kopecko, D.J. and Rubin, F.A. (1992) Molecular evolurionary genetics of the cattle-adapted serovar *Salmonella dublin*. *J. Bacteriol.* **174:** 3587-3592.

Smith, H.R., Humphreys, G.O., Grindley, N.D., Grindley, J.N. and Anderson, E.S. (1973) Molecular studies of an *fi+* plasmid from strains of Salmonella *typhimurium*. *Mol. Gen. Genet.* **126:** 143-151.

Smith, N.H., Beltran, P.and Selander, R.K. (1990) Recombination of *Salmonella* phase 1 flagellin genes generates new serovars. *J. Bacteriol.* **172:** 2209-2216.

Spika, J.S., Waterman, S.H., Soo Hoo, G.W., St.Louis, M.E., Pacer, R.E., James, S.M., Bissett, M.L., Mayer, L.W., Chiu, J.Y., Hall, B., Greene, K., Potter, M.E., Cohen, M.L. and Blake, P.E. (1987) Chloramphenicol resistant *Salmonella newport* traced through hamburger to dairy farms. *N. Engl. J. Med.* **316:** 565-570.

Spratt, B.G., Rowbury, R.J. and Meynell, G.G. (1973) The plasmid of *Salmonella typhimurium* LT2. *Mol. Gen. Genet.* **121:** 347-353.

Stanley, J., Powell, N., Jones, C. and Burnens, A.P. (1994) A framework for IS200, 16S rRNA gene and plasmid-profile analysis in *Salmonella* serogroup D1. *J. Med. Microbiol.* **41:** 112-119.

Stanley, J., Jones, C.S. and Threlfall, E.J. (1991) Evolutionary lines among *Salmonella enteritidis* phage types are identified by insertion sequence IS200 distribution. *FEMS Microbiol. Lett.* **82:** 83-90.

Stokes, J.L. and Bayne, H.G. (1958) Growth-factor-dependent strains of salmonellae. *J. Bacteriol.* **76:** 417-421.

Suziki, S., Komase, K., Matsui, H., Abe, A., Kawahara, K., Tamura, Y., Kijima, M., Danbara, H., Nakamura, M. and Sato, S. (1994) Virulence region of plasmid pNL2001 of *Salmonella enteritidis*. *Microbiol.* **140:** 1307-1318.

Taira, S. and Rhen, M. (1989) Identification and genetic analysis of *mka*A - a gene of the *Salmonella typhimurium* virulence plamid necessary for intracellular growth. *Microb. Pathog.* **7:** 165-173.

Takahashi, M., Ogino, T. and Baba, K. (1969) Estimation of relative molecular length of DNA by electrophoresis in agarose gel. *Biochim. Biophys. Acta.* **174:** 183-187.

Taylor, D.N., Wachsmuth, K., Shangkuan, Y., Schmidt, E.V., Barret, T.J., Schrader, J.S., Scherach, C.S., McGee, H.B., Feldman, R.A. and Brenner, D.J. (1982) Salmonellosis associated with marijuana - a multistate outbreak traced by plasmid fingerprinting. *N. Engl. J. Med.* **306:** 1249-1253.

Terakado, N., Sekizak, T., Hashimoto, K. and Naitoh, S. (1983) Correlation between the presence of a fifty-megadalton plasmid in *Salmonella dublin* and virulence for mice. *Infect. Immun.* **41:** 443-444.

Thong, K.L., Ngeow, Y.F., Altwegg, M., Navaratnam, P. and Pang, T. (1995) Molecular analysis of *Salmonella enteritidis* by pulsed-field gel electrophoresis and ribotyping. *J. Clin. Microbiol.* **33:** 1070-1074.

Threlfall, E.J., Rowe, B., Ferguson, J.L. and Ward, L.R. (1985) Increasing incidence of resistance to gentamicin and related aminoglycosides in *Salmonella typhimurium* phage type 204c in England, Wales and Scotland. *Vet Record.* **117:** 355-357.

Threlfall, E.J., Hall, M.L.M. and Rowe, B. (1986) *Salmonella gold-coast* from outbreaks of food poisoning in the British Isles can be differentiated by plasmid profiles. *J. Hyg. Camb.* **97:** 115-122.

Timmis, K.N. (1979) Mechanisms of plasmid incompatibility. In *Plasmids of Medical, Environmental and Commercial Importance.* Timmis, K.N. and Puhler, A (Eds). Elsevier.

Timmis, K.N., Andres, I., Slocombe, P.M. and Synenki, R.M. (1979) In *Cold Spring Harbour Symposium of Quantitative Biology*, Vol 43.

Tinge, S.A. and Curtiss III, R. (1990) Conservation of *Salmonella typhimurium* virulence plasmid maintenance regions among Salmonella serovars as a basis for plasmid curing. *Infect. Immun.* **58:** 3084-3092.

Tinge, S.A. and Curtiss, R., III (1990) Isolation of the replication and partitioning regions of the *Salmonella typhimurium* virulence plasmid and stabilization of heterologous replicons. *J. Bacteriol.* **172:** 5266-5277.

Tompkins, L.S., Troup, N., Labigne-Roussel, A. and Cohen, M.L. (1986) Cloned, random chromosomal sequences as probes to identify *Salmonella* species. *J. Infect. Dis.* **154:** 156-162.

Vandenbosch, J.L., Rabert, D.K., Kurlandsky, D.R. and Jones, G.W. (1989) Sequence analysis of rsk, a portion of the 95-kilobase plasmid of *Salmonella typhimurium* associated with resistance to the bactericidal activity of serum. *Infect. Immun.* **57:** 850-857.

Vatopoulos, A.C., Mainas, E., Balis, E., Threlfall, E.J., Kanelopoulou, M., Kalapothaki, V., Malamou-Lada, H. and Legakis, N.J. (1994) Molecular epidemiology of Ampicillin resistant clinical isolates of *S.enteritidis.. J. Clin. Microbiol.* **32:** 1322-1325.

Veron, M. and Le Minor, L. (1975) Nutrition et taxonomie des *Enterobacteriaceae* et bacteries voisines. *Ann. Pastuer (Paris)* **126B:** 111-124.

Wallis, T.S., Starkey, W.G., Stephen, J., Haddon, S.J., Osborne, M.P. and Candy, D.C.A. (1986) Enterotoxin production by *Salmonella typhimurium* strains of different virulence. *J. Med. Microbiol.* **21:** 19-23.

Ward, L.R., de Sa, J.D.H. and Rowe, B. (1987) A phage-typing scheme for *Salmonella enteritidis. Epidemiol. Infect.* **99:** 291-294.

Weide-Botjes, M., Liebisch, B., Schwarz, S. and Watts, J.L. (1996) Molecular characterizaton of *Salmonella enterica* subsp. *enterica* serovar *choleraesuis* field isolates and differentiation from homologous live vaccine strains Suisaloral and SC-54. *J.Clin.Microbiol.* **34:** 2460-2463.

Weil, A.J. and Saphra, I. (1953) *Salmonellae and Shigellae. Laboratory diagnisis correlated with clinical manifestations and epidemiology.* Springfield, Illinois: Charles Thomas.

Weinstein, D.L., Carsiotis, M., Lissner, C.R. and O'Brien, A.D. Flagella help *Salmonella typhimurium* survive within murine macrophages. *Infect.Immun.* 46: 819-825.

Whittam, T.S. (1994) Genetic population structure and pathogenicity in enteric bacteria. In: Baumberg, S., Young, J.P.W., Wellington, E.M.H. and Saunders, J.R. (Eds.) *Population genetics of bacteria,* pp. 217-245. Cambridge. Cambridge University Press

Widjojoatmodjo, M.N., Fluit, A.C., Torensma, R., Verdonk, G.P.H.T. and Verhoef, J. (1992) The magnetic immuno polymerase chain reaction assay for direct detection of Salmonellae in facael samples. *J. Clin. Microbiol.* **30:** 3195-3199.

Williamson, C.M., Baird, G.D. and Manning, E.J. (1988) A common virulence region on plasmids from eleven serotypes of Salmonella. *J. Gen. Microbiol.* **134:** 975-982.

Williamson, C.M., Pullinger, G.D. and Lax, A.J. (1988) Identification of an essential virulence region on *Salmonella* plasmids. *Microb. Pathog.* **5:** 469-473.

Williamson, C.M., Pullinger, G.D. and Lax, A.J. (1990) Identification of proteins expressed by the essential virulence region of the *Salmonella dublin* plasmid. *Microb. Pathog.* **9:** 61-66.

Willshaw, G.A., Smith, H.R., Anderson, E.S., Scotland, S.M. and Gross, J.R. (1977) Application of agarose gel electrophoresis for the characterization of plasmid DNA in drug resistant and enterotoxigenic enterobacteria. *Proc. Soc. Gen. Mircobiol.* **4:** 151-152.

Woodward, M.J., Allen-Vercoe, e. and Redstone, J.S. (1996) Distribution, gene sequence and expression *in vivo* of the plasmid encoded fimbrial antigen of *Salmonella* serotype Enteritidis. *Epidemiol. Infect.* **117:** 17-28.

Woodward, M.J., McLaren, I. and Wray, C. (1989) Distribution of virulence plasmids within Salmonellae. *J. Gen. Microbiol.* **135:** 503-511.