MOLECULAR EPIDEMIOLOGY OF *SALMONELLA ENTERICA* SEROTYPE ENTERITIDIS: CONTRIBUTION OF THE SEROTYPE ASSOCIATED PLASMID.

BY

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Abstract.

Salmonella enterica serotype Enteritidis has been consistently among the ten most prevalent serotypes in Scotland for twenty years and the dominant serotype since 1987. The study of the epidemiology of Enteritidis has become increasingly important in recent years as the increase in the number of isolations has been primarily due to one phage type. The predominance of Enteritidis phage type 4 has been attributed to clonal expansion and corresponds to the increase in poultry and egg associated infections. The capacity to discriminate between distinct strains of this serotype is an essential component of epidemiological investigations.

The use of plasmid profile and restriction endonuclease fragmentation pattern (REFP) analyses and their validity as a tool to complement epidemiological investigations is reviewed together with a wide range of molecular techniques.

Four hundred and thirty four isolates of *Salmonella enterica* serotype Enteritidis were studied. They were grouped into 5 subsets defined by either the collection criteria or the parameter which formed the basis for subsequent analysis. Three hundred and sixty two (83%) contained the 54kb serotype associated plasmid (SAP) of Enteritidis (pOG674). In two hundred and seventy five (63%) of the isolates this was the sole plasmid. Molecular variation in the SAP was detected in 17 (4%) of the isolates on the basis of REFP analysis with the restriction enzymes *Pst*I and *Sma*I. The nine SAP variants were designated pOG690, 691, 700, 701, 702, 703, 704 and 705.

The REFPs of the plasmids were analysed using the Dice coefficient of similarity. This indicated significant homogeneity between the Enteritidis plasmids and the 95kb SAP of *Salmonella enterica* serotype Typhimurium. One of the Enteritidis plasmids, pOG690, showed a greater resemblance to the SAP of Typhimurium than Enteritidis; Dice coefficients of 89% and 68% respectively for *Pst*I and 79% and 55% respectively for *Sma*I. With respect to *Pst*I this indicated that pOG690 shared 55kb of

its DNA with the Typhimurium SAP and 37kb with the SAP of Enteritidis. It was thus postulated that pOG690 was an intermediate in the evolutionary descent of plasmids from Typhimurium to Enteritidis.

To attempt to clarify this hypothesis required initially that the restriction fragments produced from each of the Enteritidis plasmids, and the Typhimurium SAP, with the enzymes *Pst*I and *Sma*I be tabulated. This allowed the identification of a 'common core' of fragments from which four were chosen to be cloned and used as probes. Two *Pst*I fragments (2.8kb and 4.4kb) and two *Sma*I fragments (1.58kb and 3.0kb) were cloned into pUC19 and pUC18 vector plasmids respectively. However, only the 2.8kb *Pst*I fragment was successfully transformed into *E.coli* DH5α. The resultant plasmid was designated pOG1001.

Hybridisation analysis revealed that the 2.8kb *Pst*I probe fragment hybridised with 2.8kb fragments from plasmids pOG691, 700, 703 and 705. A fragment of 3.3kb hybridised with the probe in plasmid pOG704 and a 3.4kb fragment hybridised with the probe from plasmids pOG690 and Typhimurium plasmid pOG660. The implications of these findings are discussed.

To further clarify the evolutionary relationship between Enteritidis plasmid pOG674 , the Typhimurium plasmid pOG660 and the variant plasmids in the study required that *Pst*I and *Sma*I restriction maps of the Enteritidis plasmid be generated. This was to allow comparison with previously published maps of the Typhimurium SAP and also to determine whether or not the *pef* (plasmid encoded fimbrial) region of Typhimurium was present on the Enteritidis plasmid.

Three restriction maps of Enteritidis plasmid, pOG674, were generated by analysis of restriction enzyme digests and double digests of pOG674 with the following enzymes, *Xba*I, *Xho*I, *Hin*dIII, *Bam*HI, *Bgl*II and *Sal*I. Extraction and re-digestion

(with a different enzyme) of restriction fragments was required in some instances. *Pst*I and *Sma*I restriction maps were constructed based on the basic map. The map data in combination with the hybridisation analysis allowed conclusions to be drawn with regard to the evolution of the Enteritidis plasmids and Typhimurium plasmid pOG660. The most important of which were i) a *Bgl*II restriction site was present in the 4.4kb *Pst*I fragment of Enteritidis plasmids pOG674, pOG690, pOG691 and pOG701 that was not seen in Typhimurium plasmid pOG660; ii) one plasmid was found (pOG701) that had no virulence region and iii) the 14kb *pef* region defined in theTyphimurium SAP was possibly not intact in the Enteritidis SAP pOG674.

Finally, three case studies were presented to demonstrate the critical application of PP and REFP analyses in epidemiological investigations of outbreaks that involved *Salmonella enterica* serotype Enteritidis, Typhimurium and Derby. The results presented showed that i) small plasmids could be used to increase discrimination within Enteritidis phage type 14b ii) molecular variants of the SAP of Typhimurium could be used to determine that two strains, with apparently different phage types, were from a common source and iii) REFP analysis of plasmids in Derby strains could be used to identify a new strain in sheep that was subsequently traced to animal feed.

The results presented in this thesis have also identified a number of areas for future research.

TABLE OF CONTENTS

Index Of Tables.

Index Of Figures.

Abbreviations.

Ap	**Ampicillin**
AP	**Alkaline phosphatase**
BAP	**Bacterial alkaline phosphatase**
BHI	**Brain heart infusion broth**
CCC	**Covalently closed circular DNA**
CIAP	**Calf intestinal alkaline phosphatase**
CLED	**Cysteine lactose electrolyte deficient agar**
DEAE	**Diethyl aminoethyl cellulose**
DIG	**Digoxigenin**
DNA	**Deoxyribonucleic acid**
dNTPs	**di nucleotide triphosphates**
DT	**Definitive phage type**
EDTA	**Ethylene diamine tetra-acetic acid**
ETs	**Electrophoretic types**
IPTG	**Isopropylthio-β-D-galactoside**
Km	**Kanamycin**
LB	**Luria broth base**
MLEE	**Multi locus enzyme electrophoresis**
NBT	**Nitroblue tetrazolium salt**
NET	**Sodium chloride/ EDTA/ Tris buffer**
OC	**Open circular DNA**
PCR	**Polymerase chain reaction**
PFGE	**Pulsed field gel electrophoresis**
PP	**Plasmid profile**
PPA	**Plasmid profile analysis**
RAPD	**Random amplified polymorphic DNA**
RDNC	**Routine dilution non conforming**

REFP	**Restriction endonuclease fragmentation pattern**
RNA	**Ribonucleic acid**
rRNA	**Ribosomal ribonucleic acid**
RNAase	**Ribonuclease A**
SAP	**Serotype associated plasmid**
S_D	**Dice coefficient of similarity**
SDS	**Sodium dodecyl sulphate**
SOC	**Super optimal catabolite medium**
SSC	**Saline sodium citrate**
SSRL	**Scottish Salmonella reference laboratory**
Su	**Sulphonamides**
TB	**Tris/ borate buffer**
TBE	**Tris/ borate/ ethidium bromide buffer**
Tc	**Tetracycline**
TE	**Tris/ EDTA buffer**
TES	**Tris/EDTA/ sodium chloride buffer**
TGE	**Tris/ glucose/ EDTA buffer**
Tp	**Trimethoprim**
UV	**Ultra violet radiation**
X-gal	**5-bromo-4-chloro-3-indolyl-β-D-galactoside**
X-phosphate	**5-bromo-4-chloro-3-indolyl phosphate toluidin salt**

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Declaration.

The experimental work and composition are the sole work of the author unless otherwise stated.

The author participated in both the experimental work and composition of the enclosed manuscripts.

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1. October 1996

CHAPTER 1.

Historical Introduction And A Review Of *Salmonella* Typing Methods.

1.1 The history of the genus *Salmonella*

In 1886, D.E. Salmon and T. Smith described an organism which was proposed as the causative agent of hog cholera. The disease was subsequently recognised as being caused by a virus but the associated bacillus was given the name *Salmonella choleraesuis*. This organism is now known to be a frequent secondary invader in hog cholera and is also particularly virulent for man. As Salmon's description of the choleraesuis bacillus antedated all other observations of paratyphoid bacilli his name was honoured when a generic term was established for the paratyphoid group of organisms.

The salmonellae are Gram negative, motile, non-sporing rod shaped organisms which belong to the family *Enterobacteriacae*. They are differentiated from other genera by the lack of fermentation of lactose, ability to utilise citrate as the sole carbon source and by the production of hydrogen sulphide in triple sugar iron agar (TSI). Salmonellae other than *Salmonella enterica* serotype Typhi, are almost always acrogenic with regard to the production of gas in the acid fermentation of carbohydrates (Le Minor, 1984).

The genus *Salmonella* contains a single species (Le Minor and Popoff, 1987) *Salmonella enterica* and is subdivided into seven sub species on the basis of biochemical tests. There are currently more than 2300 recognised serotypes within the genus (Popoff *et al*, 1994) of which many are pathogenic for man and animals.

1.2 Population genetics and epidemiology of *Salmonella*

It is important, at this stage to define what is to be inferred by the terms "clone" and "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). In this sense a clonal lineage is a closed

system that accumulates differences only through genetic processes that occur within a single cell, such as point mutations, inversions, duplications and deletions and transpositions. Clearly because of these processes members of a clone are not necessarily genetically or phenotypically homogeneous. This is the view of population genetics but an understanding of the genetic structure of microbial populations is relevant to epidemiology.

One of the primary tools for analysis of genetic variation in natural populations of bacteria is multilocus enzyme electrophoresis (MLEE) (Selander *et al*, 1986; Selander *et al*, 1987). Established more than twenty five years ago in eukaryotic population genetics (Lewontin, 1991), this technique detects protein polymorphisms caused by amino acid replacements that alter the rate of electrophoretic migration. MLEE has yielded two fundamental discoveries about the nature of genetic variation in natural populations of bacteria. First, bacterial populations are highly variable and second, the structure of populations is predominantly clonal. Thus, in most bacteria, despite gene transfer by transformation, transduction and conjugation the rate at which recombination assorts genes into new combinations in nature is very low.

In one of the first population studies of *Salmonella* (Beltran *et al*, 1988) several direct questions were addressed, notably: Does serotypic identity of strains indicate overall genetic identity or similarity? Is the population structure of *Salmonella* clonal with most cases of disease being caused by a small proportion of existing clones? The analysis demonstrated that populations of all serotypes are genetically variable, being represented by multiple electrophoretic types (ETs). In some serotypes (e.g. Typhimurium and Choleraesuis) all ETs were closely related and therefore monophyletic but in others the same antigenic structure occurred in strains which belonged to divergent ETs (i.e. were polyphyletic). The authors concluded that identity of serotype does not

necessarily indicate genetic identity and clearly invalidates the Kauffman White scheme as a method of phylogenetic classification. The results indicated that the genetic structure of *Salmonella* populations is clonal and most disease is caused by one or a few clones of global distribution. Clonality means that recombination of chromosomal genes among cell lineages is very infrequent, and occurs at a rate well below that required to randomise genes in chromosomal genomes.

Although not phylogenetic the Kauffman White scheme has been useful in epidemiology. Studies on the evolution of flagellin genes (Smith and Selander, 1989) have shown that in strains of Typhimurium which had different ETs, the DNA sequence of the central region of the H1 : i gene was identical. This suggested that the rate of evolution of the H1 flagellin gene by point mutation is not high. Therefore, new serotypes are not easily generated by mutations in this gene which allows the continued use of antigenic structure as a relatively stable marker in epidemiology. Once a serotype has been established it becomes necessary to define clonality relative to epidemiology

A working definition of the term "clone" is "any microbial isolate belonging to a set of microbial isolates that have been recovered independently from different sources, in different locations and perhaps at different times, but showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin" (Orskov and Orskov, 1983).

The epidemiologists task therefore involves the determination of phenotypic and genetic traits of isolates in order that identity may be established among a set of independent isolates. Since clonality, at least in the relative sense is, by definition, required of all involved isolates in a common source outbreak of infection, an important feature of an epidemiologic evaluation is the

determination of clonality of the suspected pathogen, regardless of the mode of transmission. Even if the clonality of recovered isolates cannot be determined unequivocally, data amassed in such analyses are almost always valuable to the epidemiologic evaluation. The judgement of non-clonality, which is often easier to make, eliminates an isolate from consideration as one involved in a particular chain of transmission.

An aspect of clonality that must now be considered is the effect of "periodic selection". In 1951, Atwood and colleagues showed that when a bacterial culture is serially transferred for many generations, new types with a selective advantage arise periodically and replace the existing population. The effect of this is to purge the population of much of the genetic variation that would otherwise accumulate through mutation. From the point of view of population genetics, an important consequence of periodic selection is that the effective population size of the organism will be much less than the actual size (Levin, 1981). From the point of view of epidemiology it means that the judgement of clonality by itself is often insufficient for identification of a common source outbreak. Since some pathogenic clones are so ubiquitous that multiple simultaneous outbreaks with that clone may occur independently and with some frequency e.g. *Salmonella enterica* serotype Enteritidis, phage type 4, clonality can rarely be absolute.

1.3 *Salmonella enterica* serotype Enteritidis and poultry

On 3 December 1988, a junior minister of health, Mrs Edwina Currie, stated that "most of the egg production of this country is, sadly, infected with salmonella". This statement aroused both public anxiety and political concern to the extent that in February 1989, the Agriculture Committee of the House of

Commons published a First Report on the role of eggs in Salmonellosis (House of Commons Agriculture Committee, 1989a and 1989b).

In common with many other countries it became apparent that eggs were frequently implicated in salmonella outbreaks, especially those in which Enteritidis was the causative organism (Perales and Audicana, 1988; Sharp, 1988; St Louis *et al*, 1988a, 1988b; Anon, 1989). Enteritidis phage type (PT) 4 predominated in the United Kingdom and was not restricted to eggs (Humphrey *et al*, 1988). Rampling and colleagues (1989) showed that Enteritidis PT4 was also present in broiler chickens on retail sale and concluded that measures to control Enteritidis PT4 should be directed at both the egg and poultry meat production aspects of the industry.

Enteritidis, unlike *Salmonella* group D serotypes Pullorum and Gallinarum, is not host adapted for poultry. However, the disease syndromes which it produces in chickens are strikingly similar to those produced by Pullorum. Chronic invasive disease in both commercial egg layers and broiler birds has been recorded and ovarian disease of boiler breeder stock can lead to vertical transmission to progeny (Lister, 1988). Although this can lead to a high death rate in chicks, by the time of slaughter, at 47 days, broiler chickens show no overt clinical signs of disease but a few birds will show mucopurulent pericarditis due to infection with Enteritidis PT4 (O'Brien, 1988).

Salmonella serotypes can be isolated from both the shells and contents of eggs and can result from either infection of the oviduct or faecal carriage. The evidence for egg shell contamination by salmonella is very variable (Humphrey *et al*, 1989a, 1989b and 1989c; Mawer *et al*, 1989; Perales and Audicana, 1989) with faecal contamination thought to be the most likely source. Although Enteritidis has been reported on eggshells Humphrey and colleagues

(1991a) reported shell contamination in the absence of faecal carriage, which suggested that for Enteritidis, infection of the reproductive tissue appears to be important. Infection of the ovaries and ovules (Barnhart *et al*, 1993) leads to contamination of the egg contents and contrary to popular belief, the principle site of contamination is not the yolk but either the albumen or outside of the vitelline membrane (Paul and Batchelor, 1988; Timoney *et al*, 1989; Shivaprasad, 1990; Humphrey *et al* 1991b).

In outbreak investigations and case control studies (Coyle *et al*, 1988; Stevens *et al*, 1989; Cowden *et al*, 1989a; Cowden *et al*, 1989b) illness due to Enteritidis PT4 was found to be significantly associated with the consumption of products that contained raw egg, cooked egg and pre-cooked hot chicken. The finding that cooked egg and poultry caused disease prompted investigation of heat resistance in Enteritidis PT4 and Humphrey and colleagues (1990) showed that this organism appeared to be more heat resistant than other poultry associated serotypes.

The economic importance of Enteritidis to the poultry industry and its prominence in human infection have meant that much work has been undertaken on the infection of chickens. A variety of tissues are involved in systemic infection and there can be a septicaemic phase. Enteritidis can spread easily from bird to bird (Gast and Beard, 1990a, 1990b, 1990c) and therefore a number of control measures have been introduced by governments in an attempt to limit risk to public health.

Serological tests for the detection of Enteritidis have been developed (Chart *et al*, 1990; Nicholas and Cullen, 1991) but have a disadvantage in that they may detect past, rather than current infection, which still retains the need for microbiological examination of culled birds, some of which may be negative.

One way to avoid the destruction of birds free of Enteritidis, is to develop more sensitive detection systems. These have included selective and differential media, enzyme immunoassay, latex agglutination and DNA probe or PCR based techniques (Scholl, 1990; Feng, 1992; Keller *et al*, 1993; Way *et al*, 1993; Swaminathan and Feng, 1994; Thorns *et al*, 1994; van der Zee, 1994; Hanes *et al*, 1995). Each of these have advantages and disadvantages and as yet no universally accepted detection system has been established. Though currently there has been much interest in PCR based detection systems, the development of which is being treated with great interest from both the poultry and food industries. In the meantime however, the prevalence of *Salmonella* serotypes, particularly Enteritidis, in these industries remains a problem.

1.4 Discrimination among salmonellae

1.4.1 Serotyping

A widely recognised scheme for the serological discrimination was developed by White in 1926 and has been expanded by Kauffman (1978). Type determination was, and still is, based on the possession of both somatic 'O' antigens and flagellar 'H' antigens. The group of an unknown organism was determined by the identification of the 'O' antigen. Greater than twenty groups, each of which was characterised by a particular combination of 'O' antigens, were recognised. The type within the group was determined by the flagellar antigens. Flagellar antigens, as did the 'O' antigens, occurred as characteristic combinations of single factors rather than as single entities. Strains with the same 'O' antigens may have completely different 'H' antigens, and the inverse may also be true; the same 'H' antigen may be encountered in strains which differ in their 'O' antigens. Many, but not all, salmonellae express in alternate phases flagella of two different antigenic types (H1 and H2) and a very small

number of them produce Vi (capsular) antigen. Thus each serotype was recognised by its unique combination of antigens.

Salmonella serotypes were traditionally given names which were accorded species status; this was later replaced by the practice of naming newly identified serotypes after the place of their first isolation. However, by 1988 more than 2250 serotypes had been recorded and doubt was expressed over the practice of according equal 'species' ranking to major pathogens and infrequently isolated serotypes (Le Minor and Popoff, 1988). Several attempts were made to change the nomenclature but disagreements regarding their appropriateness ensued. In 1972 Ewing named only three species *S.choleraesuis*, *S.typhi* and *S.enteritidis*. In this scheme *S.typhimurium* became *S.enteritidis* serotype (ser.) Typhimurium and other serotypes were similarly named. However, *S.enteritidis* became *S.enteritidis* ser. Enteritidis which led to confusion between serotype and species. The most recent advance was proposed by Le Minor and Popoff in 1987 and followed the theory that all serotypes of *Salmonella* and those of the former genus *Arizona* belonged to one species which comprised seven subspecies that were distinguished by biochemical tests (Crosa *et al* 1984; Le Minor *et al* 1982a, 1982b). The single species was named *Salmonella enterica* and six subspecies names were also designated.

Most (>99%) salmonellae isolated from man belong to subspecies I, the only one for which named serotypes was retained. Serotypes that were named as species e.g. *S.enteritidis* became *Salmonella enterica* serotype Enteritidis which could be further shortened to *Salmonella* Enteritidis or Enteritidis.

Serotypes in subspecies II to VI were designated by their antigenic structure preceded by a subspecies number. Consequent upon this

recommendation all extant serotype names from subspecies II to VI were deleted in the most recent edition of the Kauffman-White scheme (Le Minor and Popoff, 1988). This nomenclature has been adopted throughout the thesis.

1.4.2 Bacteriophage typing

This technique was devised in 1938 by Craigie and Yen for serotype Typhi, and the Vi- phage typing scheme rapidly established itself as the method of choice for epidemiological recognition of different strains that caused typhoid fever and served as a model for the development of all later schemes for the typing of bacteria by phage. Phage typing schemes have been developed for serotypes Typhimurium (Felix and Callow, 1943; Anderson and Williams, 1956; Felix, 1956; Callow, 1959; Anderson, 1964; Anderson *et al*, 1977a) Enteritidis (Ward *et al*, 1987), Infantis (Kasatiya *et al*, 1978), Hadar (De Sa *et al*, 1980) and Virchow (Chambers *et al*, 1987) among others. It remains the method of choice for the initial discrimination and typing of Typhimurium and Enteritidis.

The practicability of phage typing depended on one of the most important properties of phages, their host specificity. This property was recognised early in the study of bacteriophages (Anderson, 1958). However, it was soon realised that specific phages could be adapted to other serotypes. The resultant phages were on the whole specific for the serotypes on which they had been propagated. The term 'adaptation' was used in relation to the propagation of phages on resistant strains. Anderson (1958) pointed out that many if not all salmonellae were lysogenic and that attempts at phage adaptation always carried the risk of contamination of the original phage with phages carried by the strain on which propagation was carried out. Such contamination by temperate phage proved to be a source of confusion in the development of the later typing schemes and there were strong arguments to suspect that it played a part in the apparent phage adaptation in earlier schemes.

The Enteritidis typing scheme developed by Ward and colleagues (1987) used ten phages, five of which were adapted and identified twenty seven different types. This scheme was recently extended by the addition of a further four phages to recognise forty four different types (Threlfall *et al*, 1993).

1.4.3 Biochemical typing

In 1937 Kristensen and colleagues developed a method of typing *Salmonella* Typhimurium using the differential fermentation of nine different substrates. This was extended by Hansen (1942) and Harhoff (1948) to distinguish twenty one different biotypes. This method was less discriminatory and more laborious than phage typing, but it distinguished different biotypes within individual phage types so that in combination with phage typing it gave finer discrimination than either method alone (Kallings and Laurell, 1957; Rische and Kretzchmar, 1962; Lewis and Stocker, 1971).

In a combined study of *Salmonella* Typhimurium phage typing and biotyping, Duguid and colleagues (1975), found serious flaws in the Kristensen scheme and went on to develop an alternative two-tier biotyping scheme. This latter system defined a primary type, numbered one to thirty two, and a sub-type which distinguished within the primary type. Full biotypes were designated by primary type numbers followed by lower case Arabic letters which indicated the sub-type reactions. This system had the ability to accommodate newly discovered types, and further distinguished between them by the addition of further secondary tests.

Many studies were done which showed that the full biotype of many Typhimurium phage types remained constant, this suggested that there was evidence of phylogenic relationships among these phage types (Anderson *et al*,

typing and biotyping also made it possible to detect occasional variation in the phage type or biotype of epidemic clones during their spread e.g. phage type 49 to 204c, 56 to 193, 141 to 193 and biotype 2a to 10a, 9f to 9bf and 26a to 26f (Barker *et al*, 1980).

A long term evaluation of the ecology of *Salmonella* Typhimurium led Anderson (1971) to postulate that in any country the association of strains of a particular phage type with a host, probably indicated that that phage type was a well-established clone in that host. For example, strains of Typhimurium phage types 1, 49 and 56 were among those with long associations with bovine hosts (Anderson, 1971) and strains of phage type 141 were also bovine associated (Anderson *et al*, 1978; Barker and Old, 1979). The most likely explanation of the many strains which differed in a single (usually secondary) biotype character from the majority of strains which belonged to the same phage type, was that they were variants derived by mutation, which had led to a loss of functions in the parent strain during their spread within specific hosts. If the existence of different unrelated biotypes among cultures of a single phage type are not recognised, epidemiologically incorrect assumptions may be made.

The combined use of biotyping with phage typing was useful for confirming clonal relationships between isolates of epidemic strains. The indications of possible phage type interconversions was a further benefit obtained by biotyping (Anderson *et al*, 1978). The biotyping scheme for Typhimurium was based on fifteen characters, all of which were thought to be determined by chromosomal genes and therefore, biotype instability due to chance loss or acquisition of plasmids was not thought to be a problem.

1.4.4 Plasmid analysis

In 1987, Lewin described a plasmid as an autonomous self-replicating DNA element. Their importance in bacteria was recognised as early as 1950 with the discovery of the F-plasmid (Lederberg *et al*, 1952), but it was with the discovery of R-plasmids in the late 1950's and the recognition of their widespread distribution, that plasmid research was established Researchers discovered that antibiotic resistance was transferable *via* plasmids (Watanabe, 1963; Meynell and Datta, 1966; Meynell *et al*, 1968; Anderson, 1968) and these observations led to criteria being established for the characterisation, and thus the classification of transfer systems (Anderson, 1968). R-factor compatibility groups were devised (Grindley *et al*, 1972; Grindley *et al*, 1973) which occupied scientists until the late 1970's when the study of plasmids in epidemiological investigations was discovered. It became possible to combine information about known resistance markers carried by plasmids with their incompatibility groupings. This led to the conclusion that it was no longer sufficient in the investigation of infections with the *Enterobacteriacae*, to identify, for example, only the serotype of a salmonella. Characterisation could be rendered more precise by phage typing the organisms, where possible, and distinctive biochemical markers were sought. Isolates were routinely tested for drug resistance however, it was thought that the simple identification of resistance markers was inadequate and the plasmids which carried the markers were therefore explored. In 1977(b), Anderson and colleagues, stated that because of the enormous dispersal of resistance and other plasmids, epidemiological studies should be carried to the genetic and even the molecular level.

1.4.5 Plasmid profile analysis

Methods for the isolation of plasmid DNA were available as early as 1970 (Freifelder, 1970; Guerry *et al*, 1973; Humphreys *et al*, 1975) however, these procedures were complex, relatively lengthy and costly. The need arose for a method which was less time consuming and was also suitable for survey work. Aaij and Barst (1972) reported that the migration rates of bacteriophage and mitochondrial covalently closed circular (CCC) DNA's ranging from 3.4 x 10^6 to 10 x 10^6 daltons were related inversely to the logarithm of their mass in 0.6% agarose gels. The migration of higher molecular weight CCC DNA had until this time been overlooked. In 1976, Meyers and colleagues developed a method which allowed plasmids to be detected and their size determined. This method was suitable for the detection and estimation of plasmid DNA of molecular weights ranging from 0.6 x 10^6 to 95 x 10^6 daltons, in partially purified whole cell lysates.

Willshaw and colleagues (1979), applied this technique to the study of plasmid DNA in drug resistant enterobacteria. They examined some drug resistant strains of *Salmonella* Typhimurium which originated in several Middle Eastern countries, the strains involved had been investigated in a previous study (Willshaw *et al*, 1977). The purpose of the latter work therefore, was to highlight the limitations in the application of plasmid profile analysis (PPA) in epidemiological studies, in which it was important to determine unambiguously the number and sizes of plasmids in different wild strains. They also investigated the conditions for agarose gel electrophoresis and in particular they studied the interference of chromosomal DNA fragments and the presence of multiple DNA forms. They examined three agarose gel concentrations, 0.65%, 0.75% and 1%, and concluded that a 0.75% gel gave reliable estimates of molecular sizes over a wide range (3 x 10^6 to 80 x 10^6 daltons) for which molecular weight and relative mobility were linearly related. Within this range

plasmid sizes determined by electrophoresis were within 10% of the values obtained by electron microscopy. However, for large plasmids up to 146 x 10^6 daltons, the linear relationship was no longer valid and the resolution of plasmid species was reduced. If plasmids were suspected within or near the chromosomal region, 0.65% or 1% gels were used. Using a 0.65% gel, plasmids of molecular weights in excess of 80 x 10^6 could be determined but at this concentration the mobility of plasmids less than 10 x 10^6 were less than would have been predicted from a linear relationship between the molecular weight and relative mobility. With a 1% agarose gel, plasmid mobility was a linear function of molecular size from 3 x 10^6 to approximately 50 x 10^6 daltons and therefore these conditions were not suggested for use in the size determination of large plasmids.

The work on the presence of different plasmid DNA forms in agarose gels concluded that it may be quite common with small plasmids less than 10 x 10^6 to find open circular (OC) and/or linear forms of the plasmids present in conjunction with the CCC form. In contrast, open circular molecules were not usually detected during electrophoresis of plasmids larger than 20 x 10^6. It was presumed that large open circular molecules may not easily enter 0.75% agarose, resulting in an area of bright fluorescence at the origin of the well, after staining with ethidium bromide solution. It was concluded that the position, on 0.75% agarose, of linear plasmid DNA relative to the CCC and OC form depended on the molecular size of the plasmid. These observations were, and still are, crucial to the determination of plasmid profiles of wild type clinical isolates. Without this knowledge the application of plasmid profile analysis in epidemiological studies would have gained no impetus.

1.4.6 Restriction endonuclease fragmentation pattern (REFP) analysis of plasmids

Plasmid profile analysis was used to give an estimate of the number and sizes of plasmids present in bacterial strains. It did not, however, give any indication as to whether or not plasmids of similar size present in the same bacterial species were related. The development of REFP analysis made this possible.

Restriction endonuclease fragmentation patterns were first reported by digesting plasmid DNA with type II restriction endonucleases (Thompson, 1974). These bacterial enzymes recognised specific sequences in double stranded DNA and hydrolysed the two strands which resulted in the cleavage of DNA at this site. The linear fragments produced were separated by agarose gel electrophoresis which produced a pattern characteristic of the plasmid under investigation.

In 1983 Riley and colleagues reported the results of a study in which REFP analysis enabled the identification of pre-cooked roast beef as the vehicle of transmission in an outbreak of *Salmonella* Newport infection. The unique plasmid profile found in the isolates allowed the roast beef to be implicated in the absence of other conclusive epidemiological markers. However, this technique was not invariably useful as *Salmonella* Typhimurium was also isolated from patients in the study by Riley and colleagues (1983) and these were found to be plasmid free. Therefore, PPA could not be used to evaluate isolated Typhimurium infections, even though this was the predominant serotype in these outbreaks. Riley and colleagues (1983) suggested that phage typing may have been more useful in this epidemic as it could have added strength to the suggestion that plasmid free Typhimurium strains isolated were epidemiologically related to that isolated from the roast beef.

1.4.7 Identification of virulence associated plasmids in *Salmonella*

An important development in the study of plasmids in *Salmonella enterica* was the discovery (Jones *et al*, 1982) that the large plasmid present in Typhimurium was associated with enhanced virulence. Terakado and colleagues, 1983, demonstrated a correlation between the presence of a 50Md plasmid in Dublin and virulence for mice and by 1984 Popoff and colleagues had extended this further to include the large plasmids present in serotypes Abortusovis, Enteritidis, Paratyphi C and Newport. The molecular weight of each plasmid was thought to be typical of the serotype. Early DNA hybridisation studies (Popoff *et al*, 1984) using the entire Typhimurium plasmid as a probe indicated that the plasmids from Enteritidis and Dublin shared homologous sequences. This study also established the absence of homology between these and the plasmids found among various other serotypes and suggested that the plasmids were related. Popoff and colleagues, 1984, showed that plasmids of the same serotype usually had similar *Hin*dIII REFPs and suggested that this plasmid group constituted a single group of homology and represented a family of related plasmids that could contribute to the pathogenicity of the host serotypes. In 1985, Nakamura and colleagues proposed a possible relationship for the 36 megadalton (Md) Enteritidis plasmid to virulence in mice and suggested that this plasmid was native to Enteritidis. Helmuth and colleagues (1985) added Choleraesuis to this growing list and showed that of sixty antibiotic sensitive Typhimurium isolates 88% carried a large 60Md (95kb) plasmid. Virulence plasmid carriage in Enteritidis and Dublin was 87 and 89% respectively and a 100% carriage rate was found in Choleraesuis. Mouse virulence studies performed with these four serotypes showed that strains which possessed plasmids had LD_{50} values up to 10^6 - fold lower than plasmid free strains of the same serotype.

Helmuth and colleagues (1985) introduced the term 'serotype-specific plasmid' for these homologous plasmids which had previously been termed 'cryptic' (Jones *et al*, 1982). However, a recent report by Browning and Platt (1995) has suggested that the term serotype specific plasmid is incorrect as the same plasmid had been shown to be present in different serotypes of *Salmonella*. They have proposed the term serotype associated plasmid (SAP) and this will be adopted throughout the thesis.

Localisation of a virulence region (Williamson *et al*, 1988a, 1988b; Norel *et al* 1989a, 1989*b*, 1989c; Pullinger *et al*, 1989; Taira and Rhen 1989a, 1989b; Gulig and Chiodo, 1990; Caldwell and Gulig, 1991; Krause *et al*, 1991; Taira *et al*, 1991; Gulig *et al*, 1992) and the use of DNA probes in combination (Woodward *et al*, 1989) have confirmed the homology of a common virulence region and its wide distribution among isolates of Typhimurium, Choleraesuis, Dublin and Enteritidis. The virulence genes have been shown to be present on an 8kb region common to the SAPs of several *Salmonella* serotypes (Gulig *et al*, 1993). There are five plasmid virulence genes and under the terms of the common nomenclature these have been designated *spv*RABCD i.e. *spv* for Salmonella plasmid virulence. These genes have been shown to be induced at stationary phase and in carbon deficient media (Fang *et al*, 1991; Coynault *et al*, 1992; Valone *et al*, 1993). Krause and colleagues (1991) demonstrated that the *spv*RABCD genes formed an operon regulated by the product of the *spv*R gene. The *kat*F (*rpo*S) locus which encodes an alternative sigma factor (σ^S), in conjunction with SpvR controls the transcription of the regulatory gene *spv*R (Kowarz *et al*, 1994). Spink and colleagues (1994) showed that the SpvA protein affects *spv*R expression by a negative feedback mechanism for this operon. Since *kat*F controlled the expression of *spv* genes it has been hypothesised that chromosomal genes involved in *Salmonella* virulence may also be regulated by the *kat*F gene.

Although it has been established that the *spv*RABCD locus has an important role in *Salmonella* virulence there have been other distinct plasmid loci identified that affect serum resistance under certain circumstances and therefore enhance virulence. All of these determinants map outside the *spv* region and do not appear to be essential for virulence. They are, in Typhimurium, the *tra*T gene, (Rhen and Sukupolvi, 1988), the *rsk* region (Vandenbosch *et al*, 1989) and the *rck* locus (Hackett *et al*, 1987; Heffernan *et al*, 1992). A fourth locus has also been identified in Dublin (Terakado *et al*, 1990).

1.4.8 Plasmid profiles and epidemiology in *Salmonella*

Much of the initial work done using PPA focused on the relationship of plasmids to antibiotic resistance (Elwell *et al*, 1978; Threlfall *et al*, 1978; O'Brien *et al*, 1980; O'Brien *et al*, 1982). However, it soon became apparent that PPA could effectively be applied in the epidemiological study of infections (Bezanson *et al*, 1983; Threlfall *et al*, 1986; Ryan *et al*, 1987; Spika *et al*, 1987; Lin *et al*, 1988; Mayer, 1988).

Taylor and colleagues (1982) provided one of the first studies which utilised PPA to investigate a salmonellosis epidemic. *Salmonella* Muenchen was isolated from eighty five cases in the United States of America in a multistate outbreak. No food source could be implicated as the vehicle of transmission. It was subsequently discovered that rates of exposure to marijuana were higher in patients than in control groups. Samples of marijuana taken from patients households were found to contain as many as 10^7 *Salmonella* Muenchen per gram. All isolates, from both patients and marijuana, were fully sensitive to all antibiotics tested. Plasmids of 3.1 Md and 7.4 Md were found in every isolate epidemiologically associated with the contaminated

marijuana. None of the control strains from previous years and from unrelated sources had the same plasmid profile.

In 1984, Holmberg and colleagues provided a comparison of typing methods for the characterisation of Typhimurium isolates from outbreaks. One of the key features of this study was the diversity observed in plasmid profiles. It is unlikely that two strains with different histories will accumulate the same plasmids. It is also not necessarily true that strains with the same PP are epidemiologically related because if one or more plasmids conferred useful properties these plasmids will be disproportionately conserved. Thus two strains that are not related within an outbreak may show the same PP. The presence of a virulence associated plasmid in some serotypes of *Salmonella* can therefore pose problems in interpretation (Brown *et al*, 1986). The presence of additional plasmids, whether large or small, in salmonella isolates increases discrimination because they are diverse.

1.4.9 Ribotyping

Restriction fragment length polymorphism analysis (RFLP) is a useful method for the characterisation of several microorganisms of medical importance. However, the interpretation of RFLP patterns obtained by the digestion of chromosomal DNA with restriction enzymes can be difficult due to the large number of fragments present. Ribosomal ribonucleic acids (rRNA) have been highly conserved during evolution, and since these genes are present in all bacteria, usually in several copies in the chromosome, they can be used as probes on Southern blots (Southern, 1975) of RFLP gels (Grimont and Grimont, 1986). Several restriction fragments that carry rRNA gene sequences can be observed (Ostapchuck, 1980) and since the number and location of copies differs, a restriction pattern of rRNA genes can carry useful information. The number of hybridisation bands (usually 7-12) is small enough to allow

simple analysis of the observed patterns, additionally, restriction sites within the rRNA operon can result in more bands than anticipated. The study of bacteria using this technique is called ribotyping and it has been frequently used for both taxonomic and epidemiological investigations of many organisms including *Salmonella* (Altwegg *et al*, 1989; Martinetti and Altwegg, 1990; Olsen *et al*, 1992; Nastasi *et al*, 1991; Esteban *et al*, 1993; Nastasi *et al*, 1993; Crichton *et al*, 1996).

Many of the studies have suggested that ribotyping was useful as a tool complementary to phage typing, antibiotic sensitivity typing or plasmid analysis. This suggested that ribotyping alone may not be sufficiently discriminatory for epidemiological purposes. It is also important to state that the technique has not as yet been standardised, as in the above studies each researcher found a different restriction enzyme produced the best selection of ribotypes within a serotype. Coherence can only be achieved if each laboratory that employs a technique employs the same strategy for its use.

1.4.10 IS200 typing.

In 1983 a new insertion sequence (IS) was identified in *Salmonella* (Lam and Roth, 1983). Termed IS200, this element was found to be 708bp long which made it one of the smallest mobile elements thus characterised (Gibert *et al*, 1991). IS200 has been found in all *Salmonella* serotypes except Agona but is absent from all other *Enterobacteriacae* with the exception of a few *Shigella* strains. The number of IS200 copies found in individual serotypes can vary from one to more than eighteen (Lam and Roth, 1983). The restricted distribution of IS200 within salmonella has allowed the development of a proposed genotypic typing method that has been utilised in investigations of different *Salmonella* serotypes.

Initially IS200 distribution was investigated within the genomes of the 27 phage type strains of Enteritidis (Stanley *et al*, 1991). This study identified three distinct IS200 profiles within Enteritidis and described these as 'clonal lineages', SECLI, II and III. Stanley and colleagues (1991) stated that "in terms of population genetics these IS200 profiles correspond to clonal lineages of recent evolutionary origin, and underlie the phage-typing scheme for epidemiological subdivision of *S.enteritidis*". This analysis suggested that IS200 profiles could not be used to subdivide Enteritidis within a phage type for epidemiological purposes. Once this had been established it became necessary to use IS200 profiles in combination with other typing methods in epidemiological investigations of Enteritidis (Stanley *et al*, 1992a, 1992b).

This multi-typing approach has also been applied to serotypes other than Enteritidis (Torre *et al*, 1993; Baquar *et al*, 1994; Pelkonen *et al*, 1994). In Heidelberg, IS200 profiles and plasmid profile analysis (PPA) were used (Stanley *et al*, 1992c) and seven intra-serotype clonal lines were identified. In Typhimurium, IS200 profiles, ribotyping (variation at the 16S *rrn* loci) and PPA were used and generated sixteen distinct types based on three 16S *rrn* profiles, thirteen IS200 profiles and eleven plasmid profiles from twenty five strains (Stanley *et al*, 1993). These data confirmed previous observations of relationships between Typhimurium phage types, for example, the DT108 and DT170 strains had identical IS200 and 16S *rrn* profiles which suggested a single clonal line. However as the phage types and plasmid profiles were both different, if IS200 and/or 16S *rrn* profiles were used in an epidemiological context the results above would have led to an erroneous conclusion of relatedness. The statement by Stanley and colleagues (1993) "that IS200 profiling may be as sensitive as phage typing in *S.typhimurium*" is at the very least excessive when applied in an epidemiological context.

It can be accepted that IS200 profiles provide information on genotypic relationships within serotypes of *Salmonella*. However, it would appear to be more discriminatory as a technique applied to the population genetics of *Salmonella* serotypes than to epidemiological investigations. It has recently been shown, by independent researchers, that at least one of the clonal lineages (SECLIII) observed by IS200 analysis of the Enteritidis phage types, can be broken down using other genomic typing methods (Olsen *et al*, 1994) or phage conversion studies (Rankin and Platt, 1995).

1.4.11 Pulsed - field gel electrophoresis.

The use of restriction enzyme fragmentation pattern analysis has previously been restricted, for the characterisation of genomic DNA, by two factors, i) the procedures used to isolate genomic DNA resulted in random shearing of these large DNA molecules and ii) large DNA fragments > 40kb cannot be effectively resolved with conventional agarose gel electrophoresis. These constraints have been overcome by the development of techniques for preparing intact chromosomal DNA by *in situ* lysis of bacteria embedded in agarose and the use of pulsed field gel electrophoresis (PFGE) to resolve the large fragments generated by digestion with enzymes which are known to cleave infrequently (McLelland *et al*, 1987; Finney *et al*, 1993).

Pulsed field gel electrophoresis resolves chromosomal DNA fragments by alternating the electric field between spatially distinct electrodes. One of the most effective PFGE systems provides a hexagonal array of electrodes in which the electric field alternates at a constant angle (typically 120°) to the direction of migration (Chu *et al*, 1986) this is termed the contour-clamped homogeneous electric field (CHEF) system.

Until recently (Tenover *et al*, 1995) there were no standardised criteria for the analysis of PFGE patterns. As the results generated during an investigation could, in theory, be interpreted differently by different investigators Tenover and colleagues (1995) have defined their own set of guidelines and have suggested that these guidelines be adopted by molecular epidemiologists, to resolve this situation. Generally the analysis of PFGE patterns obtained during an outbreak should be straightforward. Strains with identical patterns are considered to be clonal. If, and when, differences are detected in strains during an outbreak the interpretation of the data can become more complex. Random genetic events e.g. mutations, insertions and deletions may occur in some isolates. The presence (or absence) of plasmid DNA can affect the PFGE profile as can the presence (or absence) of lysogenic phages. The interpretation of these events and the changes which they elicit in PFGE patterns is crucial to the correct interpretation of the data.

By chance, some epidemiologically unrelated isolates may have similar or indistinguishable patterns, particularly if there is limited genetic diversity within a species or sub-type (Barrett *et al*, 1995). For example, most strains of methicillin-resistant *Staphylococcus aureus* are derived from a small number of ancestral clones (Kreiswirth *et al*, 1993) this can make it difficult to discern outbreak associated strains with endemic strains.

This problem has been demonstrated in the analysis of Enteritidis by PFGE. In 1994, Powell and colleagues, examined thirty nine strains of Enteritidis PT4 and elucidated nine distinct PFGE patterns using the enzyme *Xba*I. Of these thirty nine strains, thirty belonged to the same PFGE pattern and eight further patterns were represented by one strain only (with the exception of PFP7 which was represented in one human and one poultry isolate). It was concluded from this data that "PFGE provides a method for discriminating

strains of *S.enteritidis* PT4 suitable for epidemiological investigations". Given that >75% of the "unrelated" strains had the same pattern this can only be described as a poor conclusion.

In a larger study by Olsen *et al*, 1994, sixty two strains of thirty three different phage types of Enteritidis were examined by four different typing methods. PFGE, using the enzyme *Not*I, showed that strains from twenty one of the thirty three phage types formed one large cluster when fragments >125kb were compared. When all fragments were compared, ten patterns were observed. Although this appears to be of some value for discrimination, it should be remembered that these PFGE patterns were generated from thirty three different phage types. It is acknowledged that phage typing alone is not an ideal system and that conversion can occur by many previously defined mechanisms (Chart *et al*, 1989; Frost *et al*, 1989; Threlfall *et al*, 1993; Rankin and Platt, 1995) however, phage types are known to be relatively stable and the application of PFGE to these strains has merely served to condense the data into seemingly less meaningful units, particularly for the epidemiologist. Olsen and colleagues (1994) have acknowledged that the data presented do not disclose how the events that led to different "types" relate to each other in time but in an epidemiological investigation the time factor should be sufficiently short that these random genetic events should rarely occur. The genome structure of an organism would be expected to vary less with time than the extra chromosomal DNA content or the phage type.

Thong and colleagues (1995) compared the PFGE patterns of one hundred and sixty one Enteritidis isolates from Switzerland and Malaysia and confirmed that epidemiologically unrelated isolates from both countries were identical and suggested that they may be clonally related. Ribotyping was also performed and although the authors concluded that both techniques were of

limited value, it was shown that ribotyping may be more sensitive than PFGE for epidemiological investigations of Enteritidis strains.

1.4.12 DNA probe and polymerase chain reaction (PCR) detection systems.

DNA probes use DNA sequences specific to an organism to detect, by nucleic acid hybridisation, the complementary DNA sequences in clinical samples and, therefore the pathogen under investigation. Since most micro-organisms possess some sequence of DNA unique to that organism a specific DNA probe should, theoretically, be available for every organism (Lowe, 1986; Eisenstein, 1990).

The first probes were invariably derived from DNA sequences extracted and cloned from the genome of the organism. However, many DNA probes are now derived from plasmid DNA or small oligonucleotide sequences which have been synthesized *in vitro* as automated processes have developed. Regardless of its origin the primary requirement of any probe is that its sequence be both unique to and conserved within the group of organisms to be identified.

The development of such probes is both laborious and costly. A sequence of DNA must first be identified, cloned and labelled in such a way that it can subsequently be detected. DNA must be isolated from the organism to be detected and immobilised on a solid support such that the detection process can be completed. These techniques are lengthy, can be technically demanding and invariably confirm only that the DNA of the organism was present in the sample. Probes are primarily used as detection tools, however, in many situations such as the food industry this is the only requirement to be met.

A major advantage of probe techniques is speed of detection since it does not require that the organism be propagated in the laboratory. However,

this approach has the disadvantage that it does not allow for subsequent antimicrobial testing or strain typing, both of which are crucial in clinical and epidemiological investigations.

Probes to *Salmonella* serotypes have been developed (Fitts *et al*, 1983; Fitts, 1985; Rubin *et al*, 1985; Tompkins *et al*, 1986; Gopo *et al*, 1988) but none have been evaluated in prospective clinical trails and thus their sensitivity and specificity are unknown.

The development of the polymerase chain reaction (PCR) by Saiki and colleagues in 1985 was a major milestone in molecular biology. PCR allows the amplification of small numbers of DNA molecules by successive rounds of denaturation and replication. Initially this technique required some knowledge of the DNA sequence of the target DNA as the process involved the use of short primers on either side of the target sequence as the starting point for amplification. The primer sequences bound to homologous DNA sequences in the sample, the double stranded DNA was denatured and DNA polymerase was then added to produce a new strand of DNA from the target DNA template. Multiple cycles resulted in an exponential increase in the number of DNA strands present and the amplified target DNA sequence could then be detected by conventional agarose gel electrophoresis. The ability of PCR to detect and amplify tiny amounts of DNA gave it the potential to be an extremely useful detection tool. The situation became more complex however when it was discovered that PCR was sensitive to inhibition by many factors present in crude clinical samples. Blood, urine and faecal materials are all known to inhibit amplification by PCR (Wilde *et al*, 1990). Assays have subsequently been developed to attempt to overcome some of these factors. Widjojoatmodjo and colleagues (1992) found that immunomagnetic separation used before PCR allowed faecal samples to be diluted twenty-fold less than assays which used

PCR alone. The magnetic immuno PCR assay (MIPA) covered *Salmonella* serogroups A to E, which account for 95% of human clinical isolates. This method was sensitive enough to detect 100 colony forming units (cfu) per gram of faecal matter without pre-enrichment in broth cultures. A quantitative PCR assay has been developed for the detection of *Salmonella* serotypes in avian faeces (Mahon and Lax, 1993). This assay claims a sensitivity of 3 cfu per gram of faecal matter but has an added disadvantage in that it is capable of detecting *Salmonella* only when the organism harbours a virulence plasmid.

In 1990, Welsh and McClelland developed a PCR technique which used arbitrary primers to amplify chromosomal DNA sequences. The usefulness of this approach was that no prior sequence information was required. This allowed fingerprints of genomes to be evaluated, and potentially allowed the comparison of polymorphisms within the genome to be used to identify and differentiate even closely related strains of the same species. This technique was applied to Enteritidis by Fadl and colleagues (1995) who found differences among patterns of common human and avian associated phage types. The seven AP-PCR patterns generated from 32 Enteritidis isolates showed that differences could be detected within the same phage types and, perhaps most importantly, that isolates of different phage types shared the same pattern. As it is known that Enteritidis phage types can convert from one to another by various mechanisms (Threlfall *et al*, 1993; Rankin and Platt, 1995) this approach may allow identification of related strains where conventional typing methods infer no relationship.

1.5. Aims and objectives.

The epidemiology of *Salmonella* serotypes is complex and although many techniques have been applied in attempts to unravel these complexities, it

is clear that for the immediate future, no single approach will provide an optimal solution. However, in context, plasmid analysis may provide adequate information to allow epidemiologists to answer many of the questions raised during an investigation.

The primary aim of this work is to provide evidence that plasmid analysis, both PPA and REFP, provides a coherent system for the epidemiological evaluation of *Salmonella* serotypes with particular emphasis on Enteritidis.

Plasmid distribution and diversity will be examined in defined sub-groups of Enteritidis to assess discrimination within this serotype. The serotype associated plasmids will be examined in detail by restriction enzyme map data and hybridisation studies with probes generated to provide information on the molecular evolution of this 'family' of related plasmids. The concepts developed in the epidemiology of sub-groups of Enteritidis will be applied to case studies of outbreaks caused by serotypes other than Enteritidis to determine whether or not the system is applicable in these cases.

CHAPTER 2.

General Materials And Methods.

2.1 Bacterial strains and plasmids.

2.1.1 Isolation of Salmonella.

The majority of isolates used in the study were sent to The Scottish Salmonella Reference Laboratory (SSRL) for confirmation of serotype, and where appropriate phage typing. The isolates were primarily from Hospitals and Veterinary laboratories in Scotland. However additional isolates were obtained from The United States of America for research purposes and this is referred to where necessary.

As isolation procedures differ from laboratory to laboratory, on arrival at SSRL all original cultures were numbered and inoculated onto MacConkey agar (Oxoid Ltd, UK). These plates were incubated overnight at 37°C and a single colony was inoculated onto a Dorset's egg slope. The slopes were incubated overnight at 37°C and stored at room temperature. All subsequent analyses were performed from sub-cultures from the Dorset's egg.

2.1.2 Maintenance and preservation of strains.

CLED agar (Mast) was inoculated from Dorset's egg slopes and incubated at 37°C overnight. The CLED plates were stored at 4°C for up to four weeks, after which they were subcultured to fresh plates. All media used for subsequent analyses were inoculated from the original CLED plate.

Dorset's egg slopes were inoculated in duplicate incubated at 37°C and stored at room temperature. For long term storage of isolates, a nutrient agar plate was harvested with a sterile swab and suspended in 1% proteose peptone number three (Difco Laboratories, Detroit, Michigan, USA) that contained 8% glycerol and frozen at -70°C. For subculture, suspensions were thawed by immersion in water at 60°C for two minutes.

2.2 Plasmid DNA preparation and agarose gel electrophoresis.

2.2.1 Plasmid DNA preparation in crude lysates.

Organisms were inoculated onto nutrient agar plates and incubated overnight at 37°C. Cells were harvested by sweeping a sterile swab over the surface of the plate and rotated several times in a 1.5ml microcentrifuge tube, which contained 600µl of TB. Three hundred microlitres of 10% SDS in TB was added and the contents were mixed gently by inversion. The tubes were placed in a heating block (Techne Dri-block, DB-1) at 50°C for ten minutes until the suspension became clear and viscous. The tubes were centrifuged at 13000g for ten minutes in a microcentrifuge (Heraeus Biofuge 13). After centrifugation the pellet was removed and the lysate, which contained the plasmid DNA, was used immediately for electrophoresis, or stored at 4°C for up to one week.

2.2.2 Purified plasmid DNA preparation.

Plasmid DNA was purified by a modification of the method of Birnboim and Doly (1979). Organisms were inoculated into 10ml BHI broth, in a universal container (Sterilin), and incubated at 37°C overnight. After centrifugation at 4000g (MSE, Mistral 1000) for ten minutes the supernatant fluid was discarded and the cell pellet was resuspended in 400µl TGE buffer using a vortex mixer (Vortex Genie 2, Scientific Industries). The cell suspension was divided equally between two 1.5ml microcentrifuge tubes and centrifuged for thirty seconds at 13000g, all subsequent centrifugations were carried out at this speed. The supernatant fluid was discarded using a Gilson micropipette. To each tube, 200µl of lysozyme/TGE (5mg/ml) was added. The pellet was resuspended and the tubes incubated on crushed ice for five minutes. After incubation, 400µl of freshly prepared alkaline SDS solution was added. The tubes were mixed by inversion and incubated on ice for five minutes. Once

the suspensions were clear and viscous, 300µl of 3M sodium acetate was added and the contents mixed gently until a white precipitate had formed. The tubes were incubated on ice for five minutes followed by centrifugation for two minutes. The supernatant fluid was removed to a fresh microcentrifuge tube and 500µl of phenol:chloroform (1:1) was added. The tubes were mixed vigorously and were centrifuged for two minutes. The upper aqueous layer was removed to a fresh tube, care being taken not to disturb the white precipitate at the interface, and 500µl of isopropanol was added. Each tube was vortexed and left to stand at room temperature for five minutes. The isopropanol precipitates were centrifuged for 10 minutes, the supernatant fluids were discarded and all traces of alcohol removed using a micropipette. The air dried pellets were resuspended in 100µl TE buffer. Duplicate tubes were pooled and 100µl of 7.5M ammonium acetate was added followed by 600µl of ice cold 100% ethanol. The tubes were vortexed and incubated at -70°C for one hour or, alternatively, at -20°C overnight.

After incubation tubes were centrifuged for ten minutes. The supernatant fluids were discarded and the pellets were resuspended in 160µl of TE. Ribonucleic acids were removed by the addition of 18µl RNAase (1mg/ml) followed by incubation at 37°C for thirty minutes. After incubation, 20µl of 2.5M sodium chloride was added followed by 500µl of phenol:chloroform, the solutions were mixed vigorously and centrifuged for two minutes. The upper aqueous layer was removed to a fresh tube, and 500µl of isopropanol was added. Each tube was vortexed and left to stand at room temperature for five minutes. The isopropanol solutions were centrifuged for ten minutes. The supernatant fluids were discarded and air dried pellets were resuspended in 100 µl TE buffer. One hundred microlitres of 7.5M ammonium acetate was added followed by 600µl of ice cold 100% ethanol. The tubes were vortexed and incubated at -70°C for one hour or, alternatively, at -20°C overnight. The tubes

were centrifuged and the supernatant fluid discarded. The pellets were allowed to air dry for ten minutes and finally 60µl of TE was added to resuspend the plasmid DNA. This 60µl was sufficient for three digestions.

2.2.3 Restriction endonuclease digestion of plasmid DNA.

Unless otherwise indicated all enzymes used were obtained from Life Technologies Ltd (Paisley, UK) and were used according to the manufacturers instructions. Each digestion was carried out in a sterile 1.5ml microcentrifuge tube which contained the following reaction mixture; 5µl of a ten times concentrated reaction buffer, 2µl of enzyme, 23µl of sterile distilled water and 20µl of plasmid DNA solution. Each tube was vortexed to thoroughly mix the contents and centrifuged for five seconds to ensure that all of the reactants were in contact. The reaction mixtures were incubated for four hours at the temperature recommended by the manufacturer. After incubation, 5µl of tracking dye was added to each tube in preparation for loading the gel.

2.2.4 Agarose gel Electrophoresis.

Plasmid profiles were run on a 0.7% agarose gel. To a 250ml Pyrex conical flask 125ml of TBE buffer was added plus 0.9g of GTG Agarose (Flowgen, UK) this was brought to the boil and allowed to cool slightly before casting the gel. The gel was allowed to set for one hour and was placed in an electrophoresis tank. To load the gel, 10µl of Blue juice was added to 100µl of each lysate and this was then added to each well. The wells were sealed with molten agarose and allowed to set for five minutes. The buffer reservoirs of the electrophoresis tank were filled with TB buffer. The gel was electrophoresed at 100V (constant voltage) for one hour followed by three hours at 200V. After electrophoresis the gel was removed from the plates and stained in ethidium bromide/TES solution for fifteen minutes.

Restriction endonuclease digests were run on a 0.8% agarose gel. The gel was prepared by adding 0.8g of GTG agarose to 100ml TEB buffer which was boiled and allowed to cool before pouring. The gel was placed in a commercial electrophoresis tank (MH1510, Flowgen, UK) which contained TEB buffer to cover the gel. The contents from each reaction tube were loaded into the wells, under buffer, using a micropipette. Gels were electrophoresed at 18mA, constant current, overnight.

2.2.5 Visualisation of plasmid DNA.

The stained gels were placed on an ultraviolet transilluminator (Fotoprep 1, Fotodyne, USA) to visualise the DNA. Gels were photographed using a Polaroid MP4 land camera on Polaroid type 552 or 665 film, following the manufacturers instructions.

2.3. Recovery of DNA fragments from agarose gels using DEAE membrane.

After electrophoretic separation in 0.8% agarose gels DNA fragments were removed using NA-45 DEAE membranes (Schleicher & Schuell Inc, Germany). Before use the membranes were cut into 3 x 0.5cm strips and treated, to increase their binding capacity, by washing for ten minutes in 10mM EDTA pH7.6, five minutes in 0.5M sodium hydroxide followed by several rapid washes in sterile distilled water. The membranes could then be stored in water at 4°C for up to four weeks.

To increase the DNA yield for further digestion, fragments were recovered from the gels in multiples of six. A strip of DEAE membrane was placed in an incision just ahead of the fragments of interest. This was done on a transilluminator set on the preparative mode to ensure that UV damage was

kept to a minimum. Electrophoresis was continued for one hour at 30mA. The strips were then washed in 1ml NET buffer to remove residual agarose.

To elute the DNA from the membrane, 250µl of High salt NET buffer was added to a microcentrifuge tube, the strip was submerged and incubated at 60°C for forty five minutes. The membrane strips were washed with a further 50µl of buffer and discarded. The DNA was precipitated by adding 600µl of 100% ethanol followed by incubation at -20°C overnight.

The tubes were centrifuged for ten minutes and the supernatant fluid was discarded. The pellet was washed in 80% ethanol followed by centrifugation for ten minutes at 13000g. The supernatant fluid was discarded and the DNA pellet allowed to air dry for ten minutes before resuspending in 20 µl of TE buffer. This was subsequently used for restriction endonuclease digestion.

2.4 Cloning and transformation of competent cells.

2.4.1 Selection of vectors.

The vector chosen for cloning with *Pst*I generated DNA fragments was pUC19 which is a small, high copy number *E.coli* plasmid cloning vector that is 2.686 base pairs in length. pUC19 is part of a series of related plasmids which contain portions of pBR322 and M13mp19. The vector DNA was supplied by Life Technologies. Cloning with *Sma*I generated DNA fragments was performed with Ready-To-Go pUC18 (Pharmacia, UK).

2.4.2 Dephosphorylation of vector DNA.

The vector DNA (5μg) was digested with the restriction endonuclease *Pst*I according to the manufacturers instructions. When digestion was complete the linearised DNA was extracted with two volumes of phenol:chloroform followed by centrifugation for two minutes at 13000g. The DNA was precipitated with two volumes of 100% ethanol for fifteen minutes at 0°C followed by centrifugation for ten minutes. The DNA pellet was resuspended in 90μl of 10mM tris-HCl, pH8.3. A small aliquot (200ng) was removed and stored at -20°C. The DNA was dephosphorylated by the addition of Calf intestinal alkaline phosphatase (CIAP). To remove 5′ cohesive termini (as generated by *Pst*I) one unit of CIAP was added per 100pmoles of vector DNA followed by 10μl of CIAP buffer as supplied by Life Technologies. The reaction was incubated for thirty minutes at 37°C. After incubation the CIAP was inactivated by heating at 75°C for ten minutes. The dephosphorylated DNA was precipitated with 0.1 volume 3M sodium acetate, pH7.0, incubated at 0°C for fifteen minutes followed by centrifugation for ten minutes. The pellet was washed in 70% ethanol and resuspended in TE buffer at a concentration of 100μg/ml. This was aliquoted and stored at -20°C.

2.4.3 Ligation.

The vector DNA was ligated to the insert fragment of plasmid DNA using T4 DNA Ligase supplied by Life Technologies. Dephosphorylated pUC19 DNA (0.1μg) and an equimolar amount of foreign DNA were added to a sterile 1.5ml tube. Water was added to a volume of 7.5μl and the tube was incubated at 45°C for five minutes to melt cohesive ends followed by rapid chilling to 0°C. The T4 DNA ligase was added according to the manufacturers instructions and the reaction was incubated for one to four hours at 16°C. The ligated DNA was used to transform competent *E.coli*.

2.4.4 Transformation.

DH5α Maximum efficiency competent cells were supplied by Life Technologies and stored at -70°C. For use the cells were thawed on ice and 100μl were transferred to chilled Falcon tubes. DNA (1μl) from the ligation reaction was added to the cells and the reaction was incubated for thirty minutes at 0°C. The cells were heat shocked for forty five seconds at 42°C followed by incubation on ice for two minutes. To each tube 0.9ml of SOC medium was added and the cells were incubated at 37°C for one hour with vigorous shaking. After incubation the reactions were diluted 1:10 and 1:100 and 100μl was spread onto an LB agar (Life Technologies) plate with Ampicillin (Sigma, UK) selection (100μg/ml). The plates were incubated overnight at 37°C.

2.4.5 Identification of transformants.

Transformants were selected on LB agar plates which contained ampicillin (100μg/ml). Each pre-poured plate was spread with 40μl of X-gal (20mg/ml in dimethylformamide) and 4μl of IPTG (200ng/ml). The plates were dried and fifty single colonies were inoculated onto each plate from the transformation plates. The selection plates were incubated at 37°C overnight and white colonies were selected for further analysis.

2.5 Labelling and detection of DNA with the DIG system.

The system used for nucleic acid labelling and detection was purchased from Boehringer Mannheim, UK, and used according to the manufacturers instructions. The DIG system used digoxigenin to label DNA for hybridisation and subsequent detection. DNA probes were labelled with DIG - 11 - dUTP via random primed labelling. The DIG-labelled probes were hybridised to nitrocellulose membrane-bound nucleic acids on a Southern blot. The hybridised probes were then immuno-detected with an alkaline phosphatase-

conjugated Anti-digoxigenin antibody and visualised with the colorimetric substrates NBT and X-Phosphate.

2.5.1 Random primed DNA labelling.

The reagents were added to a sterile microcentrifuge tube (1.5ml), on ice, in the following order; DNA template (0.5 - 150µg/ml, variable), Hexanucleotide mixture (2µl), dNTP labelling mixture (2µl), sterile distilled water to 19µl and Klenow enzyme, labelling grade (1µl). The reaction tube was incubated for twenty hours. EDTA (2µl) was added to terminate the reaction.

The labelled DNA was precipitated with 0.1 volume of 4M lithium chloride solution and three volumes of chilled 100% ethanol. This was incubated at -70°C for thirty minutes. The solution was centrifuged at 13000g for fifteen minutes. The supernatant fluid was carefully removed and the pellet was washed in 70% ethanol. This was centrifuged at 13000g for five minutes at 4°C. The 70% ethanol was decanted and the pellet air dried. The dry pellet was resuspended in 50µl of TE buffer. This was used immediately or stored at -20° C. Before use the labelled probe was denatured at 100°C in a water bath for ten minutes followed by rapid chilling on ice.

2.6 Southern blotting and hybridisation.

The VacuGene XL Vacuum blotting system (Pharmacia LKB Biotechnology, Sweden) was used according to the manufacturers instructions.

When handling agarose gels, transfer membranes, the screen and the mask, latex gloves were worn. The gel was carefully positioned onto a nitrocellulose membrane and all air bubbles were removed. The vacuum pump was switched on

and the gel was thus immobilised.

The Depurination solution was poured over the surface of the gel and the pressure was set at 50mbar for four minutes. After this time residual liquid was removed using a pipette or by vacuum suction. The Denaturation solution was added for three minutes, followed by Neutralising solution for three minutes. This solution was removed and the gel was covered to about twice its depth with Transfer solution. Transfer was allowed to continue for one hour, after which the solution was removed and the gel was gently peeled off the membrane. The vacuum pump was switched off and the membrane was removed and blotted between filter papers. The transfer efficiency was assessed by placing the membrane on a UV transilluminator, as the ethidium bromide solution was not removed prior to transfer the DNA fragments could be clearly seen. The position of the tracks was marked with a sharp scalpel and the membrane was baked at 80°C for four hours.

All prehybridisation and hybridisation steps were performed at 42°C in a Hybaid hybridisation oven according to the manufacturers instructions. Prehybridisation was performed in 15ml of DIG-Easy Hyb solution (Boehringer Mannheim, UK) for one to twelve hours. The prehybridisation solution was removed and DIG-Easy Hyb solution which contained labelled probe at a 1:1000 dilution was added. Hybridisation took place overnight. The hybridisation solution was poured into a Falcon tube and stored at -20°C for re-use. The membranes were washed twice for five minutes in 2 x wash solution (2x SSC, 0.1% SDS) at room temperature followed by two times fifteen minute washes in 0.1 x wash solution (0.1x SSC, 0.1% SDS) at 68°C.

2.6.1 Immunological Detection.

The membrane was rinsed for five minutes in maleic acid buffer followed by incubation in 100ml blocking solution (1% blocking reagent in maleic acid buffer) for at least thirty minutes. The Anti-DIG-AP conjugate was diluted 1:5000 in 10ml blocking solution and the membrane was incubated in this solution for thirty minutes. This was followed by washing twice for fifteen minutes with 100ml of maleic acid buffer and the membrane was equilibrated for five minutes in 20ml detection buffer. The colour solution was prepared by the addition of 45µl of NBT and 35µl of X-phosphate to 10ml of detection buffer. The membrane was incubated in the colour solution at room temperature in the dark overnight. Once the desired intensity was reached the membrane was washed in 50ml detection buffer for five minutes to stop further colour development. The membrane was stored in a sealable bag containing TE buffer.

2.7 R-plasmid transfer by conjugation.

2.7.1 The mating process.

Donor and recipient cells were grown in 10ml BHI broth at 37°C overnight. They were mixed in a ratio of 1:4, donor : recipient, and incubated in four volumes of fresh BHI at 37°C for four hours.

2.7.2 Selection of transconjugants.

Transconjugants were selected on CLED agar plates which were inoculated with 100µl of the mating mixture using a sterile spreader. Antibiotic discs for selection of potential transconjugants were placed onto the plates using a sterile needle. Approximately ten to fifteen discs of each of the selective antibiotics were used, placed at a distance of 0.5cm from each other. Control plates were inoculated from broth cultures of donor, recipient and mating

mixture. Antibiotic discs were also placed on these plates to ensure that i) the organisms were resistant to the appropriate antibiotics and ii) both donor and recipient cells were present in the mating mixture. All plates were incubated at 37°C overnight.

The following morning potential transconjugants were taken from the inhibition zone and inoculated onto CLED plates to check purity. Again antibiotic discs were added to the plates to check that the transconjugants were now resistant to both. Single colonies were chosen for plasmid profile analysis to confirm the presence of transferred plasmid(s) in the recipient organisms. Dorset's egg slopes and glycerol peptone broths were inoculated for storage. These were stored at room temperature and -70°C respectively.

CHAPTER 3.

Evaluation Of Plasmid Distribution And Diversity Within *Salmonella enterica* Serotype Enteritidis.

3.1 Introduction

The prevalence of individual serotypes of salmonellae associated with human infections varies with time, geographical isolation and a wide range of additional parameters. Nevertheless certain serotypes of *Salmonella enterica* tend to dominate over long periods (e.g. Typhimurium and Enteritidis). Some remain notable by their persistence at low incidence such as Infantis, whereas a third group, typified in the UK by Virchow, Hadar and Agona have each achieved temporary prominence within a five to ten year period and then declined.

Enteritidis has been consistently among the ten most prevalent serotypes isolated from humans in Scotland for 20 years and the dominant serotype since 1987 (Platt, 1987; Platt *et al*, 1988a). Before 1987 phage types 4 and 8 were isolated in similar numbers but since then phage type 4 has predominated. This has been attributed to clonal expansion and corresponds to the increase in poultry and egg-associated infections in both the UK and USA (Orskov and Orskov, 1983; St Louis *et al*, 1988a and 1988b; Humphrey *et al*, 1989a and 1989b; Rampling *et al*, 1989; Rodrigue *et al*, 1990). Enteritidis PT4 has been found in both flocks of laying hens and broiler chickens and it has been assumed that these poultry are the major source for man. It is believed by some that the role of freshly cooked shell eggs in salmonellosis is currently much exaggerated (Duguid and North, 1991).

In food animals, other than poultry, both Enteritidis and Typhimurium have been isolated in the UK with similar frequency from 1989 to 1992. The figures for 1993 and 1994 showed not only a decrease in the number of Enteritidis isolations but also an increase in the number of Typhimurium isolates (Table 3.1). In poultry however, the number of Enteritidis isolations has consistently been higher than Typhimurium. Although, it should be noted that

Table 3.1 Incidents of *Salmonella enterica* serotypes Enteritidis and Typhimurium from food animals in the United Kingdom from 1989 through 1994.

SEROTYPE	1989	1990	1991	1992	1993	1994
Enteritidis	870	946	934	865	720	377
Typhimurium	915	944	890	861	1062	1237

Adapted with permission from:- MAFF Salmonella in animal and poultry production 1994.

in 1993 and 1994 both serotypes showed an approximate three-fold reduction in numbers. This decrease does not appear to have been accompanied by any noticeable increase in the number of isolations of any other serotype (Table 3.2).

In Scotland the annual number of human isolations of Enteritidis has continued to rise steadily since 1984 (Table 3.3) although the figures for animals and poultry have shown some evidence of decline (Table 3.4).

The mechanisms that underlie these patterns of incidence remain largely unknown, although the consistently dominant serotypes contain plasmids that enhance virulence in animal models and are now termed serotype associated plasmids (SAP) (Browning and Platt, 1995). In 1984, Popoff and colleagues, showed that the large plasmid of certain *Salmonella* serotypes constituted a single group of homology and represented a family of related plasmids. The plasmids were not identical but shared a high level of DNA-sequence homology and it was proposed that their dissimilarity represented the divergence of an ancestral plasmid. Baird and colleagues (1985) provided evidence of virulence sequences in the plasmids of Dublin and Typhimurium and in 1988(a), Williamson and colleagues, identified a common virulence region on plasmids from eleven different *Salmonella* serotypes. The concept of a serotype-specific plasmid (Helmuth *et al,* 1985) was dismissed for Enteritidis by Williamson and colleagues (1988a) as they found three virulence plasmids with different REFPs and different molecular weights in their study. Given that Popoff and colleagues (1984) suggested evidence for the divergence of an ancestral plasmid it should not seem unreasonable to find virulence plasmids of different molecular weights within a serotype which may represent stages in the evolution of these plasmids.

In a study to examine the possible relationship between the large plasmid in Enteritidis and virulence in animal models Nakamura and colleagues

Table 3.2. Incidents of selected *Salmonella enterica* serotypes from poultry in the United Kingdom from 1989 through 1994.

SEROTYPE	1989	1990	1991	1992	1993	1994
Enteritidis	738	843	816	684	598	230
Typhimurium	131	107	112	103	35	32
Virchow	16	21	17	22	25	51
Seftenberg	70	162	148	62	83	93
Ohio	5	2	1	2	2	28

Adapted with permission from:- MAFF Salmonella in animal and poultry production 1994.

Table 3.3. Human Salmonellosis: Isolations of *Salmonella enterica* serotypes Enteritidis and Typhimurium in Scotland from 1984 through 1994.

SEROTYPE	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994
Enteritidis	528	619	940	1345	1402	1241	1264	1618	1797	1701
Typhimurium	689	646	679	737	552	606	503	662	527	611

Adapted with permission from SCIEH Annual Report : Salmonellosis 1993.

1994 data; Personal Communication, Mr WJ Reilly, SCIEH. Ruchill Hospital, Glasgow.

Table 3.4. Isolations of *Salmonella enterica* serotype Enteritidis from food animals and poultry in Scotland 1984-1993.

SEROTYPE	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993
Enteritidis	0	12	4	1	38	148	128	187	102	27

(1985) established a possible relationship of the large plasmid found in Enteritidis with virulence in mice. It was also observed that two duck isolates which contained a plasmid of 58Md (88kb) were shown to be less virulent for mice than strains which contained the 36Md (54kb) plasmid. In contrast, in chick virulence assays the two duck isolates were virulent, whereas two strains which contained the 36Md plasmid were less virulent for chicks. Although not suggested it would appear that the two 58Md plasmids conferred some virulence mechanism. Evidence for molecular divergence of virulence plasmids has since been demonstrated (Platt *et al*, 1988b; Woodward *et al*, 1989; Brown *et al*, 1993; Browning and Platt, 1995; Rankin *et al* 1995).

The value of plasmid profile analysis and restriction enzyme fragmentation pattern analysis in epidemiological investigations is well established and has been extensively applied in the investigation of salmonellosis; (Taylor *et al*, 1982; Farrar, 1983; Riley *et al*, 1983; Platt *et al*, 1984; Nakamura *et al*, 1986; Platt *et al*, 1986a, 1986b; Rodrigue *et al*, 1992). The presence of virulence plasmids, however, has been viewed, largely, as disadvantageous for epidemiological investigations. Plasmid profile analysis has therefore been used primarily in combination with other typing systems (e.g. phage typing) in attempts to improve discrimination (Threlfall *et al*, 1989). The strategic application of restriction enzyme fragmentation pattern (REFP) analysis (Platt *et al*, 1986a) has demonstrated both overall conservation (Brown *et al*, 1986) and divergence (Platt *et al*, 1988b) of the SAP of Typhimurium and this has also been shown in selected type strains of Enteritidis. In 1993, Brown and colleagues used REFP analysis, with the enzymes *Hin*dIII and *Pst*I, to characterise plasmids in the type strains of Enteritidis phage types. The authors found that the REFPs generated from all but one of the 38Md plasmids (SAPs) were identical but showed heterogeneity in the patterns generated from plasmids of 45 and 59Md. They concluded that REFP analysis of the SAP was unlikely

to significantly extend the degree of discrimination achieved by phage typing and plasmid profile typing.

The aims of this part of the study were to determine a) the distribution of the Enteritidis SAP in defined subsets, b) the extent of diversity which exists between homogeneous and heterogeneous populations, c) whether molecular variants analogous to those reported by Brown and colleagues (1993) were detectable and d) to evaluate the combined results in terms of strain diversity based on the molecular characterisation of Enteritidis plasmids.

3.2 Materials and methods

3.2.1 Experimental Design

Realisation of the aims and objectives of the investigation required that a large collection of Enteritidis be examined. The strains available were diverse in terms of origin, but varied considerably in respect of the availability of epidemiological information and could not justifiably be described overall as a single representative collection. They were therefore grouped into a series of subsets defined by either the collection criteria or the parameter which formed the basis of subsequent correlation. Several subsets included sporadic isolates which may have belonged to either episodes or outbreaks and were unrecognised on epidemiological grounds. However, a single strain was included where outbreaks were defined and the isolated strains were shown to be consistent with expansion of a single clone. All of the isolates were phage typed and this is referred to where necessary (Table 3.5). Enteritidis GR16485, pOG674 was used as a reference strain for comparison of restriction endonuclease fragmentation patterns.

Subset 1 contained isolates from Scotland from the period 1986 - 1989. The isolates were heterogeneous in that they were from independent outbreaks or episodes and did not belong to any particular phage type. This subset was designed to give an account of the current distribution of plasmids. Subset 2 contained only phage type 4 isolates from both human and veterinary sources and all were isolated in 1990. This subset was therefore inherently homogeneous. Subset 3 contained seventy five isolates from 1987 - 1989 that were selected to represent phage types other than phage type 4 and included phage types isolated infrequently. Multiple isolates of selected phage types were included but where possible none of these were multiple isolates from an outbreak. Subset 4 contained isolates from the United States of America, from

both a poultry and egg associated outbreak and a selection of random veterinary isolates. This subset is further subdivided into three categories; 1. Isolates were recovered from poultry, 2. Isolates were egg associated, 3. Isolates were collected from diverse geographic locations: fourteen different states and also represented the situation in animals other than poultry. Isolates were included from a mink, rat, hedgehog, pig, horse, cat, monkey and a cow; geese, ducks and chickens were also included. Subset 5 contained isolates from Scotland and from the National Collection of Type Cultures (NCTC) all of which pre-dated 1979.

3.2.2 Construction of Transconjugants

Transconjugants were constructed as described previously (Chapter 2).

3.2.3 Plasmid profiles and restriction endonuclease fragmentation patterns

Plasmid DNA was examined in crude lysates prepared as described in Chapter 2. The molecular weight of plasmids was determined by reference to plasmids of known size (kb); Rts 1 (180), RA-1 (127), R1 (93), R702 (69) and RP4 (54). Supercoiled ladder (Life Technologies, Paisley, UK) was used for the molecular weight estimation of small plasmids (<16kb). Plasmid size values incorporated into plasmid profiles were determined on a minimum of two occasions.

Restriction endonuclease fragmentation pattern analysis was carried out as described in Chapter 2. Plasmid DNA was extracted and purified from clinical isolates and transconjugants by an alkaline lysis, phenol extraction and ethanol precipitation method.

Restriction enzymes were obtained from Life Technologies and used according to the manufacturer's instructions.

REFPs were compared with each other using a coefficient of similarity (Dice, 1945) calculated from the formula,

$$S_D(\%) = \frac{2m}{a + b} \times 100$$

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 plasmid respectively after digestion by the same restriction enzyme.

3.2.4 Computer-aided analysis of restriction fragments

Restriction fragment mobility in ethidium bromide-stained agarose gels was recorded on Polaroid type 665 film and input to an Viglen 486 microcomputer via a digitiser (Summagraphics). Each gel was calibrated with restriction fragments from a *Pst*I digest of bacteriophage lambda DNA either alone or in combination with an *Sma*I or *Hin*dIII digest of the same phage DNA. The molecular weight of these fragments was fitted to a robust modified hyperbola (Plikaytis *et al*, 1986) from which the size of restriction fragments in adjacent tracks was estimated. Numerical values were stored for subsequent graphical output, which was on a logarithmic scale. Experimental variation in fragment size did not exceed 5%.

3.2.5 Interpretation of restriction endonuclease fragmentation patterns

The following general rules were applied to the interpretation of plasmid REFPs-

(1) To establish that an observed REFP represented a variant of p0G674 demanded that its recognition was initially in a strain of Enteritidis in which it was the sole plasmid or was similarly present in an *E.coli* transconjugant. In the latter situation each of the fragments detected must have been present in the original isolate of Enteritidis.

(2) If the difference between the observed variant REFP and pOG674 was solely due to an additional fragment or fragments the same result must have been obtained when the plasmid was digested with twice the standard amount of restriction enzyme to exclude the presence of the products of partial digestion.

(3) Presumptive recognition of variants in clinical or veterinary isolates that contained additional plasmids was accepted either if the additional plasmid was substantially different in copy number or the variant had lost at least one fragment, or the additional plasmid(s) were conjugative and when each fragment in the transconjugant fingerprint was matched with the donor one or more additional fragments was seen compared to those from p0G674.

3.3 Results

3.3.1 Summary of results

A total of 434 isolates of Enteritidis were studied. They were grouped into five subsets. Overall 362 (83%) contained the SAP and in 275 (63%) isolates this was the sole plasmid. Additional plasmids greater than 20kb were present in thirty nine isolates two of which co-migrated with the SAP; co-resident small plasmids less than 20kb were detected in forty nine isolates. Plasmids other than, or in the absence of the SAP were harboured by 16 (4%) isolates: 41 (9%) isolates were plasmid free. Molecular variants of the SAP were detected in 17 (4%) of the isolates and were represented in four of the five subsets (Table 3.6 and Figures 3.1 - 3.3).

3.3.2 Subset 1. Among seventy five current Scottish isolates, 65 (87%) contained plasmids and 10 (13%) were plasmid free. Of these sixty five isolates, 50 (67%) contained the 54kb SAP alone whereas all except 2 (3%) others contained the SAP together with one or more additional plasmids. The 80kb plasmid, in the two isolates that harboured a single plasmid of 80kb, was found by REFP analysis to be an SAP variant (designated p0G691: Table 3.6). Of 13 (17%) isolates that harboured more than one plasmid all included an SAP; seven contained an additional smaller plasmid of molecular weight less than 10kb and in six isolates the additional plasmids ranged between 90 and 130kb.

3.3.3 Subset 2. Among one hundred and thirty one Enteritidis PT4 isolates from 1990, 108 (82%) harboured the SAP alone, 5 (4%) were plasmid free and 18 (14%) contained the SAP together with one or more additional plasmids. No variant plasmids were found in this subset.

Table 3.5. Source, distribution and plasmid profile of *Salmonella enterica* serotype Enteritidis isolates studied.

Source (No. of isolates)	Designated Sub-set	Plasmid Profile (kb)	Frequency	Phage Type
Scotland 1986-1989 (75)	1			
		54	50	
		ND	10	
		54: 7.0	3	
		*80	2	
		54: 2.2	1	
		54: 2.7	1	
		54: 3.4	1	
		54: 5.1	1	
		90: 54	1	
		100 54	1	
		110: 54	1	
		110: 54	1	
		130: 54	1	
		110: 100: 54	1	
Scotland 1990 (131)	2			
		54	108	4
		ND	5	4
		54: 1.3	9	4
		54: 2.0: 1.3	5	4
		54: 4.0	1	4
		54: 4.5	1	4
		54: 30: 8.0	1	4
		60: 54: 2.0	1	4
Scotland 1987-1989 (75)	3			
		54	4	1
		ND	1	2
		54: 5.0	1	5
		54	1	5a
		54	1	6
		54: 8.0	1	6
		54: 10	2	6
		54: 14	1	6
		54: 50	6	6
		54: 20: 9.0	1	6
		90 : 4.0	1	6
		ND	1	6a
		54: 8.0	1	6a

		54: 40	2	6a
		90 : 7.0	1	6a
		90: 54	4	6a
		130	1	6a
		54	4	7
		*80	1	11
		54: 2.5	1	12
		54	4	13a
		7.0	1	14b
		*54	1	15
		54: 9.0	1	21
		54	1	23
		54	1	24
		54: 45	17	24
		45	5	24
		*100	1	24
		54	1	25
		54	1	26
		80: 54	1	27
		10	1	29
		ND	1	32
USA (126)	4			
	(1)	54	19	
		ND	21	
		54: 5.0	1	
		54: 6.0	1	
		54: 7.0	4	
		54: 10	1	
	(2)	54	59	
		ND	1	
	(3)	54	10	
		*80	4	
		*54: 8.0	1	
		54: 8.0	1	
		70	1	
		160: 54: 3.5	1	
		60: *54	1	
Scotland 1977-1978 (27)	5			
		54	11	
		*54	2	
		ND	1	
		100	1	
		*80	1	
		65: 54	1	
		54: 4.0	2	
		54: 4.3	1	
		54: 4.5	1	

		54: 54	1	
		3.5	1	
National Collection of Type Cultures				
	NCTC618	*70: 7.0	1	
	NCTC3045	54	1	
	NCTC5188	70:*54	1	
	NCTC8515	*80	1	

***Denotes variant plasmid.**

ND = No plasmids determined

Table 3.6. Molecular variants of the serotype-associated plasmid of *Salmonella enterica* serotype Enteritidis.

Plasmid Designation	Molecular weight (kb)	Host strain	Phage type	Comment
pOG690	80	GRI 12888	9b	Subset 4. Intermediate
	80	GRI 12988	9b	Subset 4 Intermediate
	80	GRI 13088	9b	Subset 4 Intermediate
	80	GRI 13488	9b	Subset 4 Intermediate
pOG691	80	SR881190	11	Subset 1
	80	SR882596	11	Subset 3
	80	SR893743	9a	Subset 1
	80	GRI 10688	9a	Subset 5
	80	NCTC 8515	9a	Subset 5
pOG700	54	GRI 11888	4	Subset 5
	54	GRI 12088	4	Subset 5
pOG701	54	SR881508	15	Subset 3
pOG702	54	GRI 12588	RDNC	Subset 4
pOG703	54	GRI 12388	24	Subset 4
pOG704	54	NCTC 5188	6a	Subset 5
pOG705	70	NCTC 618	6a	Subset 5
pOG706	100	SR890702	24	Subset 3 Co-integrate

FIGURE 3.1.

***Pst*I REFPs of Enteritidis and Typhimurium SAPs plus Enteritidis variant plasmids.** ***Pst*I REFPs of Enteritidis plasmids (left to right). Lanes 1, lambda phage DNA *Pst*I; 2, pOG674; 3, pOG700; 4, pOG701; 5, pOG691; 6, pOG704; 7, pOG690; 8, pOG660; 9, pOG703; 10, pOG705.**

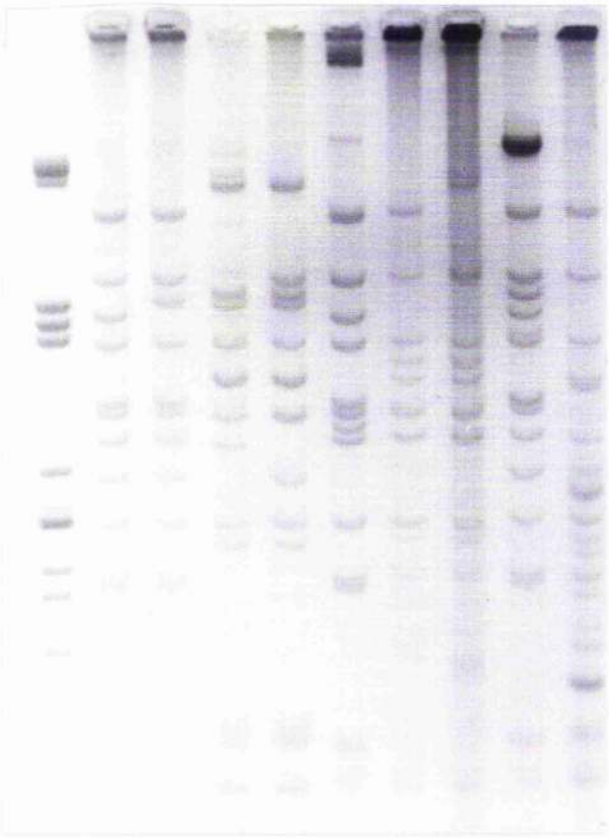


FIGURE 3.2.

_Sma_I REFPs of Enteritidis and Typhimurium SAPs plus Enteritidis variant plasmids. _Sma_I REFPs of Enteritidis plasmids (left to right). Lanes 1, _Pst_I lambda phage DNA; 2, pOG674; 3, pOG700; 4, pO704; 5, pOG701; 6, pOG691; 7, pOG690; 8, pOG703; 9, pOG705; 10, pOG660.

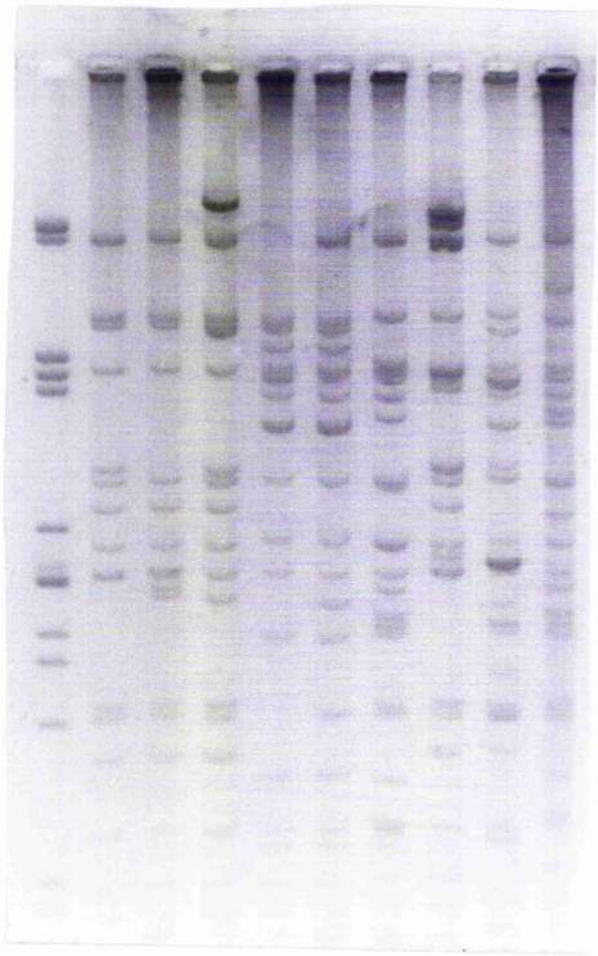
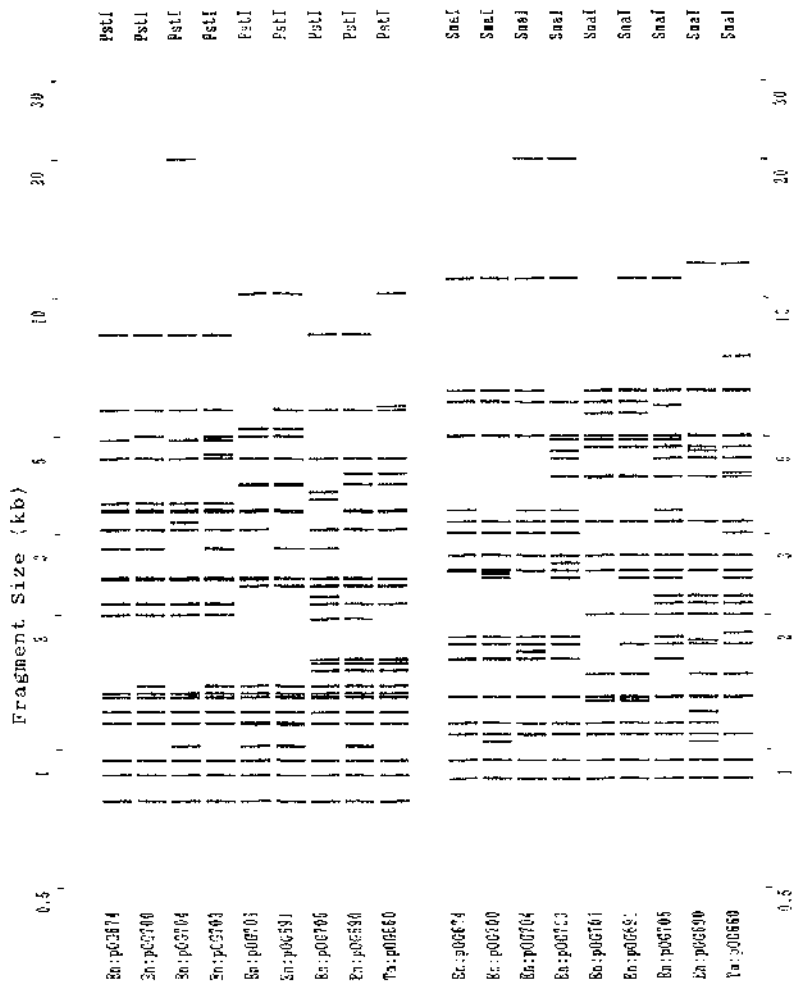


FIGURE 3.3.

Graphical output of digitised images from *Pst*I and *Sma*I REFPs of variant SAPs. Lanes 1 and 10, pOG674; 2 and 11, pOG700; 3 and 12, pOG704; 4 and 13, pOG703; 5 and 14, pOG701; 6 and 15, pOG691; 7 and 16, pOG705; 8 and 17, pOG690; 9 and 18 pOG660. Plasmids were ordered to optimise recognitions of variant fragments.



3.3.4 Subset 3. Initially, twenty isolates each of which belonged to a different phage type were examined for the presence of plasmids. Of these, 5 (25%) harboured the SAP alone, 2 (10%) were plasmid free, 2 (10%) contained a small plasmid but no SAP and in 2 (10%), variant plasmids were detected. One of the variants was identical to pOG691 (subset 1). The other was very similar but not identical to p0G691. It differed as described in detail below and was designated p0G701. Nine isolates were found to contain the SAP together with one additional plasmid.

One of twelve isolates which belonged to phage type 6 harboured the SAP alone. Six isolates showed the plasmid profile 54: 50kb and fingerprints very similar to two phage type 6a isolates with the plasmid profile 54: 40kb. The 50kb plasmid from phage type 6 isolates and the 40kb plasmid from phage type 6a isolates encoded ampicillin resistance and were therefore transferred to *E.coli* K12 to simplify analysis. The 40 and 50kb plasmids seen in these isolates appeared to be related. *Eco*RI and *Pst*I REFPs showed this (Figure 3.4 and 3.5) and from the data it was postulated that the 40kb plasmid was a derivative of one of the 50kb plasmids, possibly SR883224.

Of a further six, phage type 6 isolates, five contained an SAP together with one or more plasmid(s) and one isolate had the plasmid profile 90: 4.0kb. None of the nine phage type 6a isolates contained the SAP alone, six contained the SAP and one other plasmid (two of which were described above) one had the plasmid profile 90: 7.0kb and one contained a single plasmid of 130kb.

FIGURE 3.4.

Graphical output of digitised images of REFPs of plasmid DNA digested with *Pst*I and *Eco*RI. Ampicillin resistance plasmids were transferred from Enteritidis strains of PT6a (SR883576 & SR884424) and PT6 (all others) to *E.coli* K12 to simplify analysis. Lanes 1, SR883576; 2, SR884424; 3, SR883224; 4, SR884093; 5, SR892234; 6, SR892400; 7, SR892470; 8, SR 892607 digested with *Pst*I. Lanes 9 - 16 as above digested with *Eco*RI.

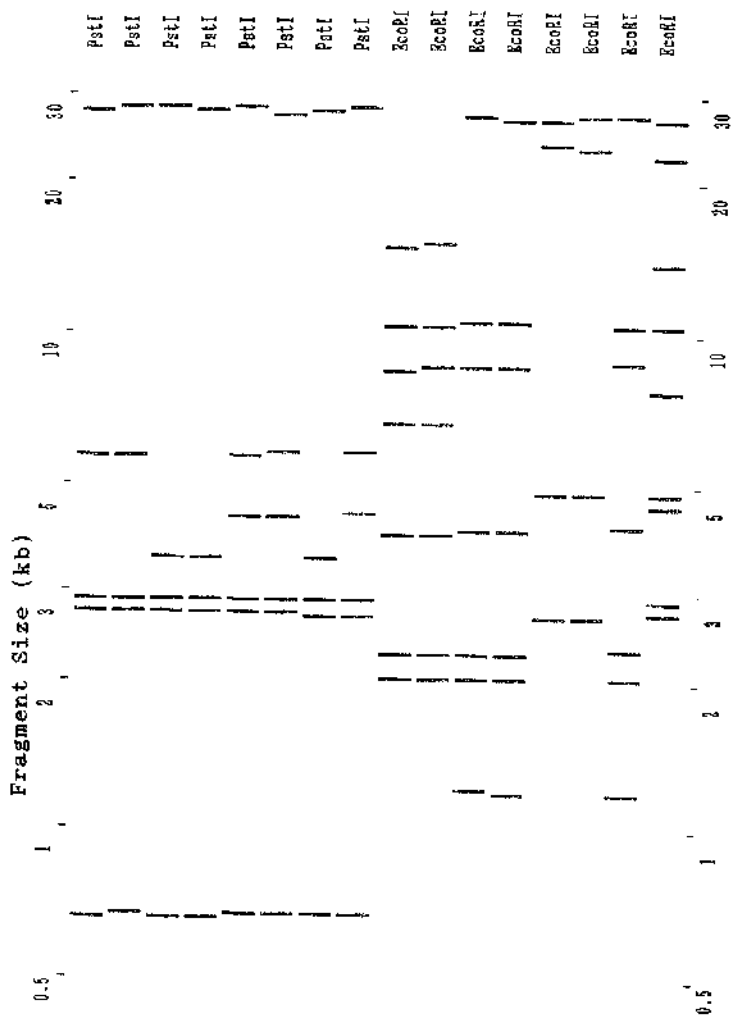
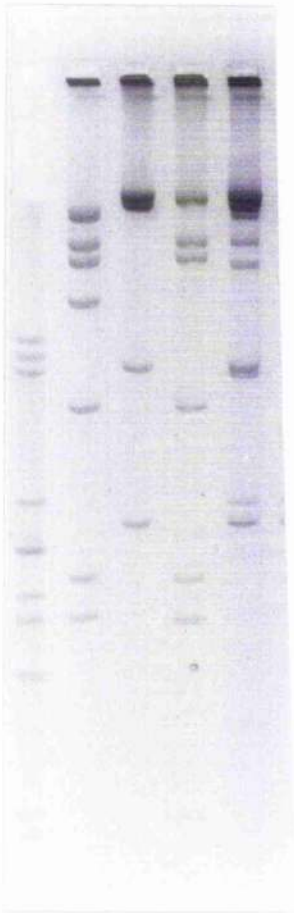


FIGURE 3.5.

***Eco*RI REFPs of ampicillin resistant transconjugants from Enteritidis strains of PT6a (SR883576) and PT6 (all others). The plasmids were transferred to *E.coli* K12. Lanes 1, Lambda DNA digested with PstI; 2, SR883576; 3, SR882234 ; 4, SR893224 and 5, SR892470.**



Twenty four isolates representative of phage type 24 showed one that contained the SAP alone, five isolates contained a single 45kb plasmid one had a 100kb plasmid and a further seventeen showed the plasmid profile 54: 45kb. The 45kb plasmid, after conjugative transfer and REFP analysis, from the seventeen isolates which also contained the SAP, was found to be identical to the 45kb plasmid from five strains in which this plasmid was present alone. The 100kb plasmid had the same REFP with four enzymes as those seventeen isolates which contained both the 54kb SAP and 45kb plasmids as distinct plasmids. However, after digestion with *HincII*, comparison of the REFPs showed minor differences in fragmentation pattern between the 100kb and the 54/45kb combination consistent with the former plasmid being a co-integrate which was designated pOG706 (Figure 3.6).

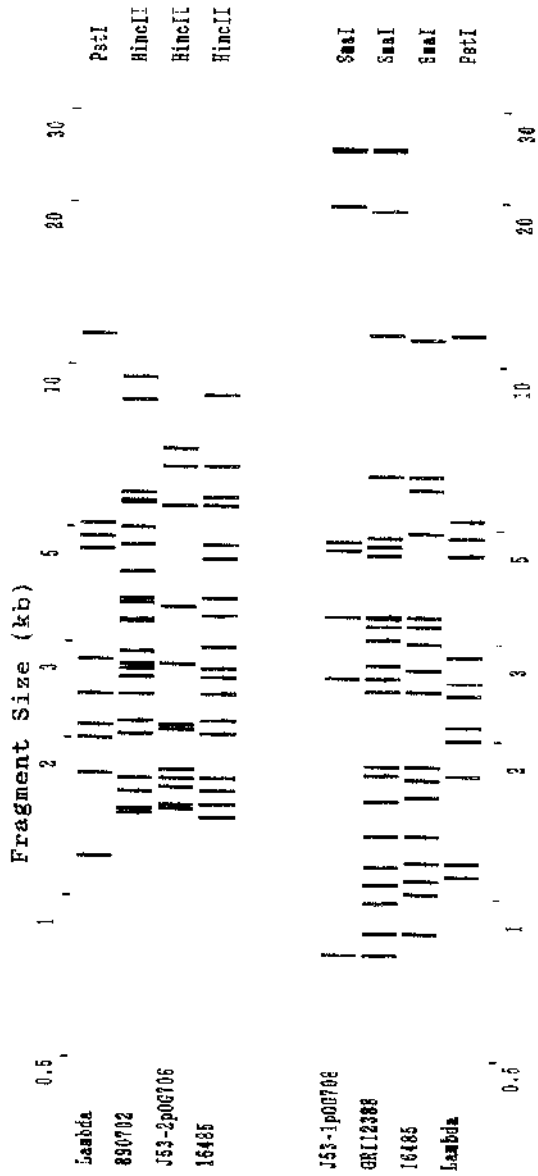
Five phage type 13a, 4 phage type 7 and 4 phage type 1 isolates contained the SAP alone.

In summary, 25% harboured the SAP only, 4% contained a variant SAP, 4% were plasmid free, 53% contained the SAP plus one or more additional plasmids and 15% harboured plasmids other than, and in the absence, of the SAP.

3.3.5 Subset 4. Episode 1 isolates recovered from a poultry associated source in the USA showed a high percentage (45%) of plasmid free strains: nineteen of the other twenty six isolates (40%) contained the SAP alone and 7 (15%) the SAP together with one additional small plasmid.

FIGURE 3.6.

Graphical output of digitised images from REFPs of plasmid DNA digested as follows. Lane 1, lambda DNA digested with *Pst*I; 2, SR890702 - 100kb co-integrate, *Hinc*II; 3, *E.coli* K12 J53-2 pOG706, *Hinc*II; 4, GRI16485, (pOG674), *Hinc*II; 5, J53-1 pOG708 *Sma*I; 6, GRI12388, *Sma*I; 7, GRI16485 (pOG674), *Sma*I; lambda DNA, *Pst*I.



Episode 2 isolates recovered from an egg associated source (egg yolk or selenite washed shells) showed 59 (98%) isolates that contained the SAP on its own and one plasmid free isolate.

Nineteen random veterinary isolates collected in the USA over a three year period from diverse geographical locations (fourteen states) were studied. Ten (53%) contained the 54kb SAP alone, three others contained the SAP in combination with one or more plasmid and one isolate had only a 70kb plasmid. The isolate with plasmid profile 54: 8.0kb contained a variant SAP designated pOG702. The isolate with plasmid profile 60: 54 kb contained two plasmids which, after REFP analysis showed the 54kb plasmid to be a variant SAP and was designated pOG703. The 60kb R-plasmid was transferred to *E.coli* K12 and showed no resemblance to the SAP with any of the enzymes used (Figure 3.4). In four further strains an 80kb SAP variant plasmid (designated pOG690) was found.

3.3.6 Subset 5. Four strains from the National Collection of Type Cultures (NCTC) showed one strain which contained an SAP alone. NCTC 618, contained a 90kb SAP variant designated pOG705, profile 90: 7.0 kb. NCTC 5188 showed the plasmid profile 70: 54 kb. The 54kb plasmid was a variant SAP, designated pOG704. The 80kb plasmid, p0G691, was also found in NCTC 8515. Twenty three strains, from the collection held by the Scottish Salmonella Reference Laboratory, isolated from 1977-78 made up the remainder of this subset. Eleven isolates contained the SAP alone, one contained a small 3.5kb plasmid, another only a 100kb plasmid and one was plasmid free. Six isolates contained the SAP together with one other plasmid; one of these was found to contain two 54kb plasmids (which co-migrated) neither of which were variant SAPs. Two other isolates contained a 54kb

plasmid alone, both of which were variants based on REFP analysis. These two isolates were Enteritidis phage type 4, and no variant SAPs have been reported in this phage type until this time. This plasmid was designated p0G700. The 80kb plasmid found in this subset was indistinguishable from p0G691. In summary, 12 (44%) isolates contained the SAP alone, 1 (3%) isolate was plasmid free, 6 (22%) contained variant SAPs, 6 (22%) contained the SAP together with one or more additional plasmids and 3 (9%) contained plasmids other than the SAP.

3.3.7 Analysis Of Variants

The SAP variants are shown in Figures 3.1 and 3.2 in which those plasmids most similar to the Enteritidis reference plasmid pOG674 are juxtaposed and those that showed a greater similarity to the Typhimurium SAP are grouped with the Typhimurium reference plasmid pOG660. The similarities found with both enzymes indicated that a gradation of relatedness was evident. This was confirmed by the similarity coefficients shown in Table 3.7

Plasmid pOG690 was detected in four American isolates of Enteritidis phage type 9b. Initial REFP analysis with *Pst*I, suggested that pOG690 bore a closer resemblance to the reference SAP from Typhimurium (p0G660) than the reference Enteritidis SAP (pOG674). Detailed comparison of the *Pst*I fingerprint of pOG690 with the SAP from both serotypes indicated that with the exception of a single restriction fragment (1.9kb) each of 24 fragments between 0.7 and 10 kb was present in either the Typhimurium or the Enteritidis reference SAP. Both of these SAPs shared only thirteen restriction fragments in common. This suggested that pOG690 was an evolutionary intermediate - *Sma*I, *Ava*II, *Hind*III and *Eco*RI fingerprints corroborated these results. Coefficients of similarity (Dice, 1945) between the reference SAPs (Table 3.7)

Table 3.7. Dice coefficients of similarity (S_D) from *Salmonella enterica* serotype Enteritidis plasmids on the basis of *Pst*I (upper) and *Sma*I (lower) REFPs.

Plasmid designation	660	674	690	691	700	701	702	703	704	705
pOG660		62	89	73	59	68	62	55	62	71
pOG674	57		68	70	93	63	92	55	92	70
pOG690	79	55		68	58	72	67	60	70	79
pOG691	74	68	83		56	86	NT	54	51	75
pOG700	73	91	62	78		60	NT	90	86	73
pOG701	73	55	78	90	60		NT	51	54	56
pOG702	64	100	61	NT	NT	NT		90	NT	NT
pOG703	59	78	65	68	81	58	NT		92	78
pOG704	68	97	63	65	89	70	NT	86		63
pOG705	81	75	74	81	71	74	NT	75	73	

NT denotes not tested

were 62% (*Pst*I) and 57% (*Sma*I). Comparisons of p0G690 with p0G674 gave S_D values of 68% (*Pst*I) and 55% (*Sma*I) and with p0G660 the values were 89% (*Pst*I) and 79% (*Sma*I). From the *Pst*I data it appeared that pOG690 shared at least 55kb and 37kb of its DNA with the SAPs of Typhimurium and Enteritidis respectively. The *Sma*I figures were 50kb and 27kb, whereas the two SAPs shared only 29kb (*Pst*I) and 30kb (*Sma*I).

p0G691. This plasmid was commonly seen in Enteritidis phage types 9a and 11. Dice coefficients of similarity indicated that this plasmid showed slightly more similarity with p0G660 than p0G674. However, this is in the order of a 2% difference with each of three enzymes.

p0G700. This plasmid was found in two isolates of Enteritidis phage type 4, from the Scottish collection of 1977-78. This plasmid showed an overall increase of 1.5kb compared to pOG674. The *Pst*I digest showed the loss of a 4.9kb fragment which was replaced by two additional fragments of 5.1kb and 1.4kb. The *Sma*I digest showed the loss of a 3.4kb fragment which had been replaced with two new fragments of 2.45 and 2.4kb, Fig 3.2. It is proposed that the additional 1.5kb is an insert or duplication that contained internal *Pst*I and *Sma*I restriction sites. The effects of this insert are seen in digests with both enzymes (Figures 3.1 and 3.2).

p0G701. This plasmid was found in Enteritidis phage type 15 isolates. It closely resembled p0G691 and Dice coefficients showed 86% and 90% similarity with *Pst*I and *Sma*I respectively. The Dice coefficients of this plasmid with the SSPs of Enteritidis and Typhimurium indicated a more marked similarity to p0G660 than p0G674.

pOG702. Preliminary data with *Pst*I and *Sma*I showed the loss of a 4.9kb fragment in *Pst*I digests however, no detectable differences were seen in the *Sma*I REFP. This plasmid was accepted as a variant SAP by previously defined criteria for the interpretation of restriction fingerprints. No subsequent work was done on this plasmid as the organism became non-viable during storage.

p0G703. *E.coli* K12 J53-1 transconjugants were characterised by plasmid profile and REFP in parallel with the parent strain, GRI12388. This confirmed that the 54kb plasmid present in the Enteritidis donor was a variant SAP. The *Pst*I digest showed an additional single fragment of 5.5kb, whereas the *Sma*I showed the loss of a 5.9kb fragment (Figure 3.6).

p0G704. This plasmid was seen in NCTC 5188 which was isolated in 1953 and was phage typed as 6a. The *Pst*I digest showed an additional 3.3kb fragment, concomitant with the loss of a 2.8kb fragment. The *Sma*I digest showed an additional 2.4kb fragment. The additional fragments seen in both digests were confirmed to have come from the 54kb plasmid by comparison with transconjugants as described above for p0G703.

pOG705. This plasmid was seen in NCTC 618 which was isolated in 1920 and was phage typed as 6a. In comparison with pOG674 the *Pst*I digest showed the loss of 4.9, 3.5 and 3.4kb fragments together with additional fragments of 3.7, 3.6, 2.3, 2.2, 1.95, 1.7, 1.6 and 1.4kb. This indicated at least 10kb of additional DNA together with several fragments of less than 1.2kb. The *Sma*I digest showed an overall increase of approximately 20kb which concurred with the *Pst*I data above.

3.4 Discussion

The results presented here demonstrated considerable diversity among the plasmids of *Salmonella enterica* serotype Enteritidis. However, the degree of diversity varied with the population studied (Rankin *et al*, 1995). In contrast to the report of Brown and colleagues (1993) a wider range of polymorphisms was demonstrated among 54kb plasmids. This was no doubt related in part to the greater number and diversity of strains examined in this study. However, close comparison indicated further contrasts, for example, the polymorphism shown in pOG701 (from Enteritidis PT15) differed from those described by Brown and colleagues (1993) and suggested that rather than being unique within this phage type, this phage type may show a propensity for polymorphism in the SAP. With regard to the 80kb plasmids, it is likely that at least some of these were analogous between the two studies, notably those contained by strains of Enteritidis PT11; unfortunately, phage types 9a and 9b were not represented among strains studied by Brown and colleagues (1993) and detailed comparison was further restricted by lack of *Sma*I fragmentation patterns. Two distinct plasmid fragmentation patterns were demonstrated in the SAP of PT6a (pOG704 and pOG705) and it was important to demonstrate that these differences were not due to the presence of additional plasmids like those seen in PT6a strains in subset 3.

The 40kb plasmid present in two phage type 6a isolates and the 50kb plasmid in six phage type 6 isolates were shown to be related, by REFP analysis. Vatopoulos and colleagues (1994) described in Enteritidis phage type 6a strains from Greece, a 34Md plasmid that encoded ampicillin resistance. This plasmid was shown to be in the N incompatibility group. Hybridisation of *Eco*RI restriction fragments of this 34Md plasmid with a TEM-type probe revealed the locus of the β-lactamase gene to be situated on a 6.6kb fragment. In this study strains SR883576 and SR884424 showed *Eco*RI REFPs that were identical to

the group I plasmids identified by Vatopoulos and colleagues (1994). The 6.6kb fragment was identified in the 40kb plasmids but was absent in the 50kb ampicillin resistance plasmids from PT6 strains although some degree of homology between the plasmids was evident (Figures 3.4 and 3.5).

The incidence of ampicillin resistance in Greece has been stated to be due primarily to the spread of PT6a. As indicated here Vatopoulos and colleagues (1994) have postulated the common evolution of ampicillin resistance plasmids in Enteritidis. The 40kb plasmid identified here was seen in two unrelated strains of PT6a with no known epidemiological relationships with those strains isolated in Greece. Further study of the two PT6a strains revealed that one of them (SR883576) may have been acquired abroad although no country was identified. The potential for country to country spread of microorganisms is vast and this raises questions with regard to both epidemiology and plasmid evolution. In the absence of the information that SR883576 had been acquired abroad the implication would have been that this strain, and particularly the R-plasmid, was endemic, in at least two European countries. In fact, at least five groups of related ampicillin resistant plasmids have been identified in Greece alone. No major studies have been done for the United Kingdom but unlike the situation we see with *Salmonella* virulence associated plasmids there are very likely a large number of groups of related ampicillin resistance plasmids.

In terms of evolution, both plasmids present in Enteritidis PT6a, would seem to offer some advantage but one (the SAP) is stable and maintained with little inherent variation, whereas the other shows variation at a greater frequency.

Plasmid profile analysis has proved to be a useful tool in epidemiological investigations of salmonellosis (Taylor *et al*, 1982; Riley *et al*, 1983; Nakamura *et al* 1986). However, this study has shown that in Enteritidis PPA offered little discrimination and could lead to the assumption that plasmids of the same molecular weight were the same plasmids. Plasmid REFP analysis resolved differences that remained undetected in PPA and demonstrated that plasmids of the same molecular weight can be unrelated and furthermore that plasmids of different molecular weights can be closely related. This study showed forty five different plasmid profiles when the isolates were examined by PPA alone but this figure increased to sixty five when the plasmid profiles were interpreted in the light of REFP analysis. Many of these would therefore have gone undetected with PPA alone. A simple pre-defined strategy (Platt *et al*, 1986a) of PPA together with REFP analysis optimised the information content and increased the level of discrimination.

Holmberg and colleagues (1984) concluded that PPA appeared to be at least as specific as phage typing in the recognition of epidemiologically related isolates of Typhimurium. Similarly, Kapperud and colleagues (1989) have stated that PPA and phage typing are valuable and convenient tools of considerable versatility in epidemiological tracing. Although useful, PPA is not without certain limitations and this study showed that the results should be interpreted, at the very least, with caution.

Six of the isolates in this study (Table 3.6) were found to contain 54kb plasmids which were molecular variants of the Enteritidis SAP. The variation would have been undetected by PPA alone. Similarly in subset 5, one isolate was found to contain two co-migrating 54kb plasmids. The presence of two plasmids was recognised only by REFP analysis. Two Enteritidis phage type 4 isolates were shown to contain molecular variants of the SAP and this was the

first report of phage type 4 isolates which showed SAP variation. However, most have been characterised by PPA and phage typing alone.

The importance of these results in the interpretation of PPs in epidemiology is twofold. The widespread presence of the 54kb plasmid among Enteritidis strains indicated that no epidemiological specificity was conferred by its recognition unless molecular variation was demonstrated by REFP analysis. Secondly, the accumulation of additional plasmids, although relatively uncommon in Enteritidis, significantly increased strain specificity because the additional plasmids were diverse.

Popoff and colleagues (1984) first suggested that the large plasmids of some serotypes of *Salmonella* constituted a family of related plasmids and concluded that they represented the divergence of an ancestral plasmid. This study described molecular variants of the Enteritidis SAP in seventeen of four hundred and thirty four isolates. Of nine variant plasmids which showed different degrees of divergence from the SAP, pOG690 showed particularly interesting features. This plasmid showed a more marked similarity to the SAP of Typhimurium than the SAP of Enteritidis. The virulence genes were assumed to be present in this plasmid based on the information that certain *Pst*I fragments have been sequenced in this region and that these fragments were present in all three plasmids (Threlfall *et al*, 1994). Beninger and colleagues (1988) concluded that despite differences in size and REFP of plasmids isolated from different *Salmonella* serotypes all of these plasmids encoded virulence functions by a common genetic mechanism. This mechanism may have arisen from a common progenitor which has proliferated and diverged to give rise to those plasmids which have been termed serotype associated: especially those of Enteritidis and Typhimurium. The findings with Dublin discussed by Platt and colleagues (1988b) suggested that the maintenance of selection for virulence

need not have co-selected for other determinants, which explained the lower level of REFP similarity of the Dublin SAP with pOG660. This explanation could be extended to the large plasmids of other *Salmonella* serotypes, many of which show similarity in the virulence region (Williamson *et al*, 1988a; Tinge and Curtiss, 1990a).

Heteroduplex analysis showed (Montenegro *et al*, 1991) that the plasmids of *Salmonella* serotypes Typhimurium, Enteritidis, Dublin and Choleraesuis shared large areas of homology. It was stated that the Enteritidis plasmid was 99% homologous to the Typhimurium plasmid and from these data a common ancestor was also suggested. It is currently considered that pOG690 is a direct evolutionary intermediate in the descent of virulence plasmids from Typhimurium to Enteritidis (Rankin *et al*, 1995). Logically, the molecular variation must have arisen by one of two general mechanisms. The currently predominant REFP was widely distributed among different phage types (Table 3.5) and was also evident in NCTC3045 isolated in 1929. Variant plasmids with a significantly higher molecular weight have therefore, either recently acquired additional DNA or alternatively the additional DNA represented an intermediate stage in the evolution of SAPs. The results presented favour the latter alternative. Nevertheless the question raised by Montenegro and colleagues (1991) of whether SAPs have evolved by ascent from the smaller plasmids of Enteritidis and Choleraesuis to the larger plasmid of Typhimurium, or the converse, remains unanswered. Although the elegant scenario proposed (Li *et al*, 1993) for the late evolution of the host adapted serotypes Gallinarum and Pullorum from an Enteritidis-like ancestor is internally consistent it seems neither to take adequate account of the non host adapted nature of Typhimurium nor the size of its virulence plasmid.

The results revealed that those populations which were epidemiologically homogeneous retained this feature after PPA and REFP analysis. There were some surprising results. The overall data showed that variant SAPs occurred among 4% of the isolates. This result was paralleled in subset 1 which contained a representative collection of current Scottish isolates. In subsets 4(3) and 5, variant SAPs occurred at 31 and 22% respectively. Both of these subsets are heterogeneous and represented situations that existed in the absence of currently predominant clones of phage types 4 and 8. It may therefore be inferred that in a dynamic, non-epidemic population, variation in the SAP occurs at a frequency far greater than that which is found when artificially created populations are examined. Randomly selected populations that include epidemic strains may therefore skew the data in favour of a less dynamic system. This is analogous to the phenomenon of 'periodic selection', an important consequence of which, is that the effective population size of a clonal organism will be much less than the actual size (Levin, 1981). To have included more than one representative isolate from the current phage type 4 'epidemic' created a situation that one aimed to avoid by initially including one representative from a known outbreak or episode. This is an important point in epidemiological investigations, particularly of 'epidemic' strains, where generally the relationship between clinical isolates and a suspected vehicle of transmission is purely by association. That the majority of isolates in an epidemic or outbreak are identical should be of no surprise. To discount any isolate which has a strong association with an outbreak, because it differs from that which is considered to be the epidemic strain - on the basis of plasmid analysis - as not being part of the outbreak may be ignoring the point that, particularly in Enteritidis, the system is not static and that evolution may occur at a rate that exceeds expectation.

The level of variation detected in this study was not unexpected and was principally a consequence of sample incomparability. Its anticipation was the reason why the strains were separated into subsets from the outset to minimise interpretative bias.

Although this work has focused on molecular variation in the SAP of Enteritidis this plasmid was highly conserved like the SAP of Typhimurium (Platt et al, 1988b) and in marked contrast to resistance plasmids (Krafl *et al*, 1983; Platt and Smith, 1991). Whereas the conservation of a virulence region might be expected and this has been shown to be about 8kb, the conservation of a further 46kb (Enteritidis) and 82kb (Typhimurium) was more surprising .

CHAPTER 4.

Analysis Of Enteritidis Variant Plasmids In Relation To *Pst*I And *Sma*I Restriction Maps Of pOG674.

4.1 Introduction

Several lines of investigation have been followed to clarify the relationship between the SAPs and virulence. Early DNA hybridisation studies (Popoff *et al*, 1984) using the entire Typhimurium plasmid as a probe indicated that the plasmids from Enteritidis and Dublin shared homologous sequences. They also established the absence of homology between these and the plasmids found among various other serotypes and suggested that the SAPs were related. Localisation of a virulence region (Williamson *et al*, 1988a, 1988b) and the use of DNA probes in combination (Woodward *et al*, 1989) have confirmed the homology of a common virulence region and its wide distribution among isolates of Typhimurium, Enteritidis and Dublin (Tinge and Curtiss, 1990a; Gulig *et al*, 1993). Whereas the application of DNA probes provides high resolution about a small proportion of the plasmid, restriction enzyme fragmentation pattern analysis allows comparison of entire plasmids at a lower resolution.

Williamson and colleagues (1988a) concluded that the concept of a serotype specific plasmid did not apply to Enteritidis on the basis of molecular divergence in a *XhoI*/*SalI* double digest of three strains that harboured plasmids of different sizes, and argued in favour of transposition of a common virulence sequence between unrelated plasmids. The findings presented thus far argue in favour of the evolution of a family of plasmids from an archetype, that may be represented by the SAP of Typhimurium, or related ancestor, and where the diversity of molecular variants within a serotype reflects both that serotype's fitness for one or more ecological niches and its ability to stably maintain a particular natural derivative of the plasmid.

These observations raise a number of questions; primarily, if an 8kb region of the plasmid common to several serotypes constitutes an essential

virulence region why is a much larger portion of the plasmid conserved within and between serotypes in the natural environment?

Previous work on the characterisation of Enteritidis plasmids revealed nine variants of the Enteritidis SAP. As discussed previously, it has been hypothesised that one of these plasmids, pOG690, may be an evolutionary intermediate between the Typhimurium SAP and that of Enteritidis. This conclusion was based on the patterns of restriction fragments produced with various endonucleases which indicated that pOG690 showed greater similarity to the SAP of Typhimurium than Enteritidis. Although molecular variants of the Typhimurium SAP have been described (Platt *et al*, 1988b) none have shown what may be the intermediate features of pOG690.

In 1991, Montenegro and colleagues performed heteroduplex analysis on the SAPs of Typhimurium, Enteritidis, Dublin and Choleraesuis and found that 99% homology was found between the Enteritidis and Typhimurium plasmids, which suggested a common ancestor. The Enteritidis plasmid could have been generated by deletion of the Typhimurium plasmid or the Typhimurium plasmid could have evolved by DNA integration into the Enteritidis plasmid. Given that pOG690 is 80kb, 10-15kb smaller than Typhimurium, and was found in Enteritidis, the former seems to be more plausible.

In 1987, Michiels and colleagues determined a physical map of the 90kb Typhimurium virulence plasmid using the restriction enzymes *Hin*dIII, *Bgl*II and *Bam*HI. This map showed the positions of the region involved in virulence and two origins of replication, *rep*A, the major replication origin and *rep*B, a minor replication origin. In 1990(a), Tinge and Curtiss confirmed the above observations but disagreed with the precise locations and incompatibility group

assignments given by Michiels and colleagues (1987). This resulted in an altered map which detailed the precise locations of *rep*A or *par* (Cerin and Hackett, 1989), *rep*B, *rep*C, the virulence region *vir* (Gulig and Curtiss, 1988; Gulig, 1990) and *tra*T (Rhen and Sukupolvi, 1988). This map also showed the restriction sites of, among others, the enzymes *Xba*I and *Xho*I.

In 1991, Sizemore and colleagues, performed Tn5 mutagenesis on the Typhimurium virulence plasmid and identified two novel regions which contributed to virulence. One of these was subsequently identified as the *pef* (plasmid encoded fimbriae) locus (Friedrich *et al*, 1993). This locus was found on a 13.9kb segment of the Typhimurium plasmid, between *rep*B and *rep*C and it has been suggested that this locus might encode a novel type of fimbria. The *rep* regions at either side of this fragment comprise regions capable of autonomous replication and an open reading frame was determined at the *rep*C end of the fragment which showed strong homology to the C-terminal regions of the RepA protein necessary for the initiation of replication in various bacterial plasmids. On the opposite DNA strand Friedrich and colleagues (1993) reported a coding region for a small polypeptide which showed strong homology to the C-terminal third of protein D of the mini-F plasmid and to replication proteins from other plasmids (Lane *et al*, 1986). Friedrich and colleagues (1993) have suggested that it is possible that the coding region for this protein was disrupted during the recombinational events leading to the formation of the Typhimurium plasmid. The Tn5 insertions generated by Sizemore and colleagues (1991) were in *Hin*dIII fragment H5 (Michiels *et al*, 1987) which is a 7.4kb fragment present in the Typhimurium virulence plasmid. Given the high degree of homology and conservation of the virulence region among SAPs it has therefore been assumed here that a region on the plasmid that encoded fimbrial genes may also be conserved. This prompted the question

of whether this region was present in whole or in part on the Enteritidis SAP and on any of the variant Enteritidis SAPs described previously.

To reconcile these observations a detailed restriction map of the Enteritidis SAP that allowed comparison with the published maps of Typhimurium was required to identify regions of homology and perhaps more interestingly, regions of non- homology. Once the map data has been completed it is proposed that fragments be isolated from which probes can be generated. The fragments will be selected to demonstrate homology and also to provide some information as to the evolution of these plasmids.

The primary aim of the study was to generate a restriction map for Enteritidis that included enzymes used previously for Typhimurium. The enzymes *Pst*I and *Sma*I are the most frequently used enzymes in the strategy developed for epidemiological REFP analysis of *Salmonella* plasmids (Platt *et al*, 1988a). If possible these enzymes should be included in a map of the Enteritidis plasmid. This should identify areas of homology between the two plasmids. The size of each fragment for Enteritidis, Typhimurium and the variant SAPs previously identified should be tabulated. This will allow the identification of a common core of fragments. Once this data has been generated fragments will be chosen to produce probes which will be hybridised with *Pst*I and *Sma*I digests of the whole plasmids. Many of these core fragments will have been identified in previously sequenced regions of the plasmid and will be located on some of the areas responsible for virulence (Gulig *et al*, 1993). The previously defined virulence region should be avoided but it may be possible to identify fragments in the *pef* region from which a probe could be generated.

4.2 Materials and methods

4.2.1 Bacterial strains and plasmids

The bacterial strains used in the study were Enteritidis GRI16485 (pOG674) and Typhimurium NCTC73 (pOG660). The following Enteritidis strains (and plasmids) from Chapter 3 (Table 3.6) were also used: GRI12988 (pOG690), SR881190 (pOG691), GRI11888 (pOG700), SR881508 (pOG701), GRI12388 (pOG703), NCTC5188 (pOG704) and NCTC618 (pOG705).

4.2.2 Experimental Design

Figure 3.3 (page 84) shows a graphical output of digitised images from *Pst*I and *Sma*I REFPs of Enteritidis variant plasmids. Stored numerical values of these images were tabulated (Tables 4.1 and 4.2) and from this data a 'common core' was postulated. This information will be further utilised at a later stage.

In 1994, Suzuki and colleagues published a restriction map of an Enteritidis plasmid (pNL2001) which included the enzymes *Hin*dIII and *Sal*I. The map also showed the location of the virulence genes. This data was used to confirm the results obtained here.

A restriction map of Enteritidis plasmid pOG674 was derived from comparison of digests and double digests with the endonucleases, *Xba*I, *Xho*I, *Hin*dIII, *Bam*HI, *Bgl*II and *Sal*I. *Pst*I and *Sma*I restriction fragments were added to this map, where possible. The position of these fragments were determined by the extraction of large restriction fragments from agarose gels followed by re-digestion with *Pst*I and *Sma*I. This technique was also employed in cases where the exact position of fragments was ambiguous from double digests.

Table 4.1

Enteritidis plasmids: *PstI* restriction fragment sizes (kb)

pOG 674	pOG 700	pOG 704	pOG 703	pOG 701	pOG 691	pOG 705	pOG 690	pOG 660
				10.10	10.10			10.10
8.3	8.3	8.3	8.3			8.3	8.3	
								5.8
5.7	5.7	5.7	5.7		5.7	5.7	5.7	5.7
	5.1		5.1	5.1	5.1			
				5.0	5.0			
4.9		4.9	4.9					
4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
							4.1	4.1
				3.9	3.9		3.9	3.9
						3.7		
						3.6		
3.5	3.5	3.5	3.5					
3.4	3.4	3.4	3.4	3.4	3.4		3.4D	3.4D
		3.3						
3.1	3.1	3.1	3.1			3.1	3.1	3.1
				3.0				
2.8	2.8		2.8		2.8	2.8		
2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
				2.3	2.3	2.3	2.3	2.3
						2.2		
2.1	2.1	2.1	2.1			2.1	2.1	2.1
2.0	2.0	2.0	2.0					
						1.95	1.95	
						1.7	1.6	1.6
						1.6	1.55	1.55
								1.5
	1.4					1.4	1.4	1.4
				1.38	1.38			
1.35	1.35	1.35	1.35	1.35	1.35	1.35	1.35	1.35
1.32	1.32	1.32	1.32	1.32	1.32	1.32	1.32	1.32
1.23	1.23	1.23	1.23	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
		1.03		1.03	1.03		1.03	
0.96	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
0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

D : Indicates a doublet.

Table 4.2

Enteritidis plasmids: *Sma*I restriction fragment sizes (kb)

pOG 674	pOG 700	pOG 704	pOG 703	pOG 701	pOG 691	pOG 705	pOG 690	pOG 660
11.0	11.0	11.0	11.0		11.0	11.0	11.0	11.0
								7.4
6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2
5.9	5.9	5.9		5.9	5.9			
						5.8		
				5.5	5.5			
							5.1	
5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
				4.9		4.9		
				4.7	4.7	4.7	4.7	4.7
						4.4	4.4	4.4
								4.1
				4.0	4.0	4.0	4.0	4.0
3.4		3.4	3.4			3.4		
3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
3.0	3.0	3.0	3.0					3.0
2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7
2.5D	2.5D	2.5	2.5D	2.5	2.5	2.5	2.5	2.5
	2.45							
	2.4	2.4			2.4	2.4	2.4	2.4
						2.2	2.2	2.2
						2.1	2.1	2.1
				2.0	2.0	2.0	2.0	2.0
								1.8
1.77	1.77	1.77	1.77			1.77		
							1.75	
								1.73
1.72	1.72	1.72	1.72		1.72	1.72		
							1.70	
1.58	1.58	1.58	1.58			1.58		
				1.46	1.46		1.46	1.46
1.32	1.32	1.32	1.32	1.32	1.32	1.32	1.32	1.32
				1.28	1.28			
							1.23	
1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	
1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08	1.08
	1.04						1.04	
0.96	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	0.87

D : Indicates a doublet.

The numerical data in Tables 4.1 and 4.2, in combination with the map data, allowed the identification of fragments as suitable candidates for the generation of probes. A 'common core' fragment, from outwith the virulence region, was chosen as a probe to demonstrate homology (4.4kb *Pst*I). Additionally, two non-core fragments were chosen from pOG674 that were not present in pOG660 (2.8kb *Pst*I and 1.58kb *Sma*I). If these fragments hybridised with any of the Typhimurium fragments this information may be used to determine whether pOG674 has increased to generate pOG660 or pOG660 has decreased to form pOG674. Finally, a fragment was chosen that was present in both pOG674 and pOG660 but absent from some of the other plasmids, notably pOG690 (3.0kb *Sma*I). This should determine whether or not this fragment in pOG674 is identical in pOG660. If the 3.0kb *Sma*I fragment hybridised with fragments from those plasmids in which it did not appear to be present, the size(s) of the fragments that hybridisd may provide some useful information.

The two *Pst*I fragments chosen were 4.4kb and 2.8kb. The 4.4kb fragment was found in all Enteritidis plasmids and also in pOG660. This fragment will be mapped on pOG674 and if homologous, should be present in the same region on all plasmids tested. This fragment was therefore chosen to demonstrate homology. A fragment of 2.8kb was chosen from pOG674, it was also present in pOG700/703/691 and 705. It was not found in pOG660 (although it has been shown to be present in Choleraesuis, but no other SAPs). The *Sma*I fragments chosen were 1.58kb and 3.0kb. The 1.58kb *Sma*I fragment from pOG674 was present in all plasmids except pOG701, pOG691, pOG690 and pOG660. The 3.0kb *Sma*I fragment was present in all plasmids except pOG701, pOG691, pOG705 and pOG690.

4.2.3 Restriction endonuclease digestion and electrophoresis of plasmid DNA.

Digestion with single enzymes followed by electrophoresis was performed as detailed in Chapter 2. Double digests were performed as recommended by Life Technologies. Enzymes which required the same REACT® buffer were digested simultaneously for four hours at the appropriate temperature. Double digests which required different buffers were digested sequentially. The enzyme that required the buffer of the lowest salt concentration was used first.

4.2.4 Recovery of DNA fragments from agarose gels.

A detailed protocol is described in Chapter 2. Briefly, plasmids were digested with restriction enzymes and electrophoresed in 0.8% agarose gels overnight. A pre-treated strip of NA-45 DEAE membrane was placed in an incision in the gel just ahead of the fragment to be removed. Electrophoresis was continued for one hour. The DNA eluted from the membrane in high salt buffer at 60°C, was precipitated and resuspended in TE buffer. This was subsequently used for digestion and cloning.

4.2.5 Cloning and digoxigenin labelled probe preparation and hybridisation.

Full details can be found in Chapter 2. Briefly, *Pst*I fragments were ligated with pUC19 vector DNA and transformed into *E.coli* DH5α Maximum Efficiency™ competent cells as described in the protocol provided by Life Technologies. Fragments from *Sma*I digests were ligated with pUC18 vector DNA using Ready-To-Go™ pUC18 *Sma*I/BAP + Ligase (Pharmacia, UK) and transformed as above. Transformants were selected on LB agar that contained, ampicillin (100μg/ml), X-gal and IPTG. The plates were inoculated and

incubated overnight at 37°C. White colonies were selected for subsequent analysis.

DNA was labelled with digoxigenin with the DIG system of Boerhinger Mannheim. The DIG-labelled probes were hybridised to nitrocellulose membrane-bound nucleic acids on a Southern blot in DIG-Easy Hyb at 42°C. The hybridised probes were immuno-detected with an alkaline phosphatase-conjugated Anti-digoxigenin antibody and visualised with the colorimetric substrates NBT and X-Phosphate.

4.3 Results

4.3.1 Construction of restriction maps.

A restriction map of Enteritidis plasmid, pOG674, is shown in Figure 4.1. Plasmid DNA was digested with restriction enzymes and the size of the fragments generated were determined both by reference to the lambda DNA standard and also by computer using the Molmatch programme (UVP Ltd, UK). The sizes of fragments generated from each enzyme can be found in Table 4.3.

The enzyme, *Xba*I, had a single restriction site in pOG674 and this site was therefore used as the origin of the map. The enzyme *Xho*I generated two restriction fragments of 39.5 and 14.5kb (Figure 4.2). Double digests of *Xba*I or *Xho*I plus one other enzyme, allowed the placement of fragments around the origin (Figures 4.3 - 4.8). Tables 4.3 - 4.9 show the results obtained from double digests of pOG674 with selected restriction endonucleases.

Table 4.4, shows the results obtained from double digests of *Xba*I plus one other enzyme. When this data was combined with that obtained from double digests of the same enzymes with *Xho*I (Table 4.5) the positions of the *Xba*I and *Xho*I restriction sites were determined on the map.

FIGURE 4.1

Restriction map of Enteritidis plasmid pOG674

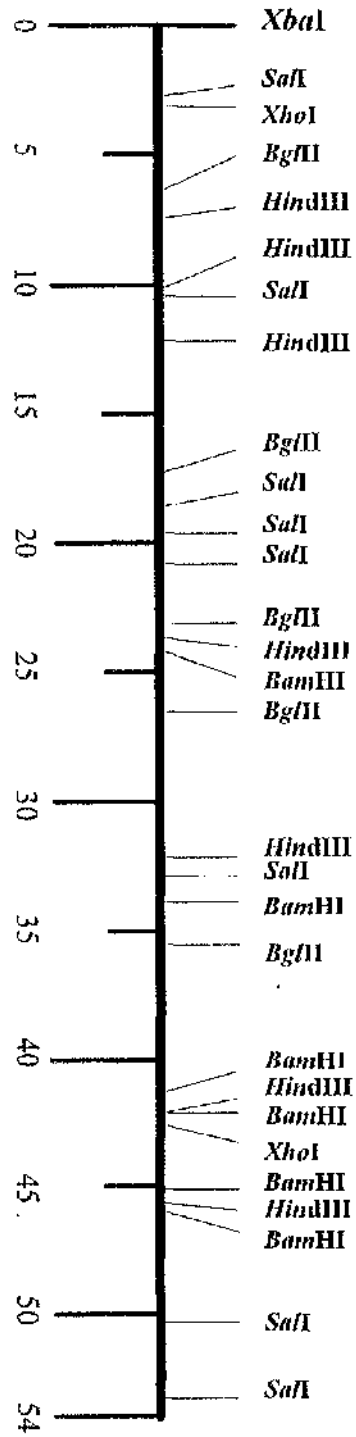


FIGURE 4.2

Enteritidis variant SAPs and Typhimurium SAP (pOG660) digested with *Xho*I. Lane 1, λ*Pst*I/*Kpn*I; 2, pOG691; 3, pOG701; 4, pOG700; 5, pOG703; 6, pOG705; 7, pOG704; 8, pOG690; 9, pOG660 and 10, pOG674.

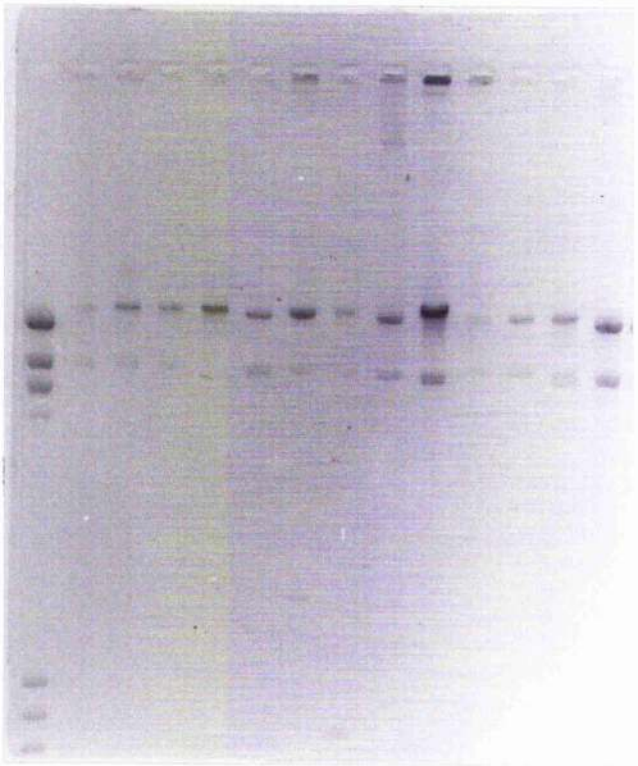


FIGURE 4.3

Enteritidis variant SAPs and Typhimurium SAP (pOG660) double digested with *Pst*I and *Xba*I. Lane 1, λ*Pst*I/*Kpn*I; 2, pOG700;3, pOG703; 4, pOG690; 5, pOG705; 6, pOG704; 7, pOG660; 8, pOG674; 9, pOG691 and 10, pOG701.

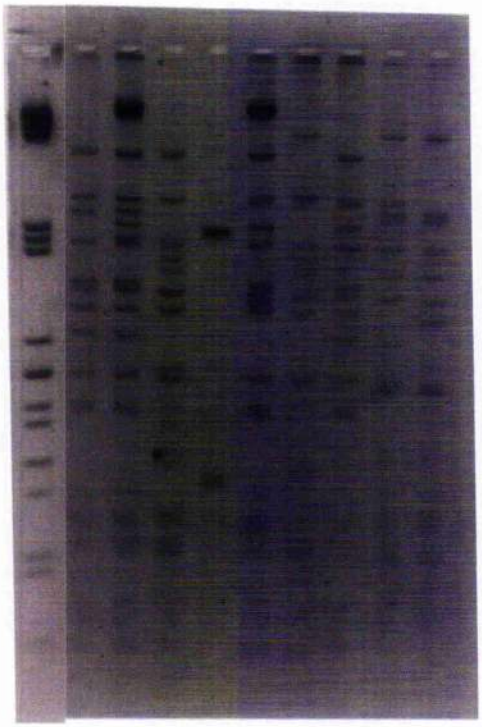


FIGURE 4.4

Enteritidis plasmid pOG674 digested and double digested with selected restriction endonucleases as shown. Lane 1, λ*Pst*I; 2, *Sma*I/*Xba*I; 3, *Sma*I; 4, *Sma*I/*Xho*I; 5, *Bam*HI; 6, *Bam*HI/*Hin*dIII; 7, *Hin*dIII; 8, *Bgl*II/HindIII; 9, *Bgl*II; 10, *Bgl*II/*Sal*I; 11, *Sal*I; 12, *Xho*I/*Pst*I and 13, *Pst*I.

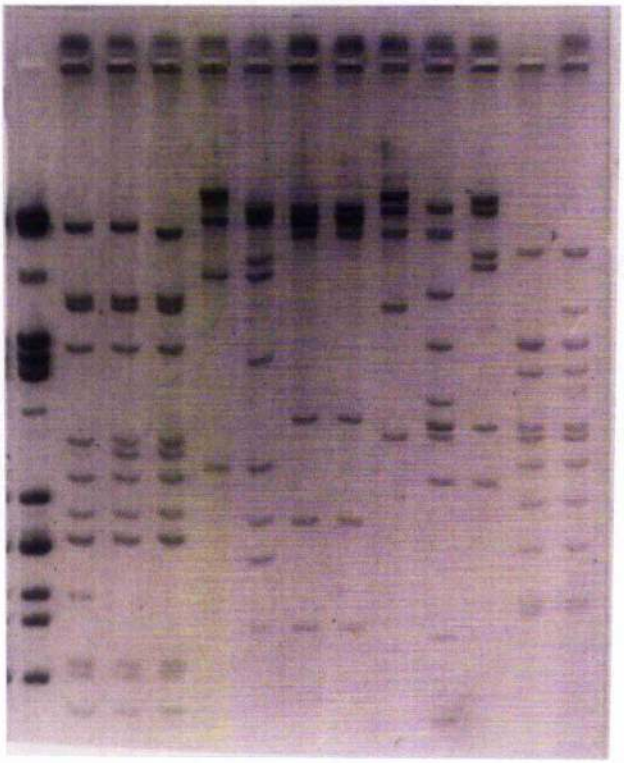


FIGURE 4.5

Enteritidis plasmid pOG674 digested and double digested with selected restriction endonucleases as shown. Lane1, λ*Pst*I; 2, *Pst*I; 3, *Xho*I/*Bgl*II; 4, *Xba*I/*Bgl*II; 5, *Bgl*II; 6, *Bgl*II/*Hin*dIII; 7, *Hin*dIII; 8, *Bgl*II/*Bam*HI; 9, *Bam*HI; 10, *Xho*I/*Sal*I; 11, *Sal*I; 12, *Xba*I/*Sal*I and 13, *Sma*I.

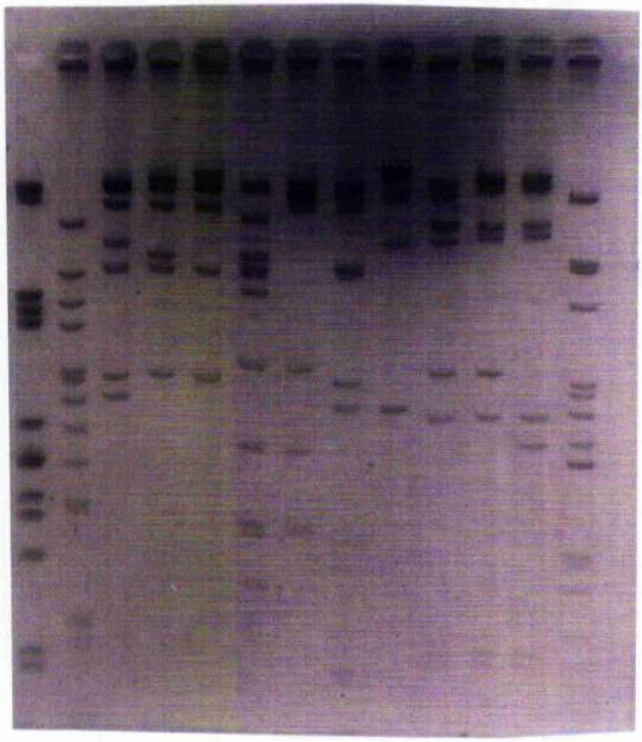


FIGURE 4.6

Enteritidis plasmid pOG674 digested and double digested with selected restriction endonucleases as shown. Lane 1, λ*Pst*I; 2, *Xho*I/*Bgl*II; 3, *Bgl*II; 4, *Xba*I/*Bgl*II; 5, *Hin*dIII/*Bgl*II; 6, *Bam*HI/*Bgl*II; 7, *Xho*I/*Bam*HI; 8, *Bam*HI; 9, *Xba*I/*Bam*HI; 10, *Bam*HI/*Sal*I; 11, *Sal*I; 12, *Xho*I/*Sal*I and 13, *Xba*I/*Sal*I.

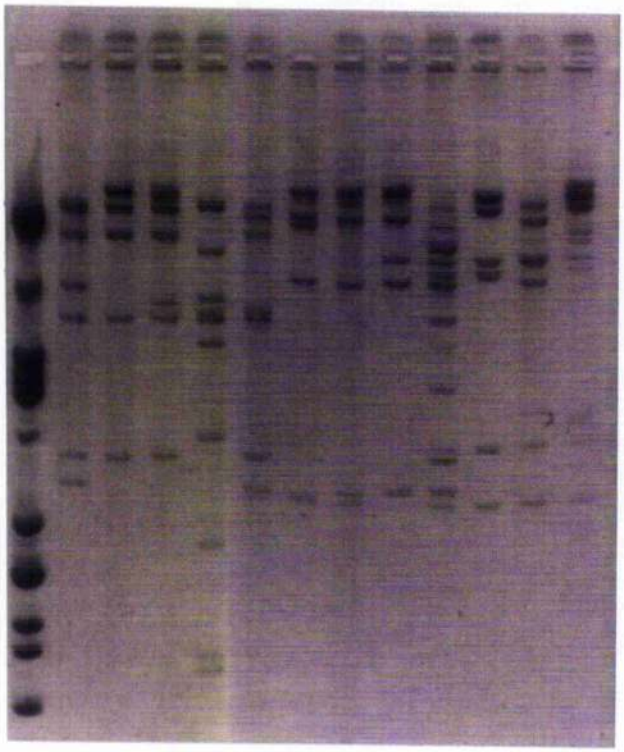


FIGURE 4.7

Enteritidis plasmid pOG674 digested and double digested with selected restriction endonucleases as shown. Also included are fragments from digests of pOG674 extracted from agarose gels and re-digested with a different endonuclease. Lane 1, λ*Pst*I; 2, *Xho*I/*Xba*I; 3, *Xho*I/*Hin*dIII; 4,*Xba*I/*Hin*dIII; 5, *Xho*I/*Sal*I; 6, 8.2kb *Sal*I / *Xba*I; 7, 3.5kb *Sal*I / *Xba*I; 8, 17.5kb *Sal*I / *Pst*I; 9, 12kb *Sal*I / *Pst*I; 10, 7.7kb *Sal*I / *Pst*I; 11, *Pst*I; 12, 16kb *Hin*dIII / *Xho*I; 13, 11.5 and 10kb *Hin*dIII / *Sal*I and 14, 8.5kb *Hin*dIII / *Bgl*II.

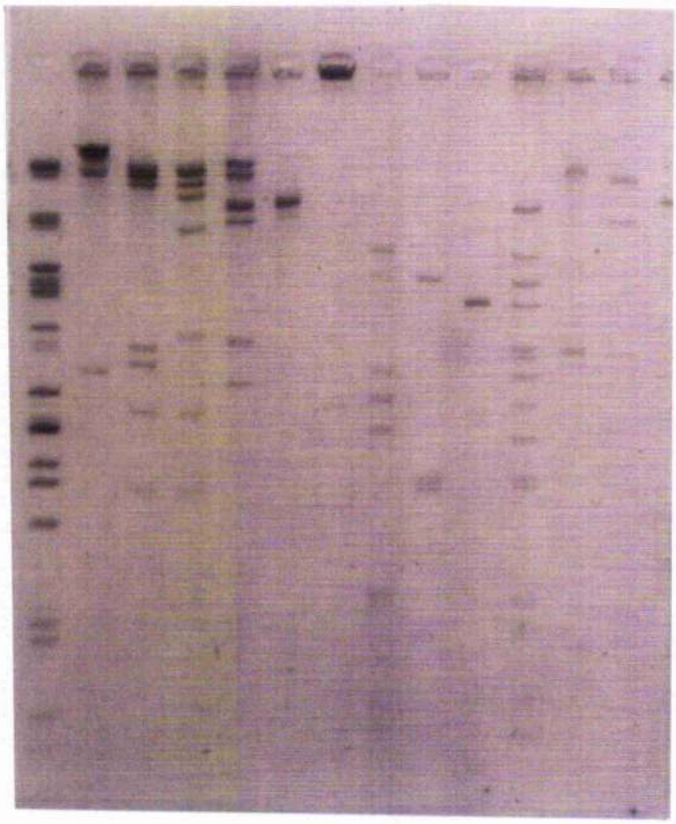


FIGURE 4.8

Enteritidis plasmid pOG674 digested and double digested with selected restriction endonucleases as shown. Lane 1, λ*Pst*I; 2, *Sma*I; 3, *Sma*I/*Sal*I; 4, *Sal*I; 5, *Hin*dIII/*Sal*I; 6, *Hin*dIII; 7, *Hin*dIII/*Bgl*II; 8, *Bgl*II/*Xho*I; 9, *Bgl*II; 10, *Bgl*II/*Xba*I; 11, *Bam*HI/*Bgl*II and 12, *Bam*HI.

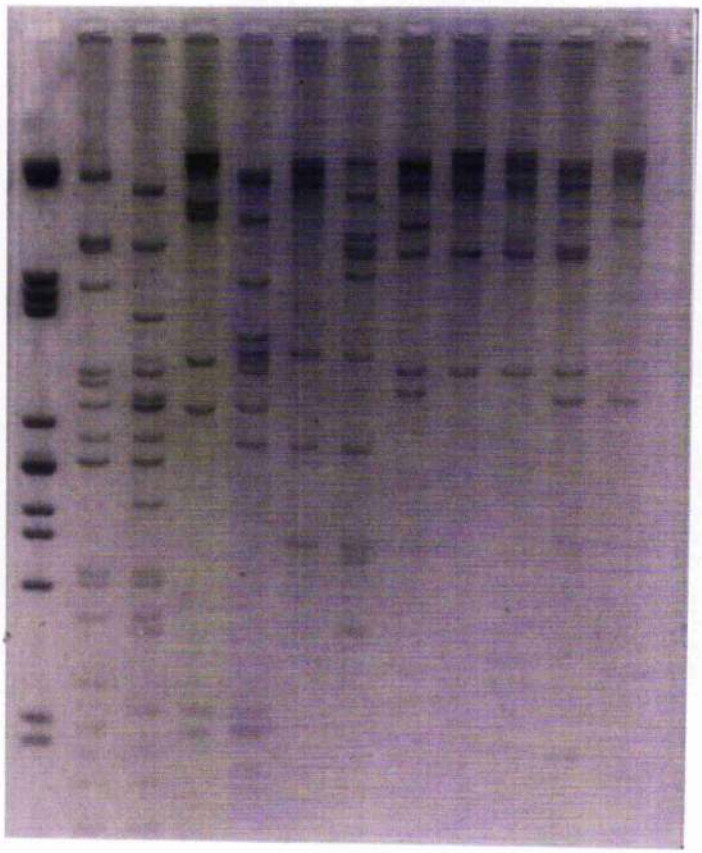


Table 4.3. Restriction fragments generated from Enteritidis plasmid pOG674 with selected endonucleases.

Enzyme	Restriction fragment sizes (kilobases)							
*Xba*I	54							
*Xho*I	39.5	14.5						
*Hin*dIII	16	11.5	10	8.5	3.5	2.7	2.0	
*Bam*HI	32	10		7.4	3.1	0.8	0.8	
*Sal*I	17.5	12	8.2	7.7	3.5	3.0	1.2	1.0
*Bgl*II	24.7	11	9	5.8	3.4			

Table 4.4 Restriction endonuclease fragments lost and gained from double digests of Enteritidis plasmid pOG674 with *Xba*1 and one other enzyme.

Enzyme 1	*Xba*1	
Enzyme 2	Enzyme 2 Fragment lost	Fragments gained
*Xho*I	14.5	11.4 : 3.1
*Hin*dIII	16	8.6 : 7.4
*Bam*HI	32	23.9 : 8.1
*Sal*I	3.5	2.7 : 0.8
*Bgl*II	24.7	18.4 : 6.3
*Pst*I	2.4	2.3 : 0.1
*Sma*I	3.2	2.1 : 1.1

Table 4.5 Restriction endonuclease fragments lost and gained from double digests of Enteritidis plasmid pOG674 with *Xho*1 and one other enzyme.

Enzyme 1	*Xho*I	
Enzyme 2	**Enzyme 2 Fragments lost**	**Fragments gained**
*Hin*dIII	16	11.7 : 4.3
	3.5	3.45 : 0.5
*Bam*HI	31	20.8 : 11.2
	3.1	3.0 : 0.1
*Sal*I	17.5	9.5 : 8.0
	7.7	7.3 : 0.4
*Bgl*II	24.7	14.5 : 7.1 : 3.1
*Pst*I	5.7	4.9 : 0.8
	<1.0	ND
*Sma*I	11	10.3 : 0.7
	<1.0	ND

ND; Fragments were too small to be determined

Table 4.6 Restriction endonuclease fragments lost from double digests of Enteritidis plasmid pOG674 with *Hin*dIII and one other enzyme.

Enzyme	*Hin*dIII Fragments lost	Enzyme 2 Fragments lost
*Bam*HI	16 : 11.5 : 8.5 : 3.5	32 : 10 : 0.8
*Sal*I	16 : 11.5 : 10 : 2.0	17.5 : 12 : 8.2 : 7.7
*Bgl*II	16 : 11.5 : 10 : 8.5	24.7 : 11 : 9 : 3.4
*Pst*I	All except 3.5	8.3 : 5.7 : 4.4 : 2.0 : 1.35 : 1.23
*Sma*I	All except 3.5 and 2.0	11.0 : 6.2 : 5.9 : 1.77 : 1.08

Table 4.7 Restriction endonuclease fragments lost from double digests of Enteritidis plasmid pOG674 with *Bam*HI and one other enzyme.

Enzyme	*Bam*HI Fragments lost	Enzyme 2 Fragments lost
*Sal*I	32 : 10	17.5 : 12
*Bgl*II	32 : 10 : 7.4	24.7 : 9 : 3.4
*Pst*I	All except 3.1	5.7 : 2.1 : 1.23
*Sma*I	All except 3.1	11 : 3.4 : 1.08

Table 4.8 Restriction endonuclease fragments lost from double digests of Enteritidis plasmid pOG674 with *Sal*I and one other enzyme.

Enzyme	*Sal*I Fragments lost	Enzyme 2 Fragments lost
*Bgl*II	17.5 : 10.5 : 8.2 : 7.7	24.7 : 5.8
*Pst*I	All except 8.2	8.3 : 3.5 : 3.4 : 2.4 : 1.16 :<1.0
*Sma*I	All except 3.0 and 1.2	11 : 6.2 : 5.0 : 3.2 : 1.32 : 1.15

Table 4.9 Restriction endonuclease fragments lost from double digests of Enteritidis plasmid pOG674 with *Bgl*II and one other enzyme.

Enzyme	*Bgl*II Fragments lost	Enzyme 2 Fragments lost
*Pst*I	All	8.3 : 4.4 : 3.5 : 2.1
*Sma*I	All	5 : 3.4 : 1.08

4.3.2 Position of *Bam*HI restriction fragments on the map.

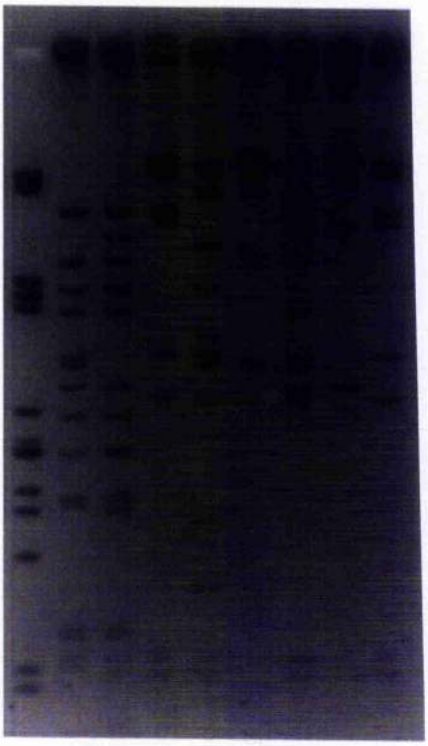
Table 4.6, showed that four *Hin*dIII fragments were lost when pOG674 was double digested with *Bam*HI (Figure 4.4). This suggested that three of the seven *Hin*dIII fragments had no internal *Bam*HI sites. Also, given that there were six *Bam*HI fragments, one of the *Hin*dIII fragments should have two *Bam*HI restriction sites within. The 32kb *Bam*HI fragment was lost after subsequent digestion with *Xba*I and generated two fragments of 23.9 and 8.1kb (Figure 4.6). The 32kb fragment was also lost after digestion with *Xho*I and generated two fragments of 20.8 and 11.2kb. From these data it was deduced that the 32kb *Bam*HI fragment began 8.1kb downstream from the *Xba*I site at 45.9' and continued 23.9kb upstream (23.9'). The second *Xho*I site was contained within the 3.1kb *Bam*HI fragment and it therefore followed that this 3.1kb fragment was located at 45.1' on the map. This fragment followed one of the 0.8kb *Bam*HI fragments. The four *Bam*HI fragments which remained were orientated as follows. The second 0.8kb fragment and the 10kb fragment both contained a single *Hin*dIII site. However, the 10kb fragment also contained a *Sal*I site (Figure 4.9), therefore, the 0.8kb fragment followed on from the 3.1kb fragment and the 10kb *Bam*HI fragment was found to be located from 23.9' to 33.9' on the map (Figure 4.1). The position of the two 0.8kb fragments and the 3.1kb fragment were later confirmed by the digestion of the 5.7kb *Pst*I fragment with *Bam*HI (Figure 4.14).

4.3.3 Position of *Bgl*II restriction fragments on the map.

Table 4.6 showed that four out of five *Bgl*II fragments were lost when pOG674 was double digested with *Bgl*II and *Hin*dIII (Figure 4.8). The *Xba*I site was in the 24.7kb *Bgl*II fragment and a double digest generated two fragments of 18.4 and 6.3kb. The *Xho*I/*Bgl*II double digest showed that both *Xho*I sites were present on the 24.7kb *Bgl*II fragment. The sizes of the

FIGURE 4.9

Enteritidis plasmid pOG674 digested and double digested with selected restriction endonucleases as shown. Lane 1, λ*Pst*I; 2, *Pst*I; 3, *Pst*I/*Sal*I; 4, *Sal*I; 5, *Sal*I/Bg*l*II; 6, *Bgl*II; 7, *Sal*I/*Bam*HI; 8, *Bam*HI and 9, *Sal*I.



fragments generated allowed the orientation of this fragment to be determined as shown (Figure 4.1). The 11kb *Bgl*II fragment had restriction sites for *Hin*dIII and *Sal*I and was therefore positioned immediately upstream from the *Xba*I site. The 5.8kb fragment had three *Sal*I sites and followed on from the 11kb *Bgl*II fragment. The 3.4kb *Bgl*II fragment had a restriction site for *Hin*dIII but no *Sal*I site, this observation allowed the position of both this fragment and the 9kb *Bgl*II fragment to be determined. Figure 4.9 showed the REFP obtained from a *SalI*/*Bgl*II double digest from which it was unclear whether or not the 9 and 11kb *Bgl*II fragments had any *Sal*I sites. To resolve this, all *Bgl*II fragments were extracted from agarose gels and were re-digested with *Sal*I. Figure 4.10, showed that all *Bgl*II fragments, except that of 3.4kb, had *Sal*I restriction sites.

4.3.4 Position of *Pst*I restriction fragments on the map.

The location of most of the *Pst*I fragments on pOG674 were derived by the analysis of double digests of *Pst*I with the other enzymes included in the map. No *Pst*I/*Sma*I double digest was done as this would have generated too many fragments to analyse. Therefore, unless otherwise indicated it can be assumed that *Sma*I was not included in this analysis.

Due to the large number of fragments generated with *Pst*I (>18) the precise location of some of the smaller fragments was not possible. The extraction of large fragments, generated with other enzymes, from agarose gels allowed some of these fragments to be placed in a given location but their exact positions could not be determined.

A *Pst*I restriction map is shown in Figure 4.11. This map also included the restriction sites for *Xba*I, *Xho*I, *Hin*dIII and *Sal*I.

The *Xba*I site was located in the 2.4kb *Pst*I fragment and generated two fragments of 2.3 and 0.1kb (Figure 4.3). The *Xho*I sites were in *Pst*I fragments of 5.7kb and a fragment <1.0kb (Figure 4.12). The 2.4kb *Pst*I fragment also had a *Sal*I site which allowed its orientation to be determined (Figure 4.9). The 5.7kb *Pst*I fragment has previously been sequenced within the virulence region and was shown to contain a *Xho*I site. The two *Xho*I fragments from pOG674 were extracted and re-digested with *Pst*I. The 14.5 kb *Xho*I fragment generated visible fragments of 4.9, 3.4, 2.4 and four fragments < 1.5kb (Figure 4.13). The 5.7kb *Pst*I fragment was also digested with *Bam*HI and showed that three *Bam*HI fragments of 3.1, 0.8 and 0.8kb were located within this 5.7kb fragment (Figure 4.14). From this information the positions of four *Pst*I fragments were determined (5.7, 3.4, 1.16 and 0.8kb).

From the *Xba*I site, the first *Pst*I site upstream was 0.1kb. This was followed by an area of approximately 2kb for which the *Pst*I sites could not be determined. The second *Xho*I site was in a *Pst*I fragment <1.0kb and this has been inserted as shown on the map. The next *Pst*I fragment was 4.4kb. This fragment was shown to be located on the 7.7kb *Sal*I fragment (Figure 4.7) and it contained a *Hin*dIII and a *Bgl*II site (Figure 4.15). No *Pst*I sites could be determined within the 2.7kb *Hin*dIII fragment at this location, but the 2.0kb *Hin*dIII fragment which followed had a *Pst*I site (10.6') which was the start of the 8.3kb *Pst*I fragment. *Hin*dIII/*Pst*I and *Bgl*II/*Pst*I double digests confirmed this position. From the end of the 8.3kb *Pst*I fragment (18.9') there was a small area (0.6kb) for which no sites could be determined. The 3.5kb *Pst*I fragment began at 19.5' and was confirmed in this position by *Sal*I/*Pst*I and *Bgl*II/*Pst*I double digests (Figures 4.9 and 4.15). The 3.5kb *Pst*I fragment was also shown to be present on the 32kb *Bam*HI fragment (45.9' - 23.9') (Figure 4.14). The 2.0 and 2.1kb *Pst*I fragments followed and their positions were confirmed by *Hin*dIII (2.0kb) and *Bgl*II double digests with *Pst*I (Figure 4.15). These two

FIGURE 4.10

Enteritidis plasmid pOG674 *Bgl*II fragments extracted from agarose gels and re-digested with *Sal*I. Lane 1, λ*Pst*I; 2, *Sal*I; 3, 3.4kb; 4, 5.8kb ; 5, 9kb; 6, 11kb; 7, 24.7kb and 8, *Bgl*II.

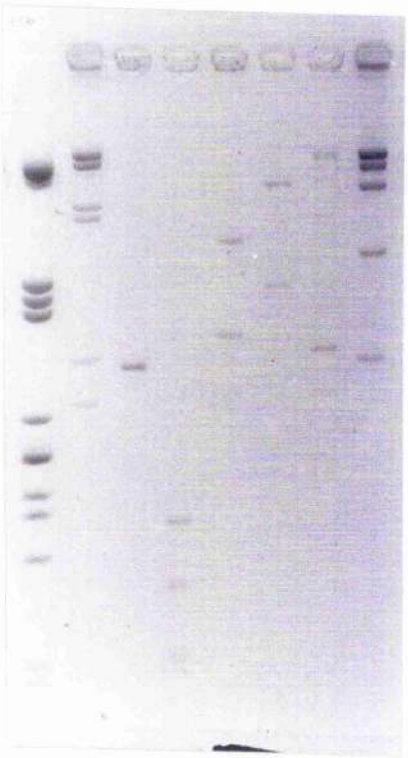


FIGURE 4.11

***Pst*I Restriction map of Enteritidis plasmid pOG674**

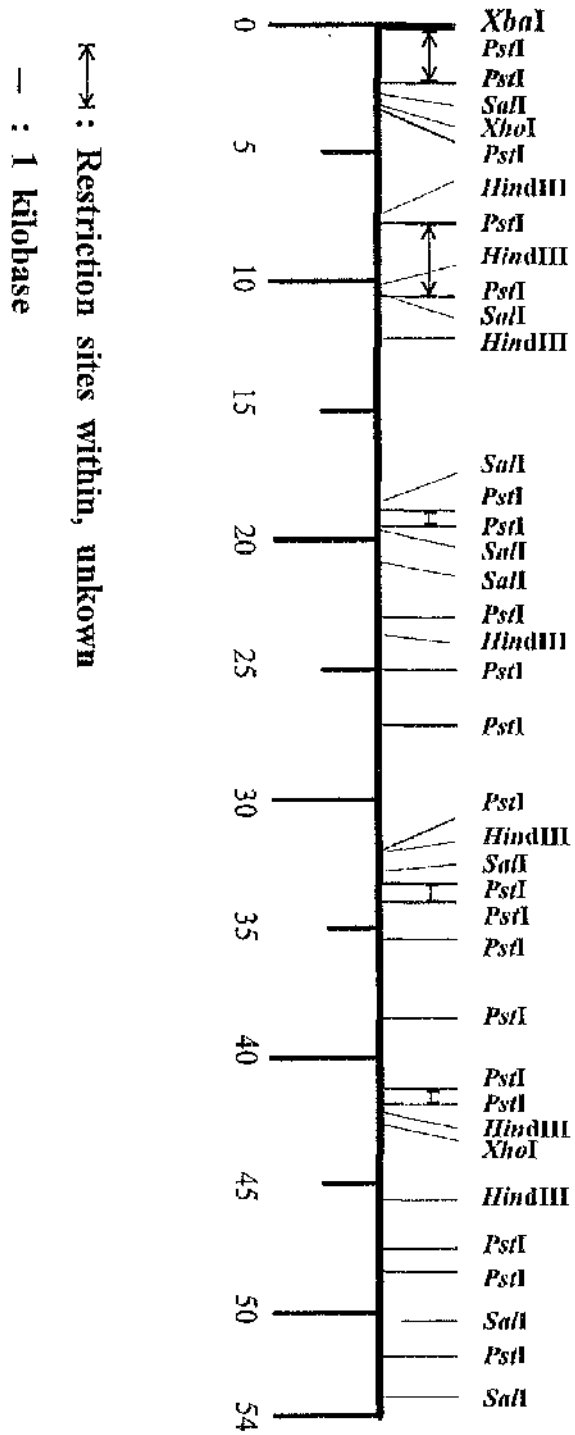


FIGURE 4.12

Typhimurium plasmid pOG660 and Enteritidis plasmids pOG674 and pOG690 digested with *Pst*I and *Xho*I and double digested with *Pst*I/*Xho*I. Lane 1, λ*Pst*I; 2, pOG674 *Pst*I; 3, pOG674 *Pst*I/*Xho*I; 4, pOG674 *Xho*I; 5, pOG660 *Pst*I; 6, pOG660 *Pst*I/*Xho*I; 7, pOG660 *Xho*I; 8, pOG690 *Pst*I; 9, pOG690 *Pst*I/*Xho*I and 10, pOG690 *Xho*I.

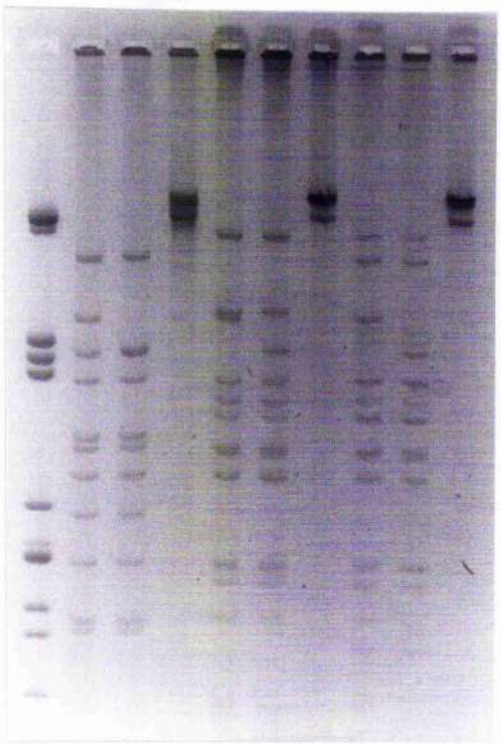


FIGURE 4.13

Enteritidis plasmid pOG674 restriction endonuclease fragments extracted from agarose gels and re-digested with a different endonuclease as shown. Lane 1, λ*Pst*I; 2, 39kb *Xho*I fragment / *Pst*I; 3, 15kb *Xho*I fragment / *Pst*I; 4, 32kb *Bam*HI fragment / *Pst*I; 5, 12kb *Bam*HI fragment / *Pst*I; 6, 7.4kb *Bam*HI fragment / *Pst*I; 7, *Pst*I; 8, *Sma*I; 9, 39kb *Xho*I fragment / *Sma*I; 10, 32kb *Bam*HI fragment/ *Sma*I; 11, 12kb *Bam*HI fragment / *Sma*I; 12, 7.4kb *Bam*HI fragment/ *Sma*I and 13, 4.4kb *Pst*I fragment / *Sma*I.

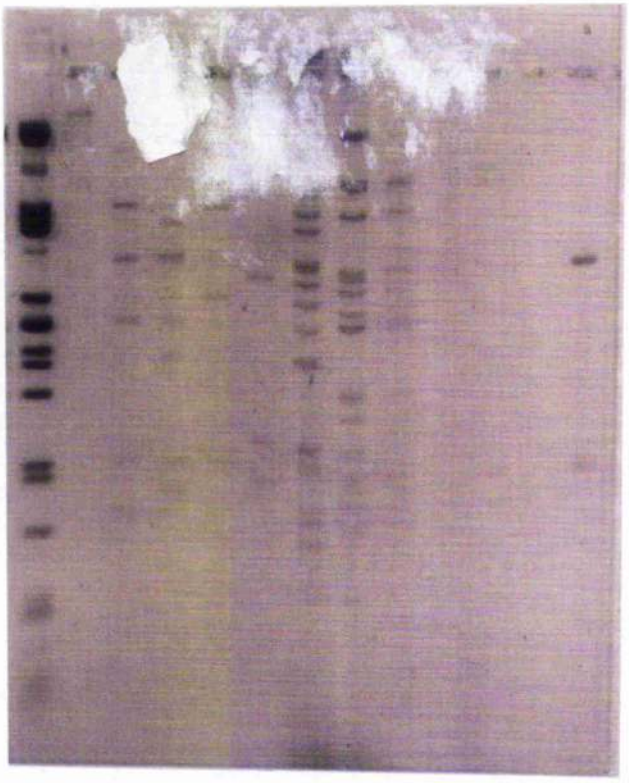
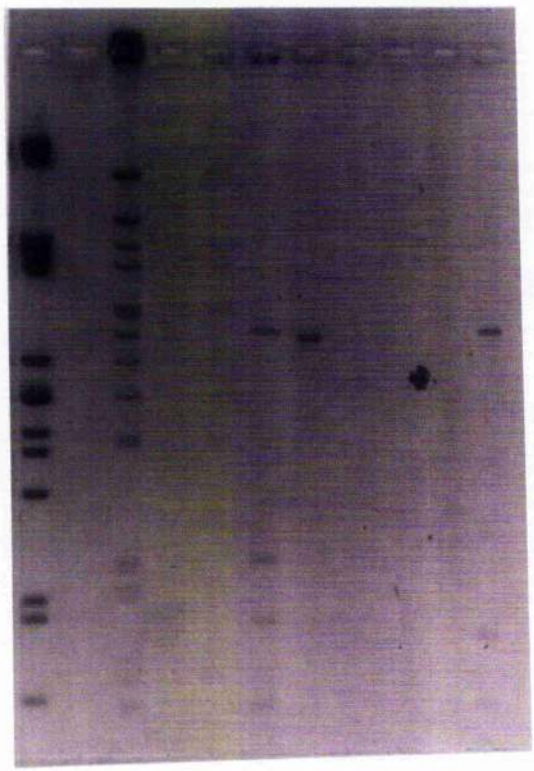


FIGURE 4.14

Enteritidis plasmid pOG674 restriction endonuclease fragments extracted from agarose gels and re-digested with a different endonuclease as shown. Lane 1, λ*Pst*I; 2, Blank; 3, *Pst*I; 4, Blank; 5, 32 and 10kb *Bam*HI / *Pst*I; 6, 7.4kb *Bam*HI / *Pst*I; 7, 3.1kb *Bam*HI / *Xho*I; 8, 8.3kb *Pst*I / *Xho*I; 9, 15kb *Xho*I / *Xba*I; 10, 39kb *Xho*I /*Bam*HI and 11, 5.7kb *Pst*I / *Bam*HI.



fragments were also shown to be present on the 12kb *Sal*I fragment (Figure 4.7). The 4.9kb *Pst*I fragment was also found on this 12kb *Sal*I fragment (Figure 4.7). The 4.9kb *Pst*I fragment had no internal sites for any of the other enzymes and therefore followed from the 2.1kb *Pst*I fragment (27.1' - 32.0'). The 1.23kb *Pst*I fragment had one *Hin*dIII restriction site and by a process of elimination followed the 2.1kb *Pst*I fragment. There followed a 0.8kb gap on the map which although 300bp too small was thought to be the 1.16kb *Pst*I fragment as this fragment was shown in double digests to have a *Sal*I restriction site (Table 4.8). This area was followed by a *Pst*I fragment of 1.32kb that was shown to have a *Bam*HI site (Table 4.7). The 2.8 and 3.1kb *Pst*I fragments were shown to be present in this area of the map from data obtained by re-digesting fragments extracted from agarose gels (Figure 4.13). A *Bgl*II restriction site was to be found at 35.5' on the map which suggested that either the 2.8kb or 3.1kb *Pst*I fragments should have a *Bgl*II site. A double digest of *Pst*I/*Bgl*II (Figure 4.15) showed that the 3.1kb *Pst*I fragment was slightly smaller and therefore these two fragments were orientated as follows, 3.1kb (35.3' - 38.4') followed by 2.8kb (38.4' - 41.2'). The 3.1kb *Pst*I fragment was confirmed in this position at a later stage. From the end of the 3.1kb *Pst*I fragment, there was a small region of 600bp within which no restriction sites could be determined.

4.3.5 Position of *Sma*I restriction sites on the map.

A *Sma*I restriction map is shown in Figure 4.16. The *Xba*I site was located in the 3.2kb *Sma*I fragment (Figure 4.4). The *Xho*I sites were located in *Sma*I fragments of 11 and 1.32kb (Figure 4.4). The 11kb *Sma*I fragment was extracted and re digested with *Bam*HI (Figure 4.17) and generated fragments of 0.8, 3.1 and 6.3kb which indicated that this fragment incorporated all or part of

FIGURE 4.15

Enteritidis plasmid pOG674 digested and double digested with selected restriction enzymes as shown. Lane 1, λ*Pst*I; 2, *Sma*I; 3, *Sma*I/*Bam*HI; 4, *Bam*HI; 5, *Sma*I/*Bgl*II; 6, *Bgl*II; 7, *Sma*I/*Hin*dIII; 8, *Hin*dIII; 9, *Pst*I/*Hin*dII; 10, *Pst*I; 11, *Bam*HI/*Pst*I and 12, *Bgl*II/*Pst*I.

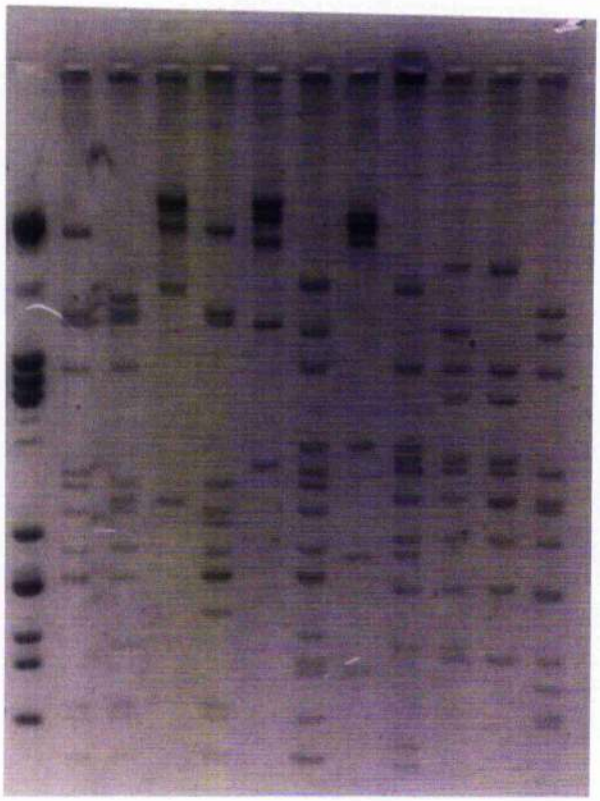
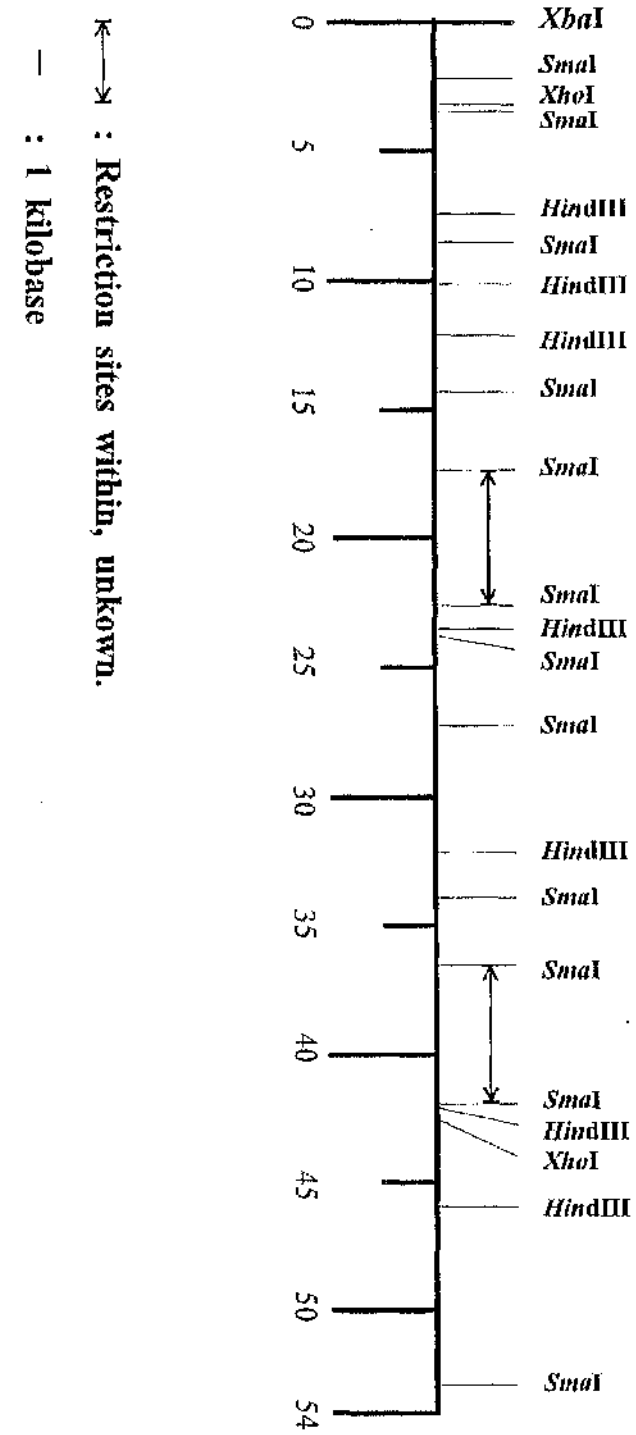


FIGURE 4.16

***Sma*I Restriction map of Enteritidis plasmid pOG674**



the virulence region. The size of the fragments generated with *Bam*HI allowed the orientation of the 11kb *Sma*I fragment which in turn allowed the orientation of the 3.2kb *Sma*I fragment around the *Xba*I site. The 1.32kb *Sma*I fragment followed on upstream from the *Xba*I site and it contained a *Xho*I site. The 5.0kb *Sma*I fragment followed on from the 1.32kb *Sma*I fragment and its position was confirmed by extraction and re-digestion with *Pst*I (Figure 4.18). This also confirmed the 4.4kb *Pst*I fragment within this area. The 5.9 and 3.0kb *Sma*I fragments respectively, followed from the 5.0kb *Sma*I fragment and were confirmed in this position by digestion of the 8.3kb *Pst*I fragment with *Sma*I (Figure 4.17). From 17.3' to 22.7' no *Sma*I fragments could be accurately determined. From 22.7' a 1.08kb fragment was found which had a single *Bgl*II and a single *Hin*dIII site (Figure 4.15). This was followed by *Sma*I fragments of 3.4, 6.2 and 2.5kb respectively. From 36.5' to 41.9' the position of the *Sma*I fragments could not be determined. However, when the 7.4kb *Bam*HI fragment was digested with *Sma*I it generated four fragments of approximately 2.7, 1.72, 1.1 and 1.0kb. This indicated that the 2.7 and 1.72kb *Sma*I fragments were in this location and as there were no *Sma*I fragments of 1.1 and 1.0kb in *Sma*I digests of pOG674 these must have been derived from fragments > 1.0kb. The 11kb *Sma*I fragment was positioned from 41.9' - 52.9' as previously determined.

4.3.6 Cloning and hybridisation.

DH5α Maximum Efficiency competent cells (Life Technologies, UK) were transformed with pUC18 or pUC19 vector DNA plus cloned insert DNA and white colonies were selected for further analysis. Potential transformants were grown overnight in 10ml Terrific broth and plasmid DNA was purified and digested as described previously. Many colonies were tested but only one had successfully transformed. The 2.8kb *Pst*I fragment from pOG674 was shown to be present in the pUC19 vector and this recombinant plasmid was designated

FIGURE 4.17

Enteritidis plasmid pOG674 restriction endonuclease fragments extracted from agarose gels and re-digested with a different endonuclease as shown. Lane 1, λ*Pst*I; 2, 11kb *Sma*I fragment / *Bam*HI; 3, 6.2kb *Sma*I fragment / *Xho*I; 4, 5.9kb *Sma*I fragment / *Xho*I; 5, 5kb *Sma*I fragment / *Bam*HI; 6, *Sma*I; 7, 8.3kb *Pst*I fragment / *Sma*I; 8, 5.7kb *Pst*I fragment / *Sma*I, 9, 4.9kb *Pst*I fragment / *Sma*I and 10, *Xho*I.

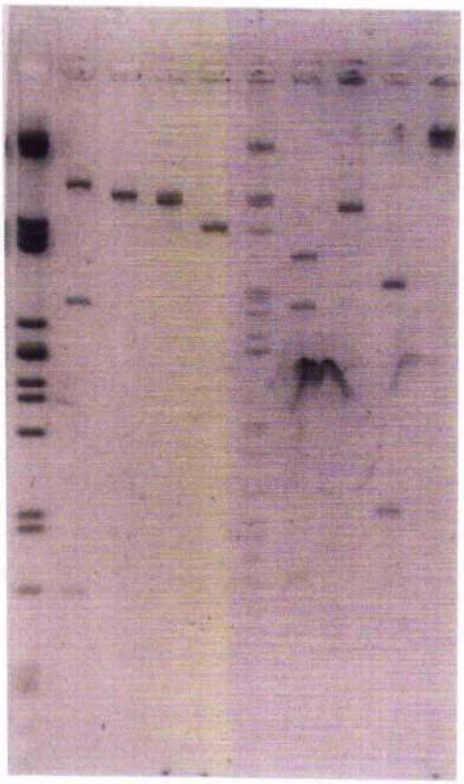
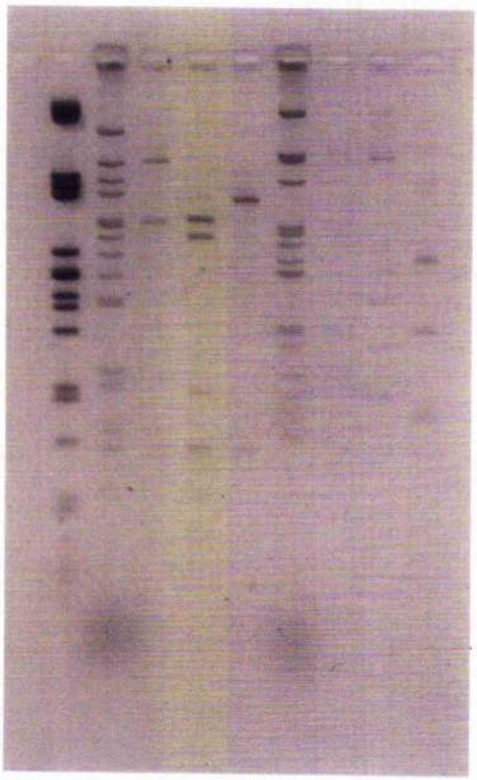


FIGURE 4.18

Enteritidis plasmid pOG674 restriction endonuclease fragments extracted from agarose gels and re-digested with a different endonuclease as shown. Lane 1, λ*Pst*I; 2, *Pst*I; 3, 11kb *Sma*I fragment / *Pst*I; 4, 6.2 and 5.9kb *Sma*I fragments / *Pst*I; 5, 5kb *Sma*I fragment / *Pst*I; 6, *Sma*I; 7, 3.2kb *Bam*HI fragment / *Sma*I; 8, 10kb *Bam*HI fragment / *Sma*I and 9, 7.4kb *Bam*HI fragment / *Sma*I.



pOG1001. Plasmid pOG1001 was digested with *Pst*I and labelled with digoxigenin as recommended by the manufacturer.

The 2.8kb insert was not purified from the pUC19 vector before it was labelled, therefore it was necessary to perform a test blot with labelled pUC19 DNA only. Plasmid DNA from two strains (NCTC618 and GRI12388) hybridised with pUC19. Both of these strains carried additional plasmids and the fragments which hybridised were shown to have come from these.

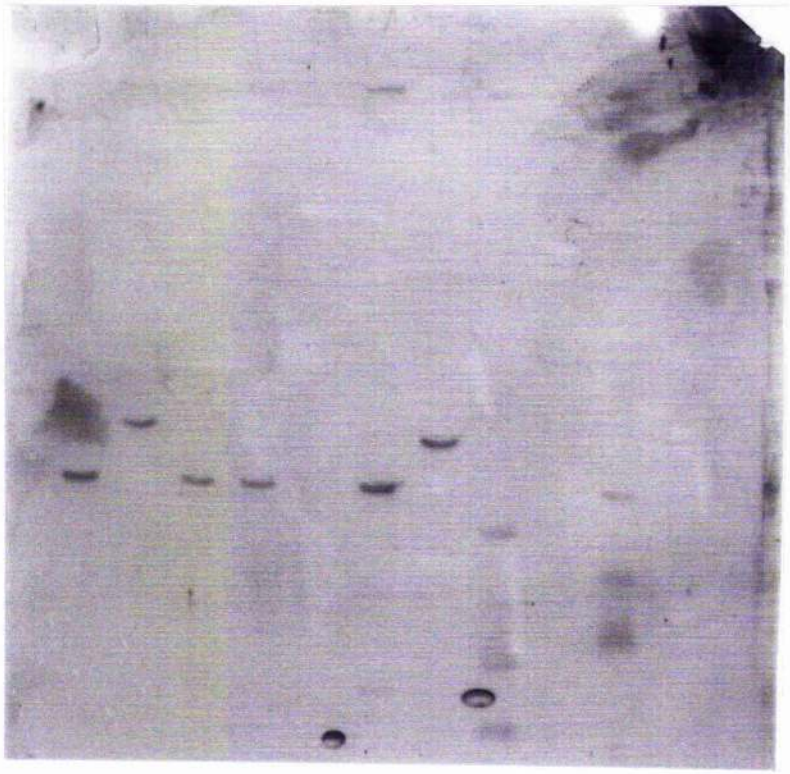
pOG1001 was hybridised overnight with a nitrocellulose blot of *Pst*I digested plasmid DNA as previously described. The results are shown in Figure 4.19. The probe hybridised to a 2.8kb *Pst*I fragment from plasmids pOG674, 691, 700, 703 and 705. A 3.4kb *Pst*I fragment from plasmids pOG660 and pOG690 hybridised with the 2.8kb probe fragment and a 3.3 kb *Pst*I fragment from pOG704. hybridised The hybridisation with pOG660 was very faint and is not clear in Figure 4.19. One plasmid pOG701 showed no hybridisation with the 2.8kb probe.

4.3.7 Analysis of hybridised fragments.

The hybridisation of 2.8kb fragments from plasmids pOG691, 700, 703 and 705 indicated that these fragments shared sequence identity with the 2.8kb *Pst*I probe fragment from pOG674 (Figure 4.19). The 2.8kb *Pst*I probe fragment has been shown on the map of pOG674, to be at position 38.4' - 41.2'. This fragment was therefore adjacent to the virulence region. Plasmid, pOG704 had no 2.8kb fragment but did have a fragment of 3.3kb which hybridised with the probe. The *Sma*I REFP of pOG704 showed a single extra 2.4kb fragment with no apparent disruption to any other fragments. Plasmid, pOG701 showed no hybridisation with any *Pst*I fragment. Both pOG690 and pOG660 (which was very faint in the figure) hybridised with a 3.4kb *Pst*I fragment which was a

FIGURE 4.19

Southern blot of Enteritidis plasmids and Typhimurium plasmid pOG660, hybridised with digoxigenin-labelled plasmid pOG1001. Lanes 1, pOG674; 2, pOG690; 3, pOG691; 4, pOG700; 5, pOG701; 6, pOG703; 7, pOG704; 8, pOG705; 9, pOG701; 10, Undesignated; 11, pOG660.



doublet in the REFP. Neither of these two plasmids had a 2.8kb *Pst*I fragment.

4.3.8 Analysis and comparison of Enteritidis plasmid pOG674 with the variant Enteritidis SAPs.

*Pst*I and *Sma*I restriction maps were determined for pOG674 (Figures 4.11 & 4.16). These maps were used with the data in Tables 4.1 and 4.2 to attempt to draw conclusions about the relationships between this plasmid, the seven variant plasmids and the Typhimurium SAP, pOG660. Although it was not possible to produce complete restriction maps for each plasmid it was attempted to logically determine the events which have led to their construction.

It has previously been hypothesised that the Enteritidis plasmid pOG674 has been derived by deletion, probably via a series of intermediates, from pOG660. This assumption here leads to the conclusion that all plasmids which are bigger than pOG674 are intermediates in the deletion process. This in turn means that the discussion which follows assumes this to be true. As it is possible that the converse is true, the final judgement for each plasmid will be stated.

pOG700. Compared with pOG674 this plasmid had lost a 4.9kb *Pst*I fragment but gained two fragments of 5.1 and 1.4kb. The *Sma*I REFPs indicated that pOG700 had lost a fragment of 3.4kb and gained two fragments of 2.4 and 2.45kb. The 4.9kb *Pst*I fragment was shown at position 27.1' - 32' on the map (Figure 4.11); the 3.4kb *Sma*I fragment was shown at 23.8' - 27.2' (Figure 4.16). It was proposed that additional DNA (approximately 1.5kb) was present in pOG700 and was responsible for the REFPs observed. This DNA could not have been inserted as a single event to generate pOG700 from pOG674 as the sizes of the restriction fragments in relation to the map positions of pOG674 are at variance with this. It has been determined that there was no one point on this

area of the map where a single 1.5kb fragment of DNA could have inserted to generate two *Sma*I and two *Pst*I fragments of the sizes observed. The converse is also true however, in that it would have taken a minimum of two deletions to derive pOG674 from pOG700.

pOG704. The *Pst*I REFPs showed that this plasmid had lost a 2.8kb fragment and gained a fragment of 3.3kb. The *Sma*I REFPs showed a single extra fragment of 2.4kb with the possible loss of a 2.5kb fragment which was a doublet in pOG674. This was of interest as the 2.8kb *Pst*I fragment from pOG674 was chosen as a probe which subsequently hybridised with a 3.3kb *Pst*I fragment in pOG704. This suggested that a small piece of DNA (600bp) may have been lost from pOG704 to generate pOG674 or that a point mutation had occurred in the 3.3kb fragment to generate a *Pst*I restriction site that resulted in a fragment of 2.8kb in pOG674.

A similar situation existed with pOG690 and pOG660 but the *Pst*I fragment which hybridised with the 2.8kb probe in those plasmids was 3.4kb and not 3.3kb. The *Sma*I REFPs however, again showed the loss of a 2.5kb fragment to generate one of 2.4kb. In pOG674 the 2.8kb *Pst*I site was shown to be positioned from 38.4' - 41.2' on the map. There was a *Bam*HI site at 41.3' which is the start of the 7.4kb *Bam*HI fragment. If pOG660 had no 2.8kb *Pst*I fragment but did have a fragment of 3.4kb that hybridised with the 2.8kb probe fragment from pOG674, then the 3.4kb *Pst*I fragment from pOG660 should have a *Bam*HI site. Figure 4.20 showed that this was the case. Although the 3.4kb *Pst*I fragment in pOG660 was a doublet, one of these fragments had a *Bam*HI site that generated a fragment of 3.2kb. This fitted well with the data and suggested that a point mutation, to create a *Pst*I site, may have been responsible for the creation of a 2.8kb *Pst*I fragment, in pOG674, from a 3.4kb

fragment in pOG660. Like pOG700, what appeared to be a simple variation was actually more complex.

pOG703. Table 4.2 showed that this plasmid had lost a 5.9kb *Sma*I fragment but did not appear to have gained any new fragments. The *Pst*I REFP showed no apparent loss of DNA but there was an additional fragment of 5.1kb. It was not possible to readily explain these data with the information currently available.

pOG705. Tables 4.1 and 4.2 suggested that pOG705 may, like pOG690, be an intermediate plasmid. There were many extra fragments in both *Pst*I and *Sma*I REFPs of pOG705 which were also present in pOG660. The fragments present in pOG674 which were not seen in pOG705, were possibly created as the result of the deletion of DNA to create pOG674 from pOG705 Therefore, this DNA should be present somewhere in pOG705. The 2.8kb *Pst*I probe hybridised with a 2.8kb fragment from this plasmid but this was faint in Figure 4.19.

pOG701. The most immediate observation from Tables 4.1 and 4.2 was that *Pst*I and *Sma*I fragments mapped in the virulence region were not present in this plasmid. The 11kb *Sma*I fragment was missing, as was the 5.7kb *Pst*I fragment. However, *Pst*I fragments of 3.4kb and 0.88kb were present which suggested the possibility that not all of the virulence region was lost. The virulence region in pOG674 had four *Bam*HI restriction sites and the entire plasmid had six sites. pOG701 however, had one single *Bam*HI restriction site, as determined from the *Pst*I/*Bam*HI double digest (Figure 4.20). This figure showed the loss of a single 3.9kb *Pst*I fragment from pOG701 to generate a fragment of 3.7kb. The *Bam*HI site from pOG701 corresponded to the *Bam*HI site of pOG674 at one of two map positions, 23.9' or 33.9'. The site at 23.9' was within the 3.4kb *Bgl*II fragment of pOG674. A *Bgl*II/*Bam*HI double digest of pOG701 (Figure

FIGURE 4.20

Typhimurium plasmid pOG660 and Enteritidis plasmids pOG690, pOG701 and pOG691 digested with *Pst*I and *Bam*HI and double digested with *Pst*I/*Bam*HI. Lane 1, λ*Pst*I; 2, pOG660 *Pst*I; 3, pOG660 *Pst*I/*Bam*HI; 4, pOG660 *Bam*HI; 5, pOG690 *Pst*I; 6, pOG690 *Pst*I/*Bam*HI; 7, pOG690 *Bam*HI; 8, pOG701 *Pst*I; 9, pOG701 *Pst*I/*Bam*HI; 10, pOG701 *Bam*HI; 11, pOG691 *Pst*I; 12, pOG691 *Pst*I/*Bam*HI and 13, pOG701 *Bam*HI.

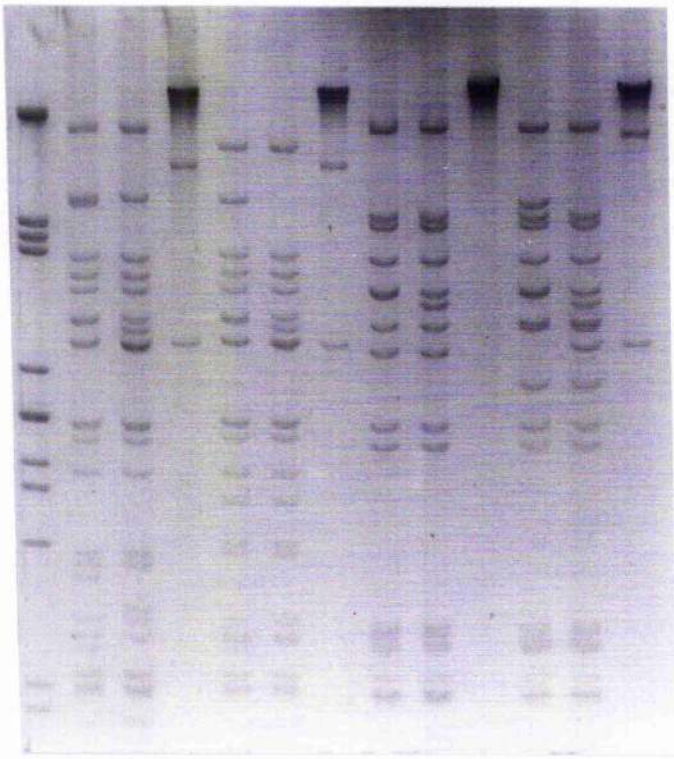
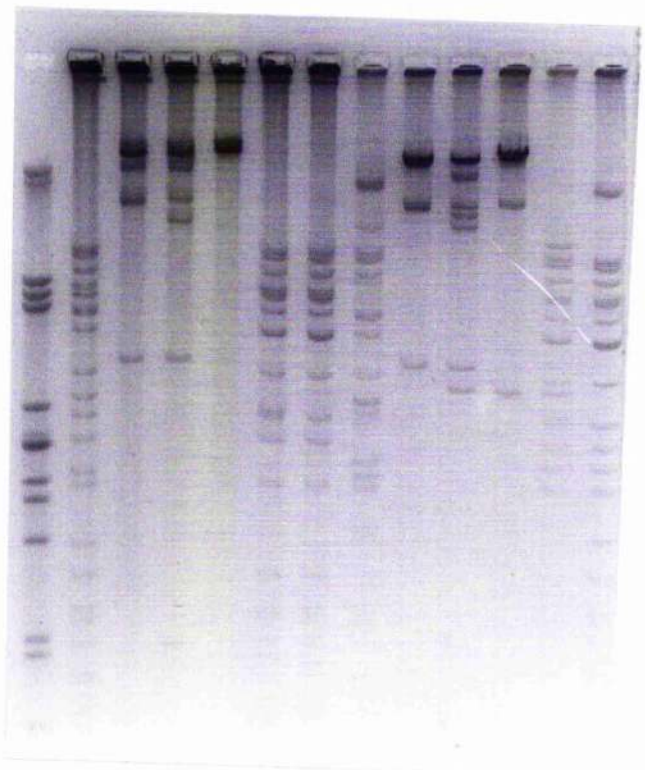


FIGURE 4.21

Enteritidis plasmids pOG701 and pOG691 digested and double digested with selected restriction endonucleases as shown. Lane 1, λ*Pst*I; 2, pOG701 *Sma*I/*Bgl*II; 3, pOG701 *Bgl*II; 4, pOG701 *Bgl*II/*Bam*HI; 5, pOG701 *Bam*HI; 6, pOG701 *Sma*I/*Bam*HI; 7, pOG701 *Sma*I; 8, pOG691 *Sma*I/*Bgl*II; 9, pOG691 *Bgl*II; 10, pOG691 *Bgl*II/*Bam*HI; 11, pOG691 *Bam*HI; 12, pOG691 *Sma*I/*Bam*HI and 13, pOG691 *Sma*I.



4.21) showed that the 3.4kb *Bgl*II fragment had no *Bam*HI site. Therefore, the *Bam*HI site in pOG701 corresponded to the site at 33.9' on the restriction map of pOG674.

pOG691. This plasmid and pOG701 were very similar and the different REFPs observed were largely due to the deletion within the virulence region of pOG701. The 2.8kb *Pst*I probe hybridised with a *Pst*I fragment of 2.8kb in pOG691. From the map data, this fragment was on the 7.4kb *Bam*HI fragment of pOG674 (Figure 4.11). Plasmid pOG660 had no 2.8kb *Pst*I fragment but did have a *Bam*HI fragment of 7.4kb. The fragments which hybridised with the probe were larger than 2.8kb in pOG660, pOG690 and pOG704 but all of these plasmids had a *Bam*HI fragment of 7.4kb. In pOG691, the probe hybridised with a 2.8kb fragment on a *Bam*HI fragment of around 10kb this suggested further heterogeneity between this plasmid and the others in the study.

pOG690. This plasmid has previously been described as an evolutionary intermediate in the descent of pOG660 to form pOG674. The Dice coefficients of similarity (Table 3.7, page 92) showed that pOG690 was more related to pOG660 than pOG674; but this could have been accounted for by the 30kb difference in size.

Between pOG690 and pOG660 there were very few differences. The *Pst*I and *Sma*I REFPs showed that pOG660 had extra fragments but as above could have been accounted for by the 15kb difference in size. The *Bam*HI REFPs of these two plasmids showed that the only difference between the plasmids was that the biggest fragment from pOG660, had lost DNA (possibly from the region in pOG674 at around 10' on the map?). This concurred with the *Sal*I REFPs (Figure 4.22) which showed that both pOG690 and pOG674 had a *Sal*I fragment of 8.2kb (in the above region of the map) which was absent

in pOG660. A *Bgl*II REFP (Figure 4.23) showed the loss of the biggest *Bgl*II fragment from pOG660 and this fragment was replaced in pOG690 by two smaller fragments. This extra *Bgl*II site in pOG690 accounted for the *Bgl*II site in a 4.4kb *Pst*I fragment. This 4.4kb *Pst*I fragment was present in all plasmids (Table 4.1) and was thought to be 'core'. However, pOG690 had a *Bgl*II site within this 4.4kb *Pst*I fragment that was not in the 4.4kb *Pst*I fragment from pOG660. This *Bgl*II site was shown to be present in pOG701, pOG691 and pOG674 also.

FIGURE 4.22

Typhimurium plasmid pOG660 and Enteritidis plasmids pOG674, pOG690 and pOG701 digested with *Pst*I and *Sal*I and double digested with *Pst*I/*Sal*I. Lane 1, λ*Pst*I; 2, pOG674 *Pst*I; 3, pOG674 *Pst*I/*Sal*I; 4, pOG674 *Sal*I; 5, pOG660 *Pst*I; 6, pOG660 *Pst*I/*Sal*I; 7, pOG660 *Sal*I; 8, pOG690 *Pst*I; 9, pOG690 *Pst*I/*Sal*I; 10, pOG690 *Sal*I; 11, pOG701 *Pst*I; 12, pOG701 *Pst*I/*Sal*I and 13, pOG701 *Sal*I.

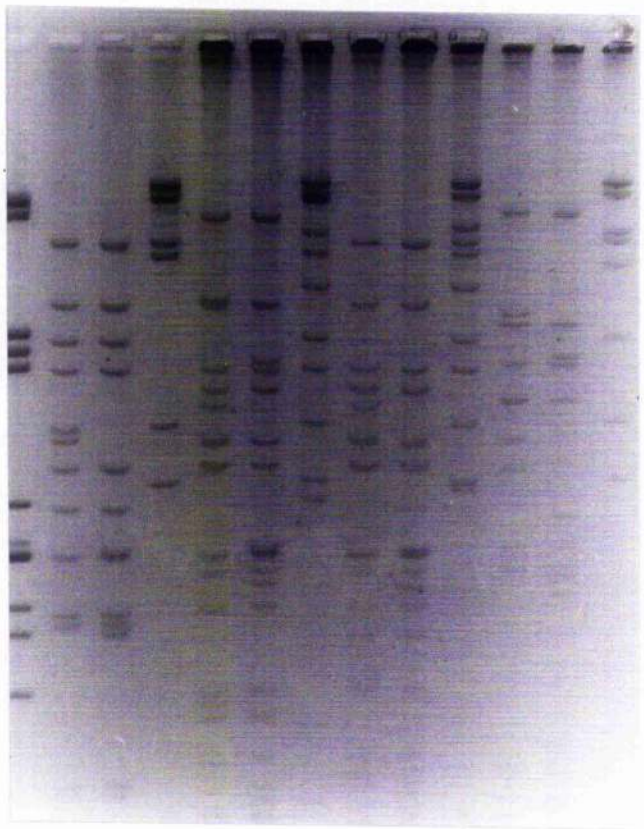
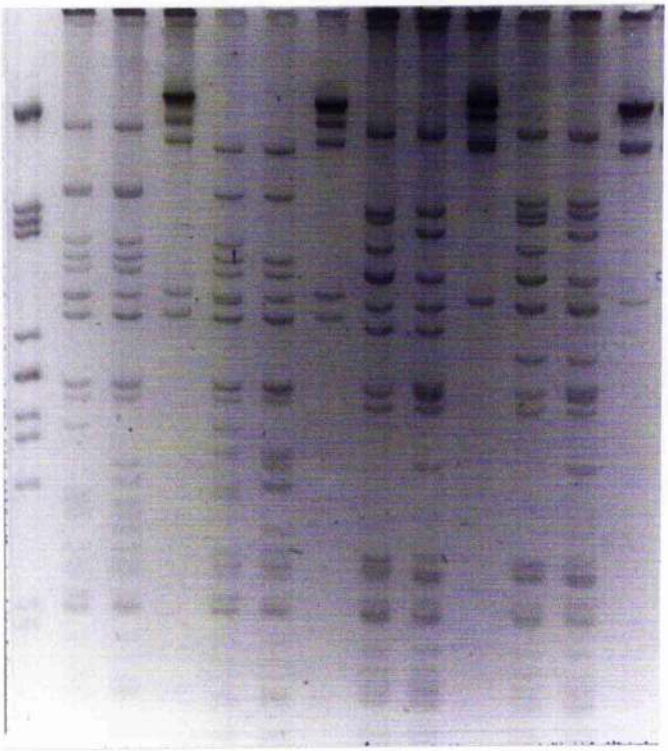


FIGURE 4.23

Typhimurium plasmid pOG660 and Enteritidis plasmids pOG690, pOG701 and pOG691 digested with *Pst*I and *Bgl*II and double digested with *Pst*I/*Bgl*II. Lane 1, λ*Pst*I; 2, pOG660 *Pst*I; 3, pOG660 *Pst*I/*Bgl*II; 4, pOG660 *Bgl*II; 5, pOG690 *Pst*I; 6, pOG690 *Pst*I/*Bgl*II; 7, pOG690 *Bgl*II; 8, pOG701 *Pst*I; 9, pOG701 *Pst*I/*Bgl*II; 10, pOG701 *Bgl*II; 11, pOG691 *Pst*I; 12, pOG691 *Pst*I/*Bgl*II and 13, pOG691 *Bgl*II.



4.4 Discussion

Partial restriction maps of the Enteritidis plasmid, pOG674, have been determined and from this data, relationships between this plasmid and the variant plasmids in the study were elucidated. Suzuki and colleagues (1994) have published a restriction map of a 55kb Enteritidis plasmid, pNL2001. This map has been adapted and used as the basis of *Pst*I and *Sma*I maps in this study (Figure 4.24).

Cloning of the 4.4kb *Pst*I fragment from Enteritidis was unsuccessful, which did not allow the demonstration of homology with this fragment that was expected. However, it became apparent from the map data that this fragment was in a region that encompassed part of the *par*/*rep*A region of the Typhimurium SAP (Cerin and Hackett, 1989; Tinge and Curtiss, 1990b). Figure 4.25 shows the position of these regions on a restriction map of a 91kb Typhimurium plasmid as described by Tinge and Curtiss (1990b). The plasmid encoded fimbrial locus (Friedrich *et al*, 1993) is also shown on this figure.

If the cloning could be successfully achieved, and the 4.4kb *Pst*I fragment from Enteritidis could be sequenced, this would allow comparison of the latter portion of this fragment with sequence data of the *par*VP region of Typhimurium plasmid pSLT (Cerin and Hackett, 1993). This sequence, of a 4332bp *Mlu*I-*Pst*I fragment from pSLT, has been shown to encode plasmid incompatibility and partition functions. The sequence showed two *Pst*I sites, one of which (3882nt) may correspond to the *Pst*I site at 7.7' on the Enteritidis map (Figure 4.11). If this were shown to be the case the sequence of the 4.4kb *Pst*I fragment from pOG674 should overlap with that of the *par*VP region from pSLT.

FIGURE 4.24

Restriction map of Enteritidis plasmid pNL2001. Adapted from Suzuki and colleagues 1994.

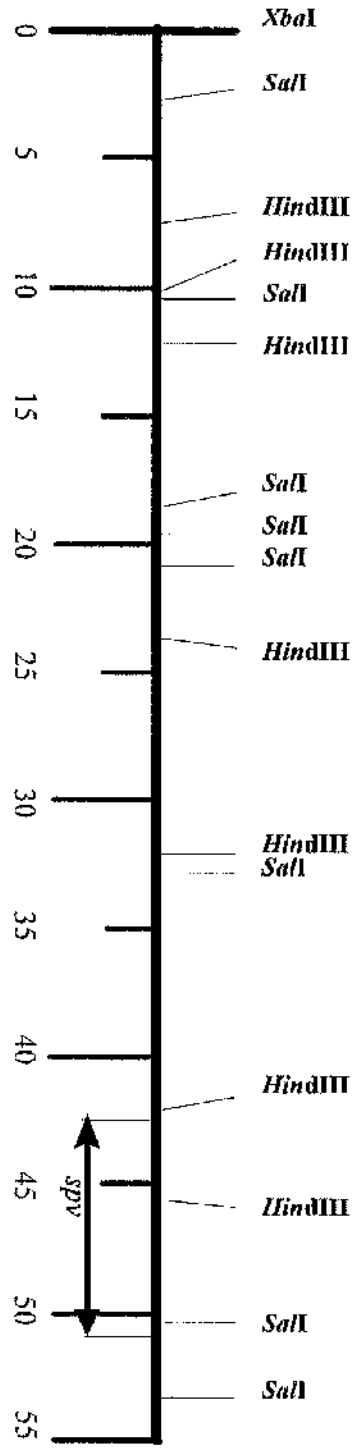
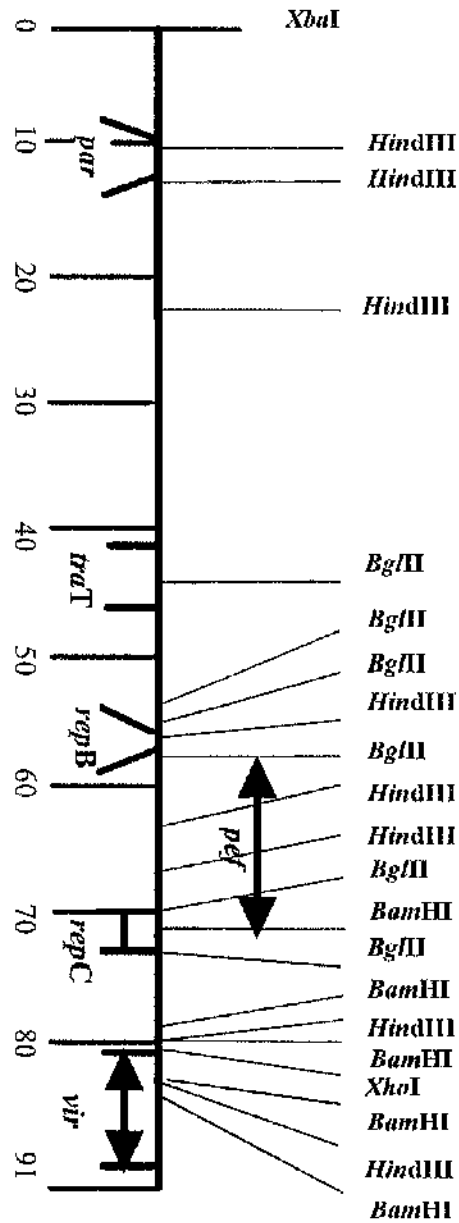


FIGURE 4.25

Restriction map of 91kb Typhimurium plasmid as described by Tinge and Curtiss 1990b. Plasmid encoded fimbriae (*pef*) locus as described by Friedrich and colleagues 1993.



Further to this, Tinge and Curtiss (1990b), revealed that probing a Southern blot of Typhimurium plasmid pStSR100 with a plasmid (pYA2027) that carried a 3.9kb insert, that covered much of the same region as the 4.4kb clone of Cerin and Hackett (1993), hybridised with 16.2 and 2.4kb *Hin*dIII, 60kb *Bgl*II and 7.3kb *Sal*I fragments. On the map of Enteritidis plasmid pOG674, the 4.4kb *Pst*I fragment has also been shown to cover these areas. Incidentally, the sequence data of Cerin and Hackett (1993) showed that the 2.4kb *Hin*dIII fragment from Typhimurium (H8, Michiels *et al*, 1987) had another *Hin*dIII site 300bp downstream. This site was absent in Enteritidis (pOG674) but the *Hin*dIII fragment in this position was 2.7kb not 2.4kb as shown in Typhimurium plasmid pStSR100 (Figure 4.1). It was therefore tentatively concluded that the probability that this fragment was non-homologous in the Enteritidis variants was unlikely.

One key observation was the presence of a *Bgl*II restriction site in the 4.4kb *Pst*I fragment of pOG690, pOG691, pOG701 and pOG674, that was not present in pOG660. In the map of pOG674, this 4.4kb *Pst*I fragment corresponded to the *rep*A region of the Typhimurium plasmid. It was possible that the *Bgl*II site in the 4.4kb *Pst*I fragments from Enteritidis plasmids could have occurred by the deletion of DNA from within this region of the Typhimurium plasmid which coincidentally resulted in a *Pst*I fragment of 4.4kb. Another mechanism was the insertion of DNA within the 4.4kb Enteritidis *Pst*I fragment that again coincidentally resulted in the formation of a 4.4kb fragment. Alternatively, this site may have arisen by point mutation in the early evolution of Enteritidis plasmids and has been conserved through time. Several attempts to clone this fragment from Enteritidis have been unsuccessful, as the transformants had apparently undergone deletion. The need to successfully achieve this appears more important now than it did in the planning stages of the study when this fragment was designated as a control of homology.

Hybridisation analysis with the 2.8kb *Pst*I probe from pOG674 showed, that with the exception of pOG701, this sequence was present in all the plasmids tested. Plasmids, pOG660, pOG690 and pOG704 showed hybridisation with fragments greater than 2.8kb. A 3.4kb fragment hybridised in *Pst*I digests of pOG660 and pOG690. This was suggested to be the result of a point mutation, and leads to the assumption that the 600bp upstream from the new site in pOG674 should have the same sequence as the extra 600bp present in the 3.4kb *Pst*I fragment from Typhimurium plasmid, pOG660. In pOG704, the *Pst*I fragment that hybridised with the probe was 3.3kb. The only observable difference in *Pst*I REFPs of pOG704 and pOG674 was this extra 3.3kb fragment. The *Sma*I REFPs of these two plasmids showed the presence of an additional single 2.4kb fragment. A fragment of this size was common to the *Sma*I REFPs of all plasmids except pOG701 and pOG674. This data suggested that pOG674 and pOG704 could have diverged recently. Cloning and hybridisation of the 3.3kb and 3.4kb *Pst*I fragments and also the 2.4kb *Sma*I fragment followed by sequence analysis may have helped to elucidate the assumptions made above.

Figure 4.20 showed that pOG691 had a *Bam*HI site in a 3.9kb *Pst*I fragment. It has been assumed that this plasmid possessed the virulence region, based on REFP data, and the 2.8kb *Pst*I probe hybridised with a fragment of 2.8kb. This suggested that all four *Bam*HI sites were preserved in the virulence region, as expected, and that the *Pst*I fragment which preceded the virulence region in pOG691 was 2.8kb as observed in pOG674. It is interesting to note here, that the *Bam*HI site in the 3.9kb *Pst*I fragment of pOG691, corresponded to the *Bam*HI site that began the 13.9kb *pef* region of the Typhimurium SAP described by Friedrich and colleagues (1993). From the sequence data provided by Friedrich and colleagues (1993) the first *Pst*I site was 3.3kb downstream from the *Bam*HI site and this fragment incorporated both *pef*B and *pef*A. If the

3.9kb *Pst*I fragment from pOG701, which has been shown to contain the only *Bam*HI site in this plasmid, corresponded to this region of the Typhimurium plasmid then due to the lack of *Pst*I fragments of this size (3.3kb) in all plasmids except pOG691, pOG690 and pOG701 could it be argued that the *pef* region was not present in full in pOG674?

The *Bam*HI site at this position in pOG674 was in a *Pst*I fragment of 1.32kb not 3.9kb. However, although there was a *Bam*HI site on a 3.9kb *Pst*I fragment on pOG691 the *Bam*HI fragment was not 7.4kb as reported by Friedrich and colleagues (1993). pOG691 had no 7.4kb *Bam*HI fragment, the *Bam*HI fragment in pOG691 was approximately 10kb (Figure 4.20). This was an unexpected anomaly and suggested further heterogeneity between this plasmid and pOG660.

The *pef* region of Typhimurium plasmid pSLT was described and sequenced by Friedrich and colleagues in 1993. As the pOG674 restriction maps suggested that this region was absent, in whole or in part, in Enteritidis, the *Pst*I and *Sma*I restriction sites were determined from the published sequence (data not shown). The 14kb sequence, from the *Bgl*II site at 45kb to the *Bam*HI site at 59kb, was cloned from the Typhimurium LT2 plasmid. It was hypothesised that the *Bam*HI site (59kb) from Typhimurium corresponded to the *Bam*HI site at 33.9' in pOG674 (Figure 4.1). This was inferred from the REFP data only and remains to be confirmed. Friedrich and colleagues (1993) showed that this *Bam*HI site was preceded by a *Bgl*II site, as it was in pOG674 (Figure 4.1), but they also showed a *Bgl*II site approximately 1.8kb upstream from the *Bam*HI site that was absent in pOG674. Two Typhimurium *Hin*dIII fragments of 3.0 and 7.4kb respectively (Michiels *et al*, 1987) were also absent from pOG674. The *Bam*HI to *Bgl*II fragment in pOG674 was 7.4kb. It was conceivable that the *Bam*HI to *Hin*dIII fragment of 1.9kb had been derived by

deletion of DNA from the 13.9kb *pef* region but further deletions must also have occurred in this region to result in the situation we currently see in Enteritidis. A direct comparison of sequence data from specific regions above would have been required to determine, conclusively, what has occurred.

Recently, Woodward and colleagues (1996) demonstrated that the 54kb Enteritidis SAP encoded the *pef*A gene. This gene showed around 76% sequence homology with the *pef*A gene of Typhimurium. A *pef*A gene probe, prepared from the Typhimurium SAP, hybridised weakly with Enteritidis isolates of many different phage types. One exception was a strain of Enteritidis of phage type 9b. This strain harboured a 90kb plasmid and showed strong hybridisation with the Typhimurium generated *pef*A probe.

A reciprocal assay that utilised an Enteritidis generated *pef*A probe showed strong hybridisation with Enteritidis plasmids but weak hybridisation with both the Typhimurium SAP and the 90kb plasmid found in Enteritidis phage type 9b. Plasmid pOG690 from this study, showed many intermediate features between Typhimurium and Enteritidis and was also phage type 9b.

Woodward and colleagues (1996) initially assumed that the Enteritidis SAP encoded the entire 13.9kb *pef* operon on the basis that homology in this area of the plasmid had previously been demonstrated by others. Buisan and colleagues (1994) provided a partial restriction map of a 61kb Enteritidis plasmid (pFM82139) and the Typhimurium SAP (pFM501) and went on to demonstrate homology based on hybridisation analysis with *Hin*dIII fragments of pFM82139. The restriction map of Typhimurium plasmid pFM501 was based on pStSR100 (Tinge and Curtiss, 1990a) which was adapted from the map provided by Michiels and colleagues (1987). Buisan and colleagues (1994) showed that a 10.3kb *Hin*dIII fragment (C) from Enteritidis hybridised with two

*Hin*dIII fragments (7.4 and 3.0kb) from Typhimurium. A 12.3kb *Hin*dIII fragment (D) from pFM82139 hybridised with a 13.2kb fragment from pFM501. The *Bam*HI and *Bgl*II sites that begin and end the *pef* region of Typhimurium were located in *Hin*dIII fragments D and B respectively. In Figure 4.1, the *Hin*dIII fragments that corresponded to fragments D, C and B of Buisan and colleagues (1994), were 10, 8.5 and 11.5kb respectively, not 12.3, 10.3 and 13.2kb as stated for pFM82139. The differences in size of the *Hin*dIII fragments in Figure 4.1 compared with fragments D, C and B of pFM82139 total 6.5kb which is approximately the difference in size of plasmids pOG674 (54kb) and pFM82139 (61kb). The figures provided by Buisan and colleagues (1994) indicated the possibility that they have either worked with an Enteritidis variant plasmid or alternatively that their estimates of the size of *Hin*dIII fragments were incorrect.

Although the hybridisation results of Buisan and colleagues (1994) suggested homology they gave no direct evidence as to the extent of the homology. Total homology should not be inferred without analysis of the sequence data. In the above example, Enteritidis probe fragments were smaller than the Typhimurium fragments to which they hybridised. This can not indicate that the smaller probe fragment was 100% homologous, in sequence, to the larger Typhimurium fragment to which it hybridised.

In 1996, Baumler and colleagues showed the position of a 3.5kb *Eco*RI/*Sal*I fragment of the Typhimurium SAP that hybridised with a PCR generated *pef*A probe. The *Eco*RI site was located 1.4kb from the *Bam*HI site that began the *pef* region therefore, the *Bam*HI/*Sal*I fragment was approximately 4.9kb. The corresponding *Bam*HI/*Sal*I fragment in Figure 4.1 was 1.1kb. Unfortunately, Buisan and colleagues (1994) did not provide *Sal*I

data as a comparison and although Suzuki and colleagues (1994) provided *Sal*I and *Eco*RI sites there were no *Bam*HII sites on their map.

Woodward and colleagues (1996), demonstrated hybridisation of Enteritidis isolates with three different genes associated with the *pef* gene cluster. In their analysis, 49 Enteritidis isolates which encoded *pef*A also encoded both *pef*B and *pef*I. It was reasoned that as the entire *pef* gene cluster was required to synthesise the fimbrial structure and this was achieved via a cosmid clone, that the entire gene cluster was present. Sequence analysis of the Enteritidis *pef*A allele, derived by PCR, indicated 76% sequence identity with the Typhimurium *pef*A allele; significant sequence divergence was located in three regions. If this level of sequence divergence was maintained within the entire 13.9kb region sequenced by Friedrich and colleagues (1993) this alone could account for the differences observed in the restriction map of Enteritidis presented here, with those published previously for Typhimurium (Michiels et al, 1987; Tinge and Curtiss, 1990a). This analysis should therefore be a high priority for future research.

The results presented have indicated a variety of plasmids that show different degrees of structural homology to both Enteritidis and Typhimurium. A few observations were of particular interest. One plasmid, pOG701, appeared on the basis of *Pst*I and *Sma*I REFPs, to have no identifiable virulence region. This plasmid is common in Enteritidis phage type 15 strains (results not shown) and clearly demands further research. As does the plasmid, pOG691, which is common in Enteritidis phage type 11 strains. It was recently shown that PT15 strains can be converted, by a phage, to PT11 (Rankin and Platt, 1995). Whether this is significant given the similarities observed in their plasmid REFPs remains to be determined. Plasmid, pOG690, is clearly an intermediate, of some description, between pOG660 and pOG674. It has been shown that at

least one restriction site (*Bgl*II) present in pOG690 has been conserved in pOG691, pOG701 and pOG674. All of these plasmids, with the exception of pOG674, have been shown by Dice coefficients of similarity (Table 3.7), based on REFPs, to bear greater similarity to Typhimurium (pOG660) than Enteritidis. Further complete maps and possibly sequence data will be required, particularly of pOG690, before this can be confirmed.

The evidence presented thus far is opposing in that the map data presented for Enteritidis plasmid, pOG674, would seem to suggest that the *pef* region described for typhimurium may not be present as a whole in Enteritidis. However, PCR and hybridisation analysis has shown three *pef* genes to be present (Woodward *et al*, 1996). Further restriction map data, and more importantly, complete sequence analysis of the region in Enteritidis plasmids that should correspond to the 13.9kb pef region of Typhimurium should be performed to complete this analysis. It is important that this approach also be extended to the variant plasmids observed in Enteritidis, particularly pOG690, to determine whether or not plasmid encoded fimbriae are expressed by these plasmids also.

The evidence presented above extended previous observations that the serotype associated plasmids of the salmonellae are part of a "family" of related plasmids. Variation such as that seen here has been identified previously in Typhimurium and Dublin (Platt *et al*, 1988b; Browning and Platt, 1995). This variation, although present overall at a low level (4%), has been observed at greater than 20% in some of the previously defined subsets. Although, as stated in Chapter Three this was dependant on the population under investigation. This variation, when observed, helps to extend the critical application of plasmid REFP analysis in Enteritidis on a day to day basis. The recognition and analysis

of further variant SAPs, as they become available, may allow further insight into the evolution of this extensive family of related plasmids.

CHAPTER 5.

Salmonella Case Studies.

5.1 Introduction

The following case studies are presented to demonstrate the practical application of plasmid analysis in routine epidemiological investigations. Three examples were chosen from a large number of investigations carried out at the Scottish Salmonella Reference Laboratory (SSRL). The aim of each study is stated in the text but the reasons for their inclusion in this chapter are given below.

The previous chapters have been primarily concerned with the application of plasmid analysis in *Salmonella enterica* serotype Enteritidis. It has been shown that diversity in plasmid content exists within Enteritidis and it has been suggested that the interpretation of plasmid restriction endonuclease fragmentation patterns are useful in an epidemiological context.

Study one gives details of an outbreak of Enteritidis phage type 14b. It has been shown previously that Enteritidis isolates predominantly harbour a single plasmid of 54kb. When present on its own this plasmid provides little epidemiological discrimination. However, the presence of one or more additional plasmids has been shown to significantly increase the discrimination achieved by plasmid analysis. This study shows that this can be the case but also highlights the benefits of the existence of a reference pool of organisms with which to conduct retrospective analysis of unrelated isolates in the course of an outbreak investigation.

Study two, highlights the significance conferred by the recognition of molecular polymorphisms within serotype associated plasmids (SAPs). *Salmonella enterica* serotype Typhimurium has, like Enteritidis, been shown to harbour molecular variants of it's SAP. The frequency of their occurrence is

low, but it is this aspect that can be used to provide conclusive evidence for relatedness in the absence of information from other typing methods.

Lastly, study three demonstrates the application of plasmid analysis in *Salmonella enterica* serotype Derby in which no SAP has previously been demonstrated. Derby is a serotype that is associated, primarily, with pigs. Its occurrence in sheep was therefore of some concern. Initially the question of cross-contamination from pigs was considered but the results obtained from plasmid analysis identified a new strain in sheep. This strain was subsequently traced to contaminated animal feed and it has recently been identified in the human population.

5.2 Case study 1

An epidemiological investigation of *Salmonella enterica* serotype Enteritidis phage type 14b.

In December 1994 the Scottish Salmonella reference laboratory (SSRL) received notification that a food poisoning outbreak had occurred at a christening party held in Glasgow. At least eighteen family members and friends were affected by diarrhoea and abdominal cramps. Faecal samples were submitted and *Salmonella enterica* serotype Enteritidis was isolated. The phage type (PT) was determined by SSRL as 14b. Antibiotic sensitivity testing revealed that all eighteen isolates were fully sensitive to all thirteen antibiotics tested at SSRL.

The previous incidence of this phage type in Scotland was low, twenty one isolates were confirmed by the SSRL prior to the above outbreak. The majority of these twenty one isolates were sporadic human cases predominantly isolated from the East coast of Scotland and two were isolated from poultry sources. All but three of these previous isolates were fully sensitive to the antibiotics tested at SSRL. These three isolates were resistant to ampicillin and sulphonamides, kanamycin and tetracycline and sulphonamides alone, respectively .

Plasmid profile (PP) and restriction endonuclease fragmentation pattern (REFP) analyses were carried out on twelve isolates that comprised five from the christening party outbreak and seven sporadic isolates (Table 5.1). All twelve isolates harboured the 54kb Enteritidis SAP together with one or more additional plasmids. The predominant plasmid profile (75%) was determined as 54: 9kb and all five isolates from the outbreak were of this type. One poultry isolate included in this analysis showed the PP 54: 9: 8: 7.5: 7kb. No source

Table 5.1 Comparison of outbreak-related and sporadic isolates of *Salmonella enterica* serotype Enteritidis isolates from 1994.

Ref No.	Source	Plasmid Profile (kb)	Antibiotic Resistance
SR940012	Poultry	54: 9: 8: 7.5: 7	ASu
SR942765	Human	54: 9	Fully Sensitive
SR943168	Human	100: 54: 9	KmTc
SR943453	Human	54: 9	Fully Sensitive
SR943470	Human	54: 9	Fully Sensitive
SR943526	Human	54: 9	Fully Sensitive
SR943586	Human	54: 9	Su
SR945998	Human	54: 9	Fully Sensitive
SR946048	Human	54: 9	Fully Sensitive
SR946085	Human	54: 9	Fully Sensitive
SR946252	Human	54: 9	Fully Sensitive
SR946279	Human	54: 9	Fully Sensitive

was established for the outbreak and it was therefore concluded, on the basis of the available epidemiological information, that the outbreak isolates were identical and had a high likelihood of being related. The predominant PP in Enteritidis is a single plasmid of 54kb, the presence of an additional plasmid is significant and would infer that the isolates had originated from a common source. However, the background isolates included in this study revealed that the PP 54: 9kb was common in Enteritidis PT14b and the evidence for relatedness between outbreak strains rested primarily on association with exposure.

In October 1995, a small outbreak of Enteritidis PT14b occurred in a geriatric nursing home and highlighted that the incidence of PT14b had increased relative to the previous year. It was thus decided that a study be undertaken to determine whether or not this increase was due to clonal expansion of a particular organism.

Thirty four isolates from 1990 to 1995 were examined by PP and REFP analysis and the results showed that 27/34 (78%) of the isolates showed the same plasmid profile, 54: 9kb (Table 5.2). Of the remaining seven isolates, two harboured both the 54 and 9kb plasmids in combination with one or more additional plasmids one showed the 54kb SAP present on its own, three isolates harboured the SAP plus one or more additional plasmids and one isolate harboured a single plasmid of 7kb. Twenty eight of the above isolates were of human origin, four were isolated from poultry sources and two others were from a dog and an environmental sample of mussels isolated from a Scottish beach respectively.

During the previous outbreak in 1994, one poultry isolate was studied. This second study examined four isolates from poultry sources, one isolated in

Table 5.2 Analysis of sporadic isolates of *Salmonella enterica* serotype Enteritidis phage type 14b.

Ref. No.	Source	Plasmid Profile (kb)	Antibiotic Resistance
SR 901842	Human	54	Fully Sensitive
SR903062	Human	7.0	Fully Sensitive
SR912766	Human	54: 40: 7	Fully Sensitive
SR913050	Human	54: 7	Fully Sensitive
SR920256	Poultry	54: 9	Fully Sensitive
SR930873	Poultry	54: 9	Fully Sensitive
SR933779	Canine	54: 9	Fully Sensitive
SR945350	Poultry	54: 9	Fully Sensitive
SR950014	Human	54: 9	Fully Sensitive
SR950763	Human	54: 9	Fully Sensitive
SR950946	Human	54: 9	Fully Sensitive
SR951123	Human	54: 9	Fully Sensitive
SR952668	Human	54: 9	Fully Sensitive
SR952852	Environment	54: 9: 8: 7.5: 7	ApSu
SR953449	Poultry	54: 9	Fully Sensitive
SR953996	Human	54: 9	Fully Sensitive
SR954202	Human	54: 9	Fully Sensitive
SR954319	Human	54: 50: 40	Ap
SR954387	Human	54: 9	Fully Sensitive
SR954443	Human	54: 9	Fully Sensitive
SR954567	Human	54: 9	Fully Sensitive
SR954607	Human	54: 9	Fully Sensitive
SR954634	Human	54: 9	Fully Sensitive

SR954642	Human	54: 9	Fully Sensitive
SR954796	Human	54: 9	Fully Sensitive
SR954916	Human	54: 9	Fully Sensitive
SR955007	Human	54: 9	Fully Sensitive
SR955008	Human	54: 9	Fully Sensitive
SR955111	Human	54: 9	Fully Sensitive
SR955191	Human	54: 9	Fully Sensitive
SR955260	Human	54: 9	Fully Sensitive
SR955389	Human	54: 9	Fully Sensitive
SR955514	Human	54: 40: 9	Fully Sensitive
SR955712	Human	54: 9	Fully Sensitive

1992, one in 1993 and two in 1995. Since 1992, when the first poultry isolate of Enteritidis PT14b was identified, the total number of isolates from all sources received by SSRL had increased (results not shown). *Salmonella* Enteritidis phage type 14b has not been isolated from any other food animal in Scotland and it would thus seem to be a reasonable conclusion that the source of this new strain may be poultry associated.

5.3 Case study 2

An investigation of a presumptive outbreak of *Salmonella enterica* serotype Typhimurium in cattle.

Salmonella enterica serotype Typhimurium is a common infectious disease in cattle. It is well recognised that the bovine reservoir is important in the foodborne transmission of salmonella to man. However, another important route of transmission to man is contact with infected animals. This study demonstrates that infection from cattle to man can occur even when an outbreak had already been recognised on a farm. In these circumstances it would have been expected that hygiene practices should have been improved.

Salmonella Typhimurium isolated in veterinary laboratories is initially identified by culture on selective media and serotyped by slide agglutination using specific antisera. Discrimination within Typhimurium is achieved primarily by phage typing. As many phage types have become ubiquitous further discrimination is often required for epidemiological investigations, and this can frequently be achieved using plasmid profile and restriction endonuclease fragmentation pattern analyses. This study highlights many of the problems encountered in the epidemiology of salmonellosis and shows how PP and REFP analyses in which SAP variation was demonstrated, were used to determine whether isolates from two bovine outbreaks, which had apparently different phage types but a history of stock movement between the two farms, were epidemiologically related.

On 12 May 1994 Farm A, a beef rearing and fattening unit, bought 9 Friesian bull calves of 3 - 4 of weeks age at auction (Batch 1). The calves came from a 110 cow, dairy herd; Farm B. They were transported directly from the mart to Farm A, placed in individual pens in a large shed and bucket-fed milk

replacer, until they progressed to pelleted dry feed. Towards the end of May one calf became depressed and died. A second calf that showed depression and blood and mucus in soft faeces was sampled on 28 May. It was treated with oral electrolytes and an oral trimethoprim/sulphadiazine combination. *Salmonella enterica* serotype Typhimurium was isolated from its faeces. The isolate was phage typed as Typhimurium DT104b.

On 23 June a further batch of 2 - 4 week old calves, including 3 from Farm B was bought and transported to Farm A (Batch 2). On 27 June 4 calves from Batch 2 were sampled, 3 exhibited malaise and had mucus and blood in the faeces; one calf was clinically well. In addition the remaining 8 calves from Batch 1 were sampled, including the first laboratory positive animal. Serotype Typhimurium was again isolated from all 4 calves of Batch 2 and from one other contact calf from Batch 1. One isolate was sent to SSRL; it was confirmed as Typhimurium and phage typed as DT104b.

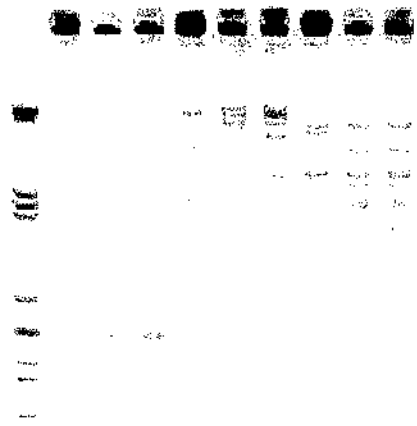
Farm B was a well managed dairy farm, calving throughout the year, it had no apparent enzootic salmonellosis problem. However, on 2 June a cow on Farm B developed scour. A dietary cause was initially suspected and supportive therapy given. With the persistence of the scour and progressive systemic involvement she was sampled on 7 June. Salmonella serotype Typhimurium was isolated and was subsequently phage typed as RDNC. The overall phage typing pattern of this isolate did not differ from that of a DT104b but consistently gave reactions which were at least ten fold less than are normally seen i.e. phages which in a DT104b produced semi confluent lysis generated 10 - 20 plaques. Thus these isolates did not conform to a recognised pattern under the strict rules which govern phage typing and were therefore designated as RDNC (Routine dilution non conforming). Other cows and some calves, on

Farm B, became similarly affected and the same organism was isolated from them.

It was noted that the isolates from Farms A & B had identical antibiotic resistance patterns. They were all resistant to ampicillin, streptomycin, sulphonamides and tetracycline (R-type ASSuT). As there was a history of stock movement between the two properties it was suggested that the isolates were examined using PP and REFPs.

Plasmid profile analysis of the isolates revealed that both types (DT104b and RDNC) harboured a single 95kb plasmid. Restriction enzyme fragmentation pattern analysis using, initially, *Pst*I and *Sma*I demonstrated that this plasmid differed from the SAP of Typhimurium strain NCTC73 (pOG660), which is used routinely as a Typhimurium SAP control. The REFP is shown in Figure 5.1 with three different enzymes. REFP analysis in contrast to hybridisation provides an indirect estimate of overall plasmid relatedness. Farrar (1983) argued that if two plasmids generate identical restriction fragments, from two or more enzymes, they may be assumed to be identical plasmids: conversely, plasmids of the same size that produce entirely different fragmentation patterns are essentially unrelated plasmids: and finally, plasmids of the same or different sizes may share homologous regions of DNA, which are detectable in restriction fingerprints. This plasmid has been recognised as a molecular variant of the Typhimurium SAP. Therefore, because this plasmid differed from both pOG660 and previously described variants it was concluded that the isolates from Farms A & B were related. SSRL has routinely examined many Typhimurium isolates using PP and REFP analyses and this is the first report of this particular molecular variant which has been designated pSRS129. The "source" of the infection has not yet been identified.

FIGURE 5.1. Restriction endonuclease fragmentation patterns of Typhimurium strains NCTC 73 (Lanes 2,5 and 8), DT 104b (Lanes 3,6 and 9) and RDNC (Lanes 4,7 and 10) digested with *Pst*I, *Sma*I, and *Eco*RV respectively. Lane 1 contains Lambda phage DNA digested with *Pst*I.



This plasmid (pSRS129) was compared to pOG660, pOG674 and pOG690 and Dice coefficients of similarity were calculated from both *Pst*I and *Sma*I REFPs (Table 5.4). It was interesting to note that pSRS129 showed greater similarity to pOG690, with both enzymes, than it did to either Typhimurium or Enteritidis SAPs. In pSRS129 the 10.1kb *Pst*I fragment seen in pOG660 was absent whereas an additional 8.3kb fragment common to both pOG674 and pOG690 was evident. This indicated that this particular variant plasmid may show polymorphisms similar to those observed for pOG674 and pOG690 which is in itself interesting as pSRS129 was seen in Typhimurium and not Enteritidis.

On 25 July while the farm outbreaks were actively under investigation a farm hand from Farm B (patient A) developed diarrhoea. Stool samples were submitted to the local hospital bacteriology laboratory and Salmonella Typhimurium was isolated. The strain was phage typed, at SSRL, as DT104b and antibiotic resistance was determined as ASSuT. This suggested that the organism isolated from patient A may have been related to the Typhimurium strain previously isolated from cattle on this farm. Plasmid profile and REFP analyses confirmed this to be the case. The 95kb plasmid harboured by this strain was indistinguishable from pSRS129.

In many cases of Typhimurium infection it is difficult to be certain that two or more isolates from different sources are related based on phage type alone. Although many phage types are ubiquitous the plasmid pool within a type may be diverse and it is important to supplement phage typing with PP and REFP when possible during epidemiological investigations. The clinical and epidemiological information available on these two outbreaks suggested as association which was confirmed by PP and REFP results. It is rare in *Salmonella* epidemiology that conclusive data is found to confirm field

observations. This case highlights the considerable advantages gained from close collaboration between laboratory and field based investigators and demonstrates the use of molecular techniques to achieve a high level of discrimination.

Table 5.3. Dice coefficients of similarity (S_D) of pSRS129 compared with *Pst*I (upper) and *Sma*I (*lower*) REFPs of pOG660, pOG674 and pOG690.

Plasmid Designation	pSRS129	pOG660	pOG674	pOG690
pSRS129	100	69	52	71
pOG660	*52*	100	68	89
pOG674	*50*	*57*	100	68
pOG690	*61*	*79*	*55*	*100*

5.4 Case study 3
Investigation of *Salmonella enterica* serotype Derby in sheep.

In May 1994 the Scottish Salmonella Reference Laboratory received a notification from the Scottish Centre for Infection and Environmental Health (SCIEH), Ruchill Hospital, Glasgow, that the incidence of serotype Derby isolated from ovine sources appeared to have increased relative to previous years. Derby is traditionally a serotype associated with porcine sources and there was concern from SCIEH of a potential problem.

In pigs infection with *Salmonella* normally follows ingestion of the organism. Pigs of all ages can be affected and outbreaks are commonest in young pigs however, this rarely results in death except in suckling pigs. The isolation of salmonellae from pigs, especially of only a few colonies after enrichment, may not indicate significant infection of the animal or involvement of the organism in any disease syndrome. It is however of concern for public health and any isolation of salmonellae from pigs must be reported under the Zoonosis Order 1975.

Many of the ovine cases in 1994 had occurred during lambing and had resulted in abortion of the foetus. There were seven ovine isolates submitted during this period all of which were isolated from different farms in different geographic locations in Scotland. A study was designed to compare these isolates with isolates of Derby from diverse sources over the previous two years. Twenty nine isolates were selected from a total of fifty two in 1992 - 1994. Details of the strains are shown in Table 5.4.

Initially, antibiotic resistance patterns and plasmid profiles (PP) were determined by previously defined methods. Eight of twenty nine isolates showed resistance to tetracycline (10μg/ml) a further two isolates showed

Table 5.4 *Salmonella enterica* serotype Derby.

Ref. No.	Source	Antibiotic resistance
SR920015	Ovine - vaginal swab	Fully Sensitive
SR920197	Environmental - sewer swab	Tc
SR920425	Human - gut	Fully Sensitive
SR920508	Porcine - weaner	Tc
SR920669	Environmental - abattoir	KmTc
SR920950	Porcine - viscera	Tc
SR924122	Human - lesion swab	Fully Sensitive
SR924916	Porcine - viscera	Tc
SR930375	Porcine - foetus	KmTc
SR932196	Pheasant - viscera	Fully Sensitive
SR932590	Human - gut	Fully Sensitive
SR940334	Environmental - abattoir, tripery	Fully Sensitive
SR940337	Environmental - lairage	Tc
SR940420	Environmental - abattoir, sewer	Tc
SR940515	Environmental - abattoir	Fully Sensitive
SR940650	Ovine - carcase	Fully Sensitive
SR940851	Ovine - placenta	Fully Sensitive
SR940870	Ovine - placenta	Tc
SR940876	Ovine - faeces	Fully Sensitive
SR940982	Ovine - abortion	Fully Sensitive
SR941053	Environmental - hospital	Tc
SR941122	Porcine - viscera	Fully Sensitive
SR941123	Not Stated	Fully Sensitive
SR941142	Porcine - abortion	Fully Sensitive
SR941164	Ovine - abortion	Fully Sensitive
SR941182	Bovine - gut & lymph nodes	Fully Sensitive
SR941203	Bovine - foetal stomach	Fully Sensitive
SR941222	Bovine - faeces	Fully Sensitive
SR941439	Ovine - viscera	Fully Sensitive

additional kanamycin resistance (20μg/ml) the other isolates were fully sensitive to all antibiotics tested (Table 5.5).

Plasmid profile analysis (PPA) showed nine different profiles (Table 5.5). A single ovine isolate was available from 1992-1993 and this showed the profile 90: 7kb. The profile 90: 7kb was common among the porcine isolates but was not observed among those from sheep in 1994. The predominant PP from ovine sources was 80kb.

Plasmid REFP analysis was performed with *Pst*I and *Sma*I. Neither of these two enzymes gave REFPs that were satisfactory for analysis. The *Pst*I REFPs showed between six and ten fragments with approximately 15kb being represented by fragments less than 10kb. The opportunity to detect molecular variation with this enzyme will be decreased substantially when less than 20% of the plasmid is visible on a gel. The *Sma*I REFPs showed more discrimination than *Pst*I with approximately ten evenly distributed fragments that encompassed around 60kb. However, 20 - 30kb was not represented with *Sma*I presumably due to a large number of *Sma*I restriction sites that generated many small fragments. Further enzymes (*Eco*RI, *Eco*RV, *Hin*dIII and *Ava*II) were tested and the most discriminatory was found to be *Eco*RV. Digestion with *Eco*RV revealed twelve different REFPs. Importantly it showed that the profile 90: 7kb, one of which was found in the 1992 ovine isolate, comprised two different REFPs. The 80kb plasmid harboured by ovine isolates from 1994 was also recovered from three independent bovine isolates. This 80kb plasmid showed no relationship to any of the 90kb plasmids isolated from pigs.

The 90kb plasmid in isolate SR940870 (REFP 9) showed a very similar fragmentation pattern to that observed in isolates of REFP 1 (90: 7kb). It was

Table 5.5 Plasmid profiles and REFPs of *Salmonella enterica* serotype Derby.

Ref. No.*	Source	Plasmid Profile (kb)	REFP No.
SR920015	Ovine	90: 7	1
SR920197	Environmental	90: 7	1
SR920425	Human	10	2
SR920508	Porcine	90: 7	3
SR920669	Environmental	90: 10: 7	4
SR920950	Porcine	90: 7	1
SR924122	Environmental	10	5
SR924916	Porcine	90: 7	1
SR930375	Human	90: 10: 7	6
SR932196	Pheasant	8	7
SR932590	Human	8: 7	8
SR940334	Environmental	10	5
SR940337	Environmental	90: 7	1
SR940420	Environmental	90: 10	9
SR940515	Environmental	80	10
SR940650	Ovine	80: 70	11
SR940851	Ovine	80	10
SR940870	Ovine	90: 10	9
SR940876	Ovine	80	10
SR940982	Ovine	80	10
SR941053	Environmental	8	12
SR941122	Porcine	90: 7	3
SR941123	NS	80	10
SR941142	Porcine	90: 7	3
SR941164	Ovine	80	10
SR941182	Bovine	80	10
SR941203	Bovine	80	10
SR941222	Bovine	80	10
SR941439	Ovine	80	10

* The first two digits of the reference number indicate the year of isolation.

deduced from the combined analysis of *Pst*I, *Sma*I and *Eco*RV that this plasmid was a variant of the 90kb plasmid in REFP 1 isolates (results not shown).

The 80kb plasmid in REFP 11 (SR940650) was identical to the 80kb plasmid seen in REFP 10. The additional 70kb plasmid in this strain digested well with *Pst*I, *Sma*I and *Eco*RV. None of the large plasmids in this study showed any similarity to the SAPs from any of the previously characterised *Salmonella* serotypes.

The 80kb plasmid appeared in isolates from 1994 only. It was found in isolates of both ovine and bovine origin and was also isolated from a vertical conductor swab taken from the Glasgow abattoir.

From January to June 1994, Derby was isolated from animal feed sources in the United Kingdom on eight occasions (Personal communication Mrs T Phillips, SVO, Scottish Office Agriculture and Fisheries Department, Edinburgh.). Three of these isolates were made available by Dr C Wray (Central Veterinary Laboratory, Weybridge) and plasmid REFP analysis was performed. One of these isolates CVLs/2922 showed an 80kb plasmid that was identical to that, previously identified as REFP 10, from ovine sources (Table 5.6 and Figure 5.2).

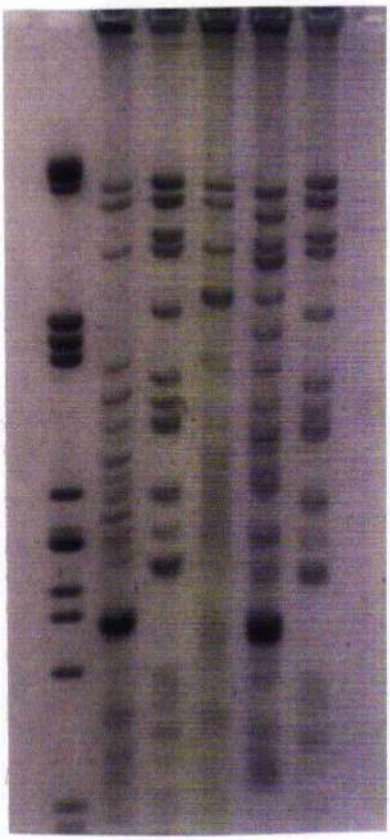
The nature and source of the feed sample from which this strain was isolated could not be determined. It was therefore concluded that a batch of contaminated feed was the most likely source for the introduction of this new strain into farm animals.

Routine monitoring at SSRL has revealed this strain in the human population which indicates that animal to human spread has occurred.

Table 5.6 *Salmonella enterica* serotype Derby isolates from animal feedstuffs.

Ref. No.	Source	Plasmid Profile (kb)	REFP No.
CVLs/1799	Feed	10	13
CVLs/2083	Feed	9	14
CVLs/2922	Feed	80	10

FIGURE 5.2. REFP analysis of plasmids from *Salmonella enterica* serotype Derby digested with *Eco*RV. Lanes 1, Lambda DNA digested with *Pst*I, 2, SR940337 (REFP1), 3, SR940515 (REFP10) 4, SR940870 (REFP9), 5, SR941122 (REFP3) and 6, CVLs/2922 (REFP10).



CHAPTER 6.

Discussion.

Epidemiology has been defined as a discipline concerned with the distribution and determinants of disease in a population (Fletcher *et al*, 1988). In clinical medicine epidemiologists are concerned that their observations represent fairly some defined group or 'population'. In general populations are large groups of people from which samples are selected for further research. When trying to determine whether exposure to 'X' produces disease 'Y' the sample should include, both people who have disease 'Y' and people who do not. It is important also, that the sample is large enough so that chance plays no part in the observed result.

Although one is interested in the characteristics of the defined population, for practical reasons these characteristics must be expressed through a sample. In doing this two fundamental questions arise. First, are the conclusions of the research correct for the sample? Second, if so, does the sample represent fairly the population of interest.

The epidemiology of transmissible disease is generally not as simplistic as stated above. In most cases the epidemiologist is trying to determine if those people with disease 'Y' have been exposed to organism 'X'; and whether or not organism 'X' is identical in all those members of the sample from which 'X' has been isolated.

Identification of pathogenic microorganisms by any means requires that the organism be distinguished from the background of non-pathogenic and non-epidemic strains (Eisenstein, 1990). This in turn requires that phenotypic traits of the organism can be used to differentiate clones within the population. Phenotypic traits are the observable characteristics of an organism e.g. serotype, bacteriophage type, biotype, antimicrobial resistance type. The phenotype of an organism is however the manifestation of gene expression in that organism, and

therefore, however indirectly, the phenotype is a reflection of the organism's genotype. The stability of phenotype can be influenced by environmental selective pressure and as isolation procedures are designed to exert selection pressure phenotypic methods of identification can be weak.

Molecular epidemiology is concerned with genotypic methods of identification. The organisms content of genetic information, either in total or with respect to one or more particular named alleles can be studied by various molecular biological techniques. Genotypes should be independent of the effects of natural selection. However, genotypes of different microbes vary in their stability. As stated by Eisenstein (1990), "Those bacteria with the most proficient conjugation systems, which promulgate the promiscuous exchange of plasmids, and the most active DNA recombination systems or transposable elements, which cause the most chromosomal mixing and rearrangements will have the most unstable DNA sequences". These organisms will be the most difficult to identify as clonal. Thus successful clone identification requires more than the ability to perform molecular techniques it requires primarily a knowledge of the organism under investigation.

Molecular epidemiology should embrace the definition of epidemiology and support its conclusions by the application of molecular biological techniques. In real terms the application of this combined approach should allow conclusions reached by epidemiology to be supported by molecular biology. Unfortunately, due to lack of understanding of both sets of basic principles by epidemiologists and molecular biologists alike, this has rarely been achieved.

Salmonella enterica serotype Enteritidis has been the dominant serotype of *Salmonella* in Scotland for almost ten years. In those years, many groups

have studied the epidemiology of this organism by many methods some of which have involved the application of molecular biological techniques. However few of these studies have examined this organism in the defined context of a population.

To strengthen this it is important to point out that the population in this case should be regarded as the entire genus *Salmonella.* In so doing it would be expected that the discrimination achieved by any typing method would be far greater than, for example, the discrimination achieved by the examination of one of the seven sub-species of *Salmonella enterica.* By extension each of the >2300 recognised serotypes (Le Minor and Popoff, 1994) would show less discrimination within them, than between them. At each stage of this process the probability that a sample of the population is heterogeneous decreases. Serotype Enteritidis should therefore be regarded as a sample of the population. To further sub-divide this sample by any other typing method requires that the limitations of the method be accepted. However, once this has been accepted it becomes clear that the information generated from any typing method will still have some validity within a defined context.

The work presented in this thesis has examined the application of plasmid analysis within serotype Enteritidis. The sample of 434 isolates was divided into sub-sets defined by the criteria set out in Chapter Three. The results were presented for each sub-set and for the sample as a whole.

Plasmid profile analysis (PPA), was used to determine the number and molecular weight of plasmids harboured by each strain. A combination of PPA and plasmid restriction endonuclease fragmentation pattern analysis (REFP) showed that overall 63% of the isolates harboured a single plasmid of 54kb. In some sub-sets the discrimination achieved by PPA was very low. Sub-set 2

showed that 82% of the strains harboured the single 54kb plasmid. On the other hand sub-set 3 showed greater diversity in that only 25% of isolates had this profile. In conclusion, and as was anticipated from the outset, plasmid profile analysis showed varying degrees of discrimination dependant on the sample of the population studied.

When dealing with a sub-set that was initially deemed to be heterogeneous by the application of a different typing method (in the case of sub-set 3, phage typing) the results obtained were highly discriminatory. In many cases plasmid analysis allowed discrimination within a phage type although as sub-set 2 showed diversity was low within phage type 4. Phage typing has become the preferred method for typing this serotype it is quick, reliable and cost effective but as can be seen from the data presented it was possible to use molecular methods with which to sub-type within a phage type.

The recent 'epidemic' of Enteritidis phage type 4 has led many on the hopeless task of developing genotypic typing methods that are capable of discrimination within this type. Since some pathogenic clones are so ubiquitous that multiple simultaneous outbreaks with that clone may occur independently, and with some frequency, the likelihood of discrimination within Enteritidis phage type 4 being achieved is low given that we are now dealing with a defined sample (phage type), of the sample (Enteritidis) , of the sample (*Salmonella*).

Pulsed field gel electrophoresis (PFGE) has been performed and as Powell and colleagues (1994) showed it was possible to differentiate within Enteritidis, to a very limited extent. Nine profiles were determined from thirty nine strains, eight patterns were represented by a single strain and six of these were from poultry associated sources. This suggested that if poultry is the main source of human infection that heterogeneity should be present in the human

population, but the evidence presented did not support this. This could have been due to many things. The sample may not have been sporadic, as the authors thought, the primary source of infection in man may no longer be poultry but person to person spread, it is also quite likely that not all strains that colonise poultry infect man equally or alternatively the use of PFGE to study Enteritidis at this level may not be applicable if the genome structure of the organism is not sufficiently diverse to allow sub-division by this technique.

Pulsed field gel electrophoresis has also been used to study Enteritidis of different phage types. Olsen and colleagues (1994) showed that twenty one of thirty three phage types formed a single cluster when bands of >125kb were used as the criterion for separation. This is consistent with the idea of insufficient diversity within the genome of Enteritidis. In the work presented by Olsen and colleagues (1994) phage typing alone gave better discrimination than PFGE. This was further corroborated by Liebisch and Schwarz (1996) who showed that PFGE could split thirty one strains of Enteritidis into nine genomic groups that corresponded closely to the assignment of isolates to different phage types.

Within the context of a reference facility, rather than a research lab, it is more cost effective to phage type thirty one isolates than to perform PFGE with three different enzymes. It has been suggested by Liebisch and Schwarz (1996) that PFGE be used for strains that are untypable by phage typing. It has been discussed previously, that in real terms a strain that is untypable with the phage typing scheme has its own significance in an outbreak situation. What Liebisch and Schwarz (1996) are therefore suggesting is an expensive surveillance system for sporadic untypable isolates which make up around 1% of Enteritidis isolates received by the Scottish Salmonella Reference Laboratory (Results not

shown). Under these circumstances plasmid analysis could serve as a more reasonable adjunct to phage typing.

Polymerase chain reaction (PCR) techniques have been rapidly developed for the study of *Salmonella* serotypes (Lin *et al*, 1996, Fadl *et al*, 1995, Lampel *et al*, 1996, Rexach *et al*, 1994). Both Fadl and colleagues (1995) and Lin and colleagues (1996) described PCR assays that allowed the differentiation of Enteritidis. However, both these studies used small samples of Enteritidis that had previously been differentiated by at least one other typing method. Fadl and colleagues (1995) showed that Enteritidis of different phage types could be differentiated by the use of a single primer (MK22).

One question from the work of Fadl and colleagues (1995) that remains unanswered is why do different phage types have the same PCR pattern? This raises many questions about the current views of Salmonella epidemiology. For example, Fadl and colleagues (1995) showed Enteritidis phage type 8 strains that had the PCR banding pattern B. This pattern was found also in strains of phage type 13a and phage type 14b. This suggested that all of these phage types were in some way related at a genotypic level. It is well recognised that phage types of salmonellae can change by a variety of different mechanisms (Chart *et al*, 1989; Frost *et al*, 1989; Threlfall *et al*, 1993, Rankin and Platt, 1995). Does this indicate that although this limited study (Fadl *et al*, 1995) suggested different phage types show the same PCR banding pattern that in real terms any range of phage types may have the same PCR pattern? And if this is the case, is it really such an unreasonable assumption. If the phage type is assumed to be stable, which has generally been thought to be the case does PCR with this primer offer any more than previously described methods for differentiating Enteritidis. The converse of the above argument is worthy of

discussion if a possibility exists that phage typing has no epidemiological significance.

The assumption that phage types are stable seems now to be regarded as an underlying principle in *Salmonella* epidemiology. However, although phage types can be stable within an episode (short term) and stable in the laboratory on subculture, they cannot be regarded as stable in the long term or there would be only one phage type. The nature of change relative to external influences is unpredictable. For example a single event may cause a change in the reaction of one phage in the typing scheme, or many phages. It follows from this that relationships between phage lysis patterns in a typing scheme cannot be discerned *a priori* whereas in principle, they can with genotypic methods.

Lin and colleagues (1996) have identified similar problems to those described above (Fadl *et al*, 1995) but they compounded them further by using six primers in their PCR assay. It was shown that isolates of the same phage type and random amplified polymorphic DNA (RAPD) profile could be differentiated by either ribotyping or PFGE. Similarly isolates of different phage types had identical ribotypes and PFGE profiles but different RAPD profiles. Two phage type 4 isolates that were clearly able to be distinguished from each other by ribotype or PFGE were identical with five of the six primers chosen. A minor difference in PCR pattern with one primer differentiated these strains. Lin and colleagues (1996) have stated that "Not at all unexpectedly these results show that the application of several methods in combination with phage typing gives the highest discriminatory power. When one considers a combination of discriminatory power and ease of application, RAPD emerges as a particularly attractive molecular technique". What a great pity that they chose to diminish the power of the first sentence by the addition of the second. As previously discussed, PCR analysis although useful in the specific detection of *Salmonella*

serotypes (Lampel *et al*, 1996) has not proved itself robust enough to stand alone as an epidemiological tool.

It has been established in this study that the majority (>80%) of Enteritidis isolates harboured a serotype associated plasmid that was required for virulence (Gulig *et al*, 1993). Therefore, discrimination among Enteritidis isolates relied on diversity in plasmid profile and REFP. Discrimination was achieved in 17% of the isolates. This figure was low but as discussed none of the methods applied to the epidemiology of Enteritidis have offered much more. And none of these methods could stand alone in epidemiological investigations. However, the adoption of a combination of suitable methods could be applied, with the practical aspects of outbreak investigations, to answer the question that is most immediate during an outbreak investigation i.e. "Have the organisms originated from a common source?" They may be indistinguishable from the majority of Enteritidis isolates but if guilty by association with the outbreak can be deemed with some certainty to be related to it.

The second part of the thesis moved from epidemiology to concentrate on the evolution of the 54kb Enteritidis SAP. Nine molecular variants of this plasmid were identified, by REFP analysis, during the course of the initial study. It was noted that on the basis of REFP analysis and Dice coefficients of similarity that one of these plasmids (pOG690) appeared closely related to the SAP of serotype Typhimurium. It was therefore hypothesised that pOG690 may be an evolutionary intermediate between the Typhimurium SAP (pOG660) and the Enteritidis SAP (pOG674). This hypothesis postulated that evolution had generated the smaller 54kb plasmid from the 95kb Typhimurium SAP via a series of intermediate plasmids. This was founded upon the observation that plasmids found in Enteritidis strains that were subsequently regarded as

intermediates between the SAPs of Typhimurium and Enteritidis were, in general, smaller than the Typhimurium SAP but larger than the Enteritidis SAP.

To attempt to discern the validity of this hypothesis required initially that a restriction map of pOG674 be generated to allow a direct comparison with the previously published maps of Typhimurium virulence plasmids (Michiels *et al,* 1987; Tinge and Curtiss, 1990a). As the map of pOG674 was being constructed Suzuki and colleagues (1994) published a map of a 55kb Enteritidis plasmid pNL2001. As this map concurred with the data generated for pOG674 it was used as a basis for the generation of *Pst*I and *Sma*I restriction maps of pOG674.

The resources available did not allow the exact positions of many of the smaller *Pst*I and *Sma*I restriction fragments to be determined and therefore the maps of pOG674 were incomplete. However, the map data presented has added sufficient information to the study of *Salmonella* plasmids to allow various conclusions to be formed. In pOG674, the established virulence region appears to be located in the same region in Enteritidis as in previously published maps of Typhimurium (Michiels *et al*, 1987; Tinge and Curtiss, 1990a). The *Pst*I fragments present in the *rep*A (Michiels *et al*, 1987) or *par*VP (Cerin and Hackett, 1993) region of Typhimurium plasmid pSLT have been shown to be the same size as fragments determined from what has been postulated from map data of pOG674 of Enteritidis to be the same region.

A 2.8kb, *Pst*I generated, probe from pOG674 showed, that with the exception of pOG701, this sequence was present in all plasmids tested. Although with three plasmids, pOG660, pOG690 and pOG704 hybridisation with the probe occurred with fragments that were greater than 2.8kb. This was subsequently postulated to be due, in pOG660 and pOG690, to a point

mutation. It has been suggested that this mutation created the 2.8kb *Pst*I fragment from the 3.4kb *Pst*I fragment of pOG660 rather than vice versa as the 2.8kb fragment was observed in plasmids of intermediate size in which further heterogeneity was also observed. However, it was possible that the mutation may have abolished a *Pst*I site in a 2.8kb fragment to create a fragment of 3.4kb.

The above argument is simplistic and for that reason all of the fragments, greater than 2.8kb, that hybridised with the 2.8kb *Pst*I probe fragment should be cloned, sequenced and compared with the 3.4kb *Pst*I fragment from Typhimurium plasmid pOG660. This is currently the best way to confirm the above observations. Sequence divergence has however been observed by Woodward and colleagues (1996) between the *pef*A gene sequences from Typhimurium plasmid S1164/1994 and Enteritidis plasmid S1400/1994 and this level of divergence may also be evident in other regions of the Enteritidis plasmid.

Most of the data generated has not proved conclusively that the Typhimurium plasmid has lost DNA to generate smaller plasmids and ultimately the 54kb Enteritidis SAP. However, some of the observations that were generated with regard, in particular, to the *pef* region (Friedrich *et al*, 1993) may have strengthened this conclusion.

The 14kb *pef* region in Typhimurium plasmid pSLT was sequenced from a *Bgl*II site at 45kb to a *Bam*HI site at 59kb. The first *Pst*I site downstream from the *Bam*HI site at 59kb was determined from the sequence data at position 3.314kb which suggested that a *Pst*I fragment of 3.3kb or greater should be located downstream from the *Bam*HI site at 33.9kb on the map of pOG674 (Figure 4.1). Figure 4.11 showed that there were at least two small (1kb) *Pst*I

fragments in this location followed by a 4.9kb *Pst*I fragment from 32.0' - 27.1'. This was preceded by a *Bgl*II site at 26.5', that began a 3.4kb fragment (to 23.1'). None of the *Pst*I fragments determined from the sequence of Friedrich and colleagues (1993) are found in the *Pst*I map of pOG674. However the sizes of these *Pst*I fragments are closely matched by fragments seen in *Pst*I digests of pOG660. Details from *Sma*I digests confirmed these observations. The *Sma*I fragments in this region of pOG674 were 6.2kb (33.9' - 27.2') and 3.4kb (27.2' - 23.8').

Woodward and colleagues (1996) have established by PCR and hybridisation that some of the *pef* genes were present in the Enteritidis SAP although the restriction map data presented here did not concur with this view. It has previously been stated that sequence divergence was observed between the *pef*A genes of Typhimurium and Enteritidis plasmids that could perhaps have accounted for this anomally.

In the restriction map of the Typhimurium SAP (Michiels *et al*; 1987) the largest of five *Bam*HI fragments was shown to be 78kb. This fragment therefore encompassed a very large part of the plasmid from a site within the virulence region to the site that began a 7.4kb *Bam*HI fragment, that co-incidentally was also the site from which the *pef* region was sequenced (Friedrich *et al*, 1993). The corresponding *Bam*HI fragment in the Enteritidis SAP (pOG674) was 32kb and it was suggested that DNA had been lost from within the 78kb Typhimurium fragment to create the 32kb Enteritidis fragment. This was consistent with the hypothesis that the Enteritidis plasmid had been created by deletion of DNA from the Typhimurium plasmid rather than insertion of DNA into the Enteritidis SAP to generate the larger plasmid of Typhimurium. It was considered improbable that newly inserted DNA (approximately 40kb) would contain no new *Bam*HI restriction sites. This was

substantiated by similar findings with regard to the largest *Bgl*II fragments from both of these plasmids

A similar hypothesis was proposed for the *pef* region of Enteritidis in that if this region was present in pOG660 and pOG690 it would seem unreasonable to expect that it was acquired by Enteritidis intermediate plasmids as a series of insertions that created the larger Typhimurium plasmid(s). Given that a *Bgl*II site 1.8kb from the *Bam*HI site, that began the sequence, was not present in pOG674 it was suggested that deletions may have occurred in the Typhimurium SAP that have retained some of the *pef* genes but allowed unnecessary DNA to be lost. This may also have resulted in the loss of *rep*C from Enteritidis and this should be considered for further research.

Friedrich and colleagues (1993) have also shown the *rck* sequence (Heffernan *et al*, 1992) to be present on the *pef* operon from position 11416bp to 11973bp. This region has been shown by hybridisation to be present on a 61kb Enteritidis plasmid (Buisan *et al*, 1994). A *rck* probe hybridised with a *Bam*HI/*Sma*I fragment that was on a *Sma*I fragment of approximately 3kb. This correlates with the *Bam*HI site in Figure 4.1 at 23.9' which is on a *Sma*I fragment of 3.4kb (Figure 4.15). Whether this *rck* sequence is present on pOG674 and any of the other Enteritidis variants remains to be determined.

From this data it is clear that there is still much to be done in order to elucidate further the relationship between pOG674 and pOG660. It is important that the area from the *Bgl*II site at 23.1' to the *Bgl*II site at 35.5' be studied further. Sequence analysis of this region should be done to determine how it relates to the published sequence data for Typhimurium. This sequence analysis could be further extended to the *Bam*HI site at 42.1' to determine unequivocally

that the 2.8kb *Pst*I fragment from pOG674 was generated from a point mutation in the 3.4kb *Pst*I fragment of pOG660.

The presence of variant plasmids in Enteritidis may also be useful in the interpretation of the relationship between Enteritidis and Typhimurium plasmids. Plasmids pOG691 and pOG701 would provide interesting material and work is underway to determine *Pst*I and *Sma*I maps of both these plasmids and the other plasmids in the study. The construction of maps of Typhimurium plasmid pOG660 are currently in progress at SSRL and it is believed that the combination of all of this information may provide a conclusive answer to the question of how these plasmids have evolved.

Appendix.

Media And Reagents.

Media.

Autoclaving of media and reagents.

Unless otherwise stated all media and reagents which required to be sterilised were autoclaved at 121°C at fifteen pounds per square inch (psi) pressure for fifteen minutes.

Cystiene lactose electrolyte deficient (CLED) agar.

Pre-weighed sachets (Mast, UK) were resuspended in one litre of distilled water in an autoclavable bottle, and steamed for one hour to dissolve. The bottles were autoclaved. The agar was allowed to cool to 65°C and poured into sterile plastic Petri dishes (Sterilin,UK). After drying at room temperature the plates were stored at 4°C.

Nutrient agar.

Twelve grams of nutrient broth (CMI, Oxoid Ltd, UK) and 10g of Bacteriological agar (Oxoid Ltd) were resuspended in one litre of distilled water, steamed and autoclaved.

Brain heart infusion broth.

Thirty seven grams of brain heart infusion (Oxoid Ltd) was resuspended in one litre of water in a Pyrex conical flask. This was boiled to dissolve the contents. The broth was aliquoted into 100ml amounts in autoclavable bottles and autoclaved.

Dorset's egg slopes.

To a sterile stainless steel mixing bowl were added 750ml of beaten egg mixture and 250ml of sterile nutrient broth (Difco). These were beaten using a sterile whisk and aliquoted in 1ml amounts into 3ml glass bottles. The bottles were racked and incubated at an angle of approximately 45° at 170°C in an hot air oven for two hours until the egg mixture had set. The slopes were further incubated at 37°C overnight to check for contaminated slopes, if any were found they were discarded.

Glycerol peptone broths (8% glycerol, 1% proteose peptone).

Five grams of proteose peptone (Oxoid,UK) and 40ml of glycerol were added to 400ml distilled water and heated until all the peptone had dissolved. This solution was made up to 500ml and aliquoted in 2.5ml amounts in glass vials. The vials were autoclaved and stored at room temperature.

Isosensitest agar.

Isosensitest agar was obtained from Oxoid,UK (CM471); 31.4g was added to one litre of distilled water this was subsequently steamed and autoclaved.

LB agar.

Luria broth base (Millers LB broth) was obtained from Life Technologies,UK. Twenty five grams of Luria broth base and ten grams of Bacteriological agar (Oxoid, UK) were resuspended in one litre of distilled water, steamed and autoclaved.

SOC broth.

SOC broth was obtained from Life Technologies, UK in 10ml amounts. Its formulation was given as follows: Bactotryptone (2%), Yeast extract

(0.5%), NaCl (10mM), KCl (2.5mM), $MgCl_2$, $MgSO_4$ (20mM, 10mM each) and Glucose (20mM).

Reagents.

Electrophoresis buffer - TB

Tris base	89mM	53.9g
Boric acid	89mM	53.9g
Disodium EDTA	1.25mM	2.3g
Distilled water		5l

pH 8.2

The powders were dissolved with heat and stirring in 2.5l of distilled water, the volume was made up to 5l and the buffer was stored at room temperature.

Electrophoresis buffer - TEB

Tris base	89mM	53.9g
Boric acid	89mM	53.9g
Disodium EDTA	1.25mM	2.3g
Distilled water		5l
Ethidium Bromide solution		0.8ml

pH 8.2

The powders were dissolved with heat and stirring in 2.5l of distilled water, the volume was made up to 5l and the buffer was stored at room temperature. Ethidium bromide stock solution was added.

Ethidium bromide - stock solution

Stock solution 0.495mM. Ethidium bromide solution was purchased from Life Technologies Ltd, at a concentration of 10mg/ml. The 0.495mM stock solution was achieved by diluting the ethidium solution in 40ml sterile distilled water. The solution was stored at 4°C.

TES buffer x 10 concentrate

Tris base	50mM	60.6g
Sodium chloride	50mM	29.2g
Disodium EDTA	5mM	18.6g
Distilled water		1000ml

pH 8.0

The powders were dissolved with heat and stirring in 900ml of distilled water. The pH was adjusted to 8.0 with hydrochloric acid and the volume made up to 1000ml. The solution was autoclaved and stored at 4°C.

TGE buffer

Tris base	25mM	1.5g
Disodium EDTA	10mM	1.85g
Glucose	50mM	4.5g
Distilled water		500ml

pH 8.0

To 100ml of distilled water were added 1.5g Tris base and 1.85g disodium EDTA. This was dissolved and the pH adjusted to 8.0. The volume was made up to 480ml with distilled water and this was autoclaved. The glucose was added to 20ml sterile distilled water this was dissolved and added to the above solution when cooled using a 0.45μm filter. The solution was stored at room temperature.

Sodium hydroxide solution (2M)

Sodium hydroxide : 8g

Distilled water.

The solution was dissolved in 50ml of distilled water, made up to 100ml, autoclaved and stored at room temperature.

Sodium dodecyl sulphate (10%)

SDS : 10g

Distilled water : 100ml

The SDS was dissolved at 37°C and stored at room temperature.

Sodium acetate solution (3M)

Anhydrous sodium acetate : 123g

Distilled water : 500ml

The sodium acetate was added to 100ml of distilled water. This was dissolved by heating to 95°C and the pH was adjusted to 4.8 with glacial acetic acid. This was made up to 500ml, autoclaved and stored at 4°C.

Ammonium acetate solution (7.5M)

Ammonium acetate : 57.8g

Distilled water.

The ammonium acetate was added to 50ml of distilled water. This was dissolved and the pH adjusted to 8.0 with glacial acetic acid. The solution was made up to 100ml with distilled water and stored at room temperature.

Alkaline SDS

2M NaOH : 1ml

10% SDS : 1ml

Distilled water : 8 ml

This solution was prepared immediately prior to use.

Sodium chloride solution (2.5M)

Sodium chloride : 14.6g

Distilled water.

The sodium chloride was added to 50ml of water. This was made up to 100ml and autoclaved. The solution was stored at room temperature.

Phenol chloroform (1:1)

Phenol : 250g

Chloroform : 250ml

TGE : 50ml

The phenol was dissolved in the chloroform in a fume cabinet. The TGE was added and mixed well. The solution was decanted into a dark bottle and the aqueous layer allowed to separate. The solution was stored at 4°C.

RNAase solution (1mg/ml).

Ribonuclease A (Sigma, UK) was added to 10ml sterile distilled water in a universal container. This was placed in a boiling water bath for ten minutes aliquoted and stored at 4°C.

TE buffer

Tris base	10mM	0.605g
Disodium EDTA	1mM	0.186g
Distilled water		500ml

pH 8.0

The powders were dissolved in 400ml of distilled water and the pH of the solution adjusted to 8.0 with concentrated hydrochloric acid. The solution was made up to 500ml, autoclaved and stored at room temperature.

Gel loading buffer (Blue juice).

Sucrose	25g
Sodium acetate	60mg
Sodium dodecyl sulphate	100mg

Bromophenol blue	50mg
Distilled water	100ml

The powders were dissolved in the distilled water and stored at room temperature.

TGE - Lysozyme (5mg/ml)

The lysozyme (Sigma, UK) was dissolved in TGE buffer immediately prior to use.

Ethidium bromide stain.

Ethidium bromide for staining plasmid profile gels was prepared by adding 1ml stock ethidium bromide solution to 300ml 1xTES buffer.

EDTA solution (10mM)

Disodium EDTA : 0.3722g

Distilled water.

The disodium EDTA was added to 80ml of water. This was dissolved and the pH adjusted to 7.6. The solution was made up to 100ml, autoclaved and stored at room temperature.

Sodium hydroxide solution (0.5M)

Sodium hydroxide : 2g

Distilled water.

The sodium hydroxide was dissolved in 50ml of water, made up to 100ml, autoclaved and stored at room temperature.

NET buffer

Sodium chloride : 8.77g

Disodium EDTA : 0.037g

Tris : 2.422g

Distilled water : 1000ml

The reagents were dissolved in 500ml distilled water, the pH was adjusted to 8.0, made up to 1000ml, autoclaved and stored at room temperature.

High salt NET

Sodium chloride : 58.44g

Disodium EDTA : 0.037g

Tris : 2.422g

Distilled water : 1000ml

The reagents were dissolved in 500ml distilled water, the pH was adjusted to 8.0, made up to 1000ml, autoclaved and stored at room temperature.

Hybridisation Solutions For Vacuum Blotting

Depurination solution

2.9M (0.25N) Hydrochloric acid

Denaturation solution

Sodium chloride	1.5M	87.6g
Sodium hydroxide	0.5M	20.0g
Distilled water		1000ml

The reagents were dissolved in distilled water to give a final volume of one litre. Filter sterilised using a 0.45µm filter, store at room temperature.

Neutralising solution

Tris	1.0M	121.1g
Sodium chloride	2.0M	116g
Distilled water		1000ml

pH5.0

The reagents were dissolved in 800ml of distilled water. The pH was adjusted to 5.0 with concentrated hydrochloric acid (75 - 100ml) and the volume made up to 1litre. The solution was filter sterilised before use using a 0.45μm filter. The solution was stored at room temperature.

Transfer solution (20x SSC)

Sodium chloride	175g
Trisodium citrate	88.2g
Distilled water	1000ml

The reagents were dissolved in 500ml distilled water. The pH was adjusted to 7.0-7.2 with citric acid and sterilised using a 0.45μm filter before use. The solution was stored at room temperature.

2x Wash solution.

2x SSC : 1L

SDS : 1g

1g of sodium dodecyl sulphate was added to one litre of 2x SSC solution. The SDS was allowed to dissolve and the solution was autoclaved and stored at room temperature.

0.1x Wash solution.

0.1x SSC : 1L

SDS : 1g

1g of sodium dodecyl sulphate was added to one litre of 0.1x SSC solution. The SDS was allowed to dissolve and the solution was autoclaved and stored at room temperature.

Blocking reagent (Stock solution).

Blocking reagent, as supplied by Boerhinger Mannheim, is a powder of proteolytic fragments of caesin. This powder was dissolved in maleic acid buffer to a final concentration of 10%(w/v) in a microwave oven (650W) on full power for two minutes. This solution was autoclaved and stored at 4°C.

Maleic acid buffer.

Maleic acid	100mM
Sodium chloride	150mM
Distilled water	1000ml

pH7.5

The powders were dissolved in 500ml distilled water. The pH was adjusted to 7.5 with solid sodium hydroxide pellets and the volume made up to 1000ml. The solution was autoclaved and stored at room temperature.

Blocking solution.

A 10% solution of blocking reagent in maleic acid buffer. The solution was autoclaved and stored at 4°C.

Detection buffer.

Tris base	0.1M
Sodium chloride	0.1M
Magnesium chloride	50mM
Distilled water	1L

pH9.5

The powders were dissolved with heat and stirring in 900ml of distilled water. The pH was adjusted to 9.5 and the volume made up to 1000ml. The solution was autoclaved and stored at room temperature.

Hybridisation solution.

Hybridisation was carried out in DIG Easy Hyb as supplied by Boehringer Mannheim, UK.

Colour substrate solution.

This solution is prepared fresh immediately before use. To 10ml of detection buffer add 45µl NBT solution and 35µl X-phosphate solution (both supplied by Boehringer Mannheim).

IPTG solution (200mg/ml).

1g of IPTG was dissolved in 5ml sterile water. The powder was dissolved and the solution was aliquoted in 500µl amounts into eppindorf tubes and stored at -20°C.

X-gal (20mg/ml).

100mg of X-gal was dissolved in 5ml of dimethylformamide in a dark bottle. The powder was dissolved and the solution stored at -20°C.

Antibiotics.

Ampicillin (Ap)

Stock solution 10mg/ml. 577mg of ampicillin (Sigma, UK) was dissolved in 20ml 0.1M hydrochloric acid in warm water to aid solution. This was made up to 50ml with distilled water, this was filter sterilised and stored at -20°C.

Chloramphenicol (C)

Stock solution 2mg/ml. 100mg of chloramphenicol (Sigma, UK) was dissolved in 50ml distilled water, this was filter sterilised and stored at -20°C.

Gentamicin (Gm)

Stock solution 1mg/ml. 76.9mg of gentamicin sulphate (Sigma, UK) potency 650μg/mg was dissolved in 50ml distilled water, this was filter sterilised and stored at -20°C.

Kanamycin (Km)

Stock solution 2.5mg/ml. 167mg ofkanamycin sulphate (Sigma, UK) potency 750μg/mg, was dissolved in 50ml distilled water, this was filter sterilised and stored at -20°C.

Nalidixic acid (Na)

Stock solution 2.5mg/ml. 125mg of nalidixic acid (Sigma, UK) was dissolved in 0.1M sodium hydroxide solution (5 - 10ml) made up to 50ml with distilled water, this was filter sterilised and stored at -20°C.

Trimethoprim (Tp)

Stock solution 3mg/ml. 150mg of trimethoprim (Sigma, UK) was dissolved in 50ml distilled water, this was filter sterilised and stored at -20°C.

Rifampicin (Rif)

Stock solution 5mg/ml (with 0.5mg/ml ascorbic acid). 250mg of rifampicin (Sigma, UK) and 25mg ascorbic acid was added to a small beaker with 15ml distilled water. 3 - 4 drops of 10M hydrochloric acid was added and the solution was mixed gently to dissolve. The solution was made up to 50ml with distilled water, filter sterilisd and stored at -20°C.

Streptomycin (St)

Stock solution 1.5mg/ml. 81mg of streptomycin sulphate (Sigma, UK) was dissolved in 50ml distilled water, filter sterilised and stored at -20°C.

Sulphamethoxazole (Su)

Stock solution 10mg/ml. 500mg of sulphamethoxazole (Sigma, UK) was dissolved in 20ml 0.1M sodium hydroxide solution. Made up to 50ml with distilled water, filter sterilised and stored at -20°C.

Tetracycline (Tc)

Stock solution 1mg/ml. 54mg of tetracycline hydrochloride (Sigma, UK) was dissolved in 50ml distilled water, filter sterilised and stored at -20°C.

Antibiotic concentrations incorporated in isosensitest agar plates tested at the Scottish Salmonella Reference Laboratory.

Antibiotic	Final Concentration
Ampicillin	50µg/ml
Chloramphenicol	20µg/ml
Ciprofloxacin	0.5µg/ml
Furazoladone	20µg/ml
Gentamicin	20µg/ml
Kanamycin	20µg/ml
Nalidixic acid	40µg/ml
Netilmycin	20µg/ml
Spectinomycin	100µg/ml
Streptomycin	20µg/ml
Sulphamethoxazole	100µg/ml
Tetracycline	10µg/ml
Trimethoprim	2µg/ml

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