Molecular typing of *Salmonella enterica* serovars of significance in Australia using MLVA and bacteriophage genes

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LIST OF ABBREVIATIONS

aa	Amino acid
ATCC	American type culture collection
AFLP	Amplified fragment length polymorphism
AGRF	Australian Genomic Research Facility
AIDS	Acquired immune deficiency syndrome
ASRC	Australian *Salmonella* Reference Centre
bp; kb	Base pair(s); kilobase(s)
BHI	Brain heart infusion
BSA	Bovine serum albumin
CHEF	Clamped homogeneous electric field
DI	Simpson's index of diversity
DNA	Deoxyribonucleic acid
DOP-PCR	Degenerate-oligonucleotide primed – PCR
dNTP	Deoxyribobucleic acid
dsDNA	Double-stranded DNA
DT	Definitive type
EDTA	Ethylene-diaminetretra-acetic acid
g; mg; µg	gram; milligram; microgram
HBA	Columbia horse blood agar
IMVS	Institute of Medical and Veterinary Science
IS	Insertion sequence
l; ml; µl	Litre(s); millilitre(s); microlitre(s)
LB	Luria Bertani
LMP	Low melting point
M; mM; µM	Molar; millimolar; micromolar
MAPLT	Multiple amplification of prophage locus typing
MH	Mueller Hinton
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequencing typing
MLVA	Multiple-locus variable-number tandem repeat analysis
MOI	Multiplicity of infection
mPCR/RLB	Multiplex PCR-based reverse line blot hybridisation
MPU	Media Production Unit
n/a	Not applicable

NCTC	National collection of type cultures
NGS	Next generation sequencing
nt	Nucleotide
O/B	Outbreak
ORFs	Open reading frames
O/S	Overseas
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pfu	Plaque forming units
PT	Phage type
RAPD	Randomly amplified polymorphic DNA
RDNC	Reacts does not conform
Rep-PCR	Repetitive element PCR
RFLP	Restriction fragment length polymorphism
SNP	Single-nucleotide polymorphism
SPI	*Salmonella* pathogenicity island
TAE	Tris- Acetate-EDTA
TE	Tris-EDTA
Tris	(hydroxymethyl) aminomethane
TRF	Tandem Repeat Finder
TSI	Triple sugar iron
TTSS	Type III secretion system
UN	Untypable
UPGMA	Unweighted-pair group method with arithmetic averages
VNTR	Variable-number tandem repeat
WGS	Whole genome sequencing
v/v	Volume per volume
w/v	Weight per volume
XLD	Xylose-lysine-desoxycholate

DECLARATION

I Chun Chun Young declare that the work described herein contains no material that has been previously submitted for the award of any degree or diploma in any university and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference is made in the text.

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PUBLICATIONS

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SUMMARY

Salmonella enterica subspecies *enterica* is the leading causes of food-borne infections and outbreaks worldwide and are therefore routinely monitored in many countries. Both epidemiological surveillance and tracking outbreaks require typing systems to characterise bacterial isolates. Pulsed-field gel electrophoresis (PFGE) is the most widely applied molecular method for *Salmonella* outbreak epidemiological typing despite that the method being relatively labour-intensive, generating restriction patterns that are subjective in nature and can be difficult to analyse. Furthermore, the recent emergence of homogeneous *Salmonella* phage groups (e.g. *S.* Typhimurium DT104) has hampered the discriminatory power of PFGE in outbreak settings.

The combination of these factors has led to the exploitation of alternative methods. Two of these emerging typing methods are multiple-locus variable-tandem repeat analysis (MLVA) and multiple amplification of prophage locus typing (MAPLT) that have been developed for the globally significant serovars: *S.* Typhimurium and *S.* Enteritidis. In this study, MLVA and MAPLT were being further investigated for use in other *Salmonella* serovars that are of significance to Australia. These serovars include *S.* Virchow, *S.* Bovismorbificans and *S.* Heidelberg. Overall, the developed MAPLT schemes demonstrated a comparable differentiating ability with PFGE for the serovars as a whole. However development of discriminative MLVA methods in the study was not always successful using the published MLVA loci, therefore prompting the need of examining the MLVA loci from the complete genomes of the serovars.

Differentiation within predominating phage types of the studied serovars was also examined. Both MAPLT and PFGE displayed similar levels of intra-phage type differentiation suggesting the usefulness for outbreak investigations. However strains were separated optimally when using MAPLT in association with discriminative MLVA primers. The composite assay of MAPLT/MLVA for *S.* Bovismorbificans and *S.* Virchow were further examined in the retrospective outbreak studies. It was observed that outbreak isolates generally showed identical or highly similar MAPLT/MLVA profiles (one locus difference). The results suggested that MAPLT / MLVA could confirm the close genetic relationship between outbreak isolates. However result interpretation guidelines might need to be established individually for each serovar due to the differences in the relative genetic similarity displayed within each serovar. It is expected that further enhancement of the composite assays could easily be carried out even when the complete genomic data of the *Salmonella* serovars are lacking. It is an advantage that MAPLT could be improved through using a DOP-PCR (degenerate oligonucleotide primed-PCR) procedure to detect additional prophage loci for further strain differentiation. Overall, the

study suggested that composite assays can also be developed for fine levels of differentiation of other *Salmonella* serovars to meet local epidemiological needs when required.

Development of MAPLT involved genetic characterisation of phages residing in the studied serovars. It was noted that DNA elements shared between phages Gifsy-1 and Gifsy-2 were frequently detected from these serovars. Complete genome analysis were subsequently performed on a *S.* Virchow phage (PV10) and a *S.* Heidelberg phage (PH03), which demonstrated their high DNA similarity to Gifsy-1 and Gifsy-2 respectively. Further analysis demonstrated that Gifsys-related phages are possibly widespread within *Salmonella*. Since the phages were observed to carry different sets of virulence genes between serovars, this study hypothesised that Gifsy-related phages may have a significant role in shaping the epidemiology of *Salmonella*.

CHAPTER 1 INTRODUCTION

1.1 *SALMONELLA*

The genus *Salmonella* is classified in the family of *Enterobacteriaceae* (Darwin and Miller, 1999). The bacterium was first discovered by Eberth and cultured by Gaffky in 1884 from a patient who died from typhoid fever (Todar, 2008). However it was named after Salmon and Smith in 1885 when they first successfully isolated *Salmonella choleraesuis* from pigs with hog cholera (McDermott, 2006). Morphologically, members of *Salmonella* are characterised as gram-negative, non-spore forming bacilli that are usually 0.7-1.5 x 2-5 μm in size (Cruickshank *et al.*, 1975). All members of *Salmonella* are motile by peritrichous flagella, with the exceptions of *Salmonella enterica* serovars Gallinarum and Pullorum (Bopp *et al.*, 2003). Biochemically, members of *Salmonella* are described as facultative anaerobes that are oxidase negative and can grow well on simple media (Bopp *et al.*, 2003). Laboratory identification of *Salmonella* usually begins with culturing samples in enrichment broths (e.g. selenite or tetrathionate broths) to maximise the recovery of *Salmonella* bacteria and inhibit the growth of other bacteria (Bopp *et al.*, 2003). Subsequently they are isolated on selective plating media such as bismuth sulphite agar and xylose lysine deoxycholate agar (Bopp *et al.*, 2003). Biochemical identification of *Salmonella* colonies can be performed using triple sugar iron (TSI) agar slopes and most *Salmonella* isolates will ferment glucose, and produce gas and hydrogen sulphide (H_2S) (Bopp *et al.*, 2003). Alternatively, *Salmonella* colonies can be confirmed using slide agglutination with antisera for *Salmonella* O (somatic) antigen groups (Bopp *et al.*, 2003).

1.1.1 *Salmonella* nomenclature

The nomenclature within the genus *Salmonella* has changed many times. Formally, *Salmonella* was divided into subgenera based on their distinct biochemical characteristics, and further subdivision into species was based on serological differences (Kauffmann, 1966). In 1982, Le Minor and co-workers proposed that genus *Salmonella* should only consist of a single species *Salmonella choleraesuis* (cited in Davos 2005). In 1987, Le Minor and Popoff suggested the use of *Salmonella enterica* instead of *Salmonella choleraesuis* as the species name to avoid confusion since choleraesuis is also used as a serovar name (Le Minor and Popoff, 1987). In addition, they proposed to refer the designated subgenera as subspecies (I, II, IIIa, IIIb, IV, V, VI) since members of subgenera were indeed closely related at species level as demonstrated by the DNA-DNA hybridisation study (Crosa *et al.*, 1973; Le Minor and Popoff, 1987). Later in 1989, Reeves *et al.* demonstrated by using multilocus enzyme electrophoresis (MLEE) that subspecies V should be elevated to be a separate species (Reeves *et al.*, 1989).

1.1.2 *Salmonella* species and subspecies

The nomenclatural system currently used for *Salmonella* is the combined version of Le Minor and Popoff and Reeves *et al.* in which two species are recognised: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies which are listed as follows: *Salmonella enterica* subsp. *enterica* (subsp. I), *Salmonella enterica* subsp. *salamae* (subsp. II), *Salmonella enterica* subsp. *arizonae* (subsp. IIIa), *Salmonella enterica* subsp. *diarizonae* (subsp. IIIb), *Salmonella enterica* subsp. *houtenae* (subsp. IV) and *Salmonella enterica* subsp.*indica* (subsp. VI) (Le Minor and Popoff, 1987; Reeves *et al.*, 1989; Tindall *et al.*, 2005).

1.1.3 *Salmonella* serovars

Further division from each subspecies of *Salmonella enterica* as well as species *Salmonella bongori* is performed through detection of the immunologically distinct structures on the cell surfaces. Currently there are 2610 *Salmonella* serovars being recognised and the majority of which belong to subspecies I (1547 serovars) (Guibourdenche *et al.*, 2010).

Traditionally serovar names were designated indicating the syndromes observed from the infected hosts, e.g. serovars Typhi and Abortusovis. However since not all serovars are host-specific e.g. serovars Typhimurium and Enteriditis, serovar names are now designated reflecting the geographical location where the first strain of the new serovars is isolated e.g. serovars London and Heidelberg (Le Minor, 1988). Currently only serovars belonging to subspecies I are designated names and written with the first letter capitalised following the genus name (Le Minor, 1988). For example, *Salmonella enterica* subsp. *enterica* serovar Typhimurium is simplified as *Salmonella* Typhimurium or *S.* Typhimurium. Since 1966, serovars of the other subspecies and *S. bongori* are only referred to by their antigenic formula following by the subspecies name (Le Minor, 1988). This naming system clearly highlights the significance of subspecies I serovars as they account for the majority (99.5%) of *Salmonella* infections in humans and warm-blooded animals (Le Minor, 1988). Conversely, serovars of the other subspecies and *S. bongori* are commonly isolated from the environment and cold-blooded animals and are rare in humans (Brenner *et al.*, 2000).

1.1.4 Natural habitat and clinical significances

The primary habitats of *Salmonella* are the intestinal tracts of humans and animals (Todar, 2008). *Salmonella* bacteria can also be found in the environment where contamination via faeces of infected humans and animals has occurred (Bopp *et al.*, 2003; Todar, 2008). With regard to serovars of subspecies I that cause most cases of human *Salmonella* infections, most are host-ubiquitous and have been isolated from humans and nearly all warm-blooded animals (Brenner *et al.* 2000). However, there are also host-specific serovars. For example, humans are the only reservoir found for *S.* Typhi (Uzzau

et al., 2000). *S.* Choleraesuis and *S.* Dublin are considered host-adapted serovars that are most commonly associated with infections in pigs and cattle respectively but occasionally cause diseases in other hosts such as humans (Uzzau *et al.*, 2000).

1.1.5 Typhoidal salmonellosis

Infections caused by *Salmonella* can be grouped into typhoidal salmonellosis and non-typhoidal salmonellosis. Typhoidal salmonellosis is commonly known as typhoid or enteric fever. This is a severe systemic disease typically accompanied with sustained high fever and headache with no diarrhoea (Boyd *et al.*, 1996; Bopp *et al.*, 2003). A related less severe disease is caused by *Salmonella* paratyphi A, B and sometimes C (Bhan *et al*., 2005). Humans are the only reservoir for *S*. Typhi and *S.* Paratyphi, transmission of the organisms is through direct contact with the infected patients or consumption of the contaminated foods or water that are faecally-contaminated by the infected patients (Bopp *et al.*, 2003). In developed countries, infections of *S.* Typhi or *S.* Paratyphi are rarely reported and are usually associated with overseas travel to the developing countries where the infections are endemic (Ackers *et al.*, 2000; OzFoodNet, 2007).

1.1.6 Non-typhoidal salmonellosis

In contrast, non-typhoidal salmonellosis most commonly presents as a self-limiting gastroenteritis that usually resolves within a week (Darwin and Miller, 1999). The typical symptoms are fever, abdominal cramps and diarrhoea. Nausea and vomiting are also common (Darwin and Miller, 1999; Bopp *et al.*, 2003). The more severe forms of non-typhoidal *Salmonella* infections are those of the localised suppurative infections including oesteomyelitis, endocarditis, arthritis and bacteraemia (Bopp *et al.*, 2003; Murray *et al.*, 2005). These infections are commonly seen in immuno-compromised patients including long-term hospitalised patients and patients with acquired immune deficiency syndrome (AIDS) (Bopp *et al.*, 2003). As opposed to typhoid fever, a large number of *Salmonella* serovars have been isolated from human non-typhoidal *Salmonella* infections but only a small number of them are common (Brenner *et al.*, 2000). For example, *S.* Typhimurium and *S.* Enteriditis have caused the most cases (77%) of human *Salmonella* gastroenteritis worldwide (Galanis *et al.*, 2006). Food animals are among the usual reservoirs of the non-typhoidal *Salmonella* serovars, thus transmission of these causative agents to humans is commonly through consumption of foods of animal origin and animal products such as poultry, pork, beef, milk and eggs (Humphrey, 2000; Bopp *et al.*, 2003; Murray *et al.*, 2005). Recently there has also been an increasing trend for fruits and raw vegetables such as bean sprouts (Pouhiniemi *et al.*, 1997; Rimhanen-Finne *et al.*, 2011) to be vehicles of *Salmonella* outbreaks.

1.1.7 Epidemiology of non-typhoidal *Salmonella* infections

Non-typhoidal *Salmonella* represents a significant public health problem and is among the notifiable food-borne pathogens in many parts of the world. In Australia, *Salmonella* accounted for 35% cases of the reported food-borne infections in 2007 (OzFoodNet, 2008). A slightly higher rate of food-borne *Salmonella* infections was recorded in the United States in the same year (38.0%) and in 2008 (40.2%) (CDC, 2008, 2009). In addition, *Salmonella* is the most common causative agent for food-borne outbreaks in Australia (OzFoodNet, 2007, 2008).

Despite the fact that mild cases of gastroenteritis are usually reported, non-typhoidal *Salmonella*, together with other enteric pathogens, cause food-borne illnesses leading to a significant economic burden in many countries (WHO 2003). In Australia it has been estimated that there are 5.4 million cases of food-borne diseases annually and the associated economic loss is approximately $1.2 billion Australian dollars per year (cited in OzFoodNet 2007). Whereas in the United States, it was specifically estimated that there are 1.4 million cases of food-borne non-typhoidal *salmonella* infections annually which lead to the loss of $3 billion US dollars annually (WHO, 2003; Voetsch *et al.*, 2004).

1.2 BACTERIAL TYPING SYSTEMS

As non-typhoidal *Salmonella* is among the most common causes of food-borne diseases in many countries; its distribution is monitored locally in a community and on a national basis. As a consequence, changes in the relative frequency of *Salmonella* strains can be monitored to facilitate outbreak detection.

Routine surveillance and outbreak investigations of non-typhoidal *Salmonella* infections both employ typing systems to differentiate between *Salmonella* strains. Apart from being used for epidemiological purposes, typing systems are also essential for studying taxonomy, phylogeny and population genetics of the bacteria of interest (van Belkum *et al.*, 2001). Based upon the nature of the biological characteristics being analysed, typing systems can be categorised into phenotypic or genotypic methods.

1.2.1 Phenotypic versus molecular methods

Phenotypic methods differentiate bacterial strains by comparing their observable biological characteristics resulting from gene expression (van Belkum *et al.*, 2007). Phenotypic characteristics detected for bacterial strain differentiation include biochemical, serological and physiological characteristics (Riley, 2004; van Belkum *et al.*, 2007). In contrast molecular methods differentiate

bacterial strains by detecting variations in their bacterial genomes (van Belkum *et al.*, 2007). The measured genomic variations may be specifically located in the selected gene loci or more broadly by analysing the composition or the overall structure of the bacterial genomes (Riley, 2004). Methodologies employed to demonstrate such genomic variations include restriction enzyme digestion, and/ or post-hybridisation, DNA sequencing and PCR assays.

Currently, molecular methods are preferred over phenotypic methods for typing purposes. This is mainly because molecular methods can generally provide a higher differentiating ability within the species level. In addition a number of limitations associated with phenotypic methods can be overcome or at least improved by using molecular methods. For example, genetic traits of bacteria (genotypes) are comparatively less susceptible to changes than phenotypic traits, which can be readily influenced by external factors such as growth conditions and environmental changes (Arbeit, 2003). Furthermore, use of molecular methods simplifies procedures in typing organisms that are extremely slow growing or require special culture medium (e.g. cell culture), and they make possible differentiation of bacteria that are not culturable (Arbeit, 2003). Nevertheless phenotypic methods have provided the important foundations in understanding the evolutionary relationships between strains of the same species (e.g. multilocus enzyme electrophoresis). In addition, some phenotypic methods are of great value in studying the epidemiology of bacterial infections. For example, serotyping is widely carried out on *Salmonella* and *Escherichia coli* for surveillance purposes.

1.2.2 Evaluation criteria for typing methods

A number of criteria have been proposed to demonstrate the performance and convenience values of a typing system. Performance criteria address the intrinsic properties, whereas convenience criteria measure the practical advantages or disadvantages of a typing system (Maslow and Mulligan, 1996; Struelens, 1998). Eventually, comparisons between typing systems can be made with ease, in accordance to these criteria, to select the most suitable typing methods for different purposes (e.g. outbreak epidemiological studies, long-term surveillance or population genetic studies).

1.2.3 Performance criteria

Several criteria for evaluating the performance of typing systems include typeability, discriminatory power, reproducibility, stability and epidemiologic concordance (Maslow and Mulligan, 1996; Struelens, 1998; van Belkum *et al.*, 2007).

1.2.3.1 Typability

Typability refers to the ability of typing systems in assigning definite types to tested bacterial isolates (van Belkum *et al.*, 2007). Non-typable results are produced when the characteristics being analysed

are not present in the tested isolates. For example phage types cannot be assigned to *Salmonella* isolates that do not possess the cell surface receptors for the typing bacteriophages to bind. Therefore these isolates are reported as non-typable but this does not mean that these non-typable strains are related or of the same type. Typability of a method can be expressed as percentage of typable isolates over the total number of tested isolates (Maslow and Mulligan, 1996).

1.2.3.2 Discriminatory power

Discriminatory power or differentiating ability refers to the ability of a method to measure the relatedness of two bacterial strains (van Belkum *et al.*, 2001). This is usually expressed as the Simpson's index of diversity (DI), indicating the probability of two unrelated strains being recognised as different by a method (Hunter and Gaston, 1988). Depending on the purpose of typing, different levels of discrimination are required. A high level of discrimination is needed for outbreak epidemiological studies which investigate the transmission of the outbreak isolates within a short period of time (days to months) (Struelens *et al.*, 1998). Due to the fact that the outbreak strains and the sporadic strains are derived from a confined area such as a hospital or a community, they are likely to show high genetic similarity. As a consequence, the typing method employed should be able to distinguish the variations displayed by the micro-evolutionary markers such as microsatillites and insertion sequences (*IS*) (Struelens *et al.*, 1998).

In comparison to outbreak epidemiological studies, epidemiological surveillance of geographic spread of different clones of bacteria over a long period of time (years to decades) may require typing systems with a lower discriminatory power such as ribotyping, multilocus sequencing typing (MLST) and more recently single-nucleotide polymorphism (SNP) typing. This is because bacteria are derived from the same or different places over a long period of time. As a result, they would have undergone episodes of genetic alternation to enhance their ability to survive and respond to different environmental pressures (Struelens *et al.*, 1998). Therefore typing systems detecting slowly changing evolutionary markers are needed to reveal the clonal lineages to which these bacterial strains belong to (Struelens *et al.*, 1998).

1.2.3.3 Stability of typing markers

The biological characteristics (typing markers) being analysed are considered stable when they do not change over the study period from isolation to laboratory storage and in subcultures (van Belkum *et al.*, 2007). Such *in vitro* stability of typing markers may be tested by subculturing the bacterial strains on the laboratory culture media a number of times then subjecting each subculture to the typing method. The high stability of typing markers is indicated when identical typing data is obtained from each subculture (Sabat *et al.*, 2006). On the other hand, high *in vivo* stability of typing markers may be indicated when identical typing results are obtained from consecutive bacterial isolates from the same animals or

humans over time (Sabat *et al.*, 2006). Due to the fact the stability of the typing markers is inversely proportional to the evolving rate of the typing markers, typing methods providing high differentiating ability are likely to generate typing data with minor variations in epidemiologically-linked isolates (Kahl *et al.*, 2005; Hopkins *et al.*, 2007). Despite this, the methods are often still acceptable to use in bacterial epidemiology in combination with the result interpretation guidelines, which determine the epidemiological relationships between the isolates in reference to the genetic relationships inferred by the methods (Hopkins *et al.*, 2007).

1.2.3.4 Reproducibility

Reproducibility refers to the ability of a typing method to produce the same results repeatedly on a bacterial strain (Singh *et al.*, 2006). This property is largely influenced by the variations of testing parameters including the protocol used, the brand of reagents, the types of equipment and the operators carrying out the procedures (van Belkum *et al.*, 2007). As a consequence, many efforts have been made to standardise protocols to eventually facilitate reliable comparison of typing data generated from the same laboratories and between laboratories over time for surveillance of epidemic strains, such as a standardised pulsed-field gel electrophoresis (PFGE) protocol for *Salmonella* (Murchan *et al.*, 2003; Ribot *et al.*, 2006).

1.2.3.5 Epidemiological concordance

Epidemiological concordance describes the capacity of a typing method to provide results consistent with the available epidemiological information (van Belkum *et al.*, 2007). During outbreak investigations the epidemiological data recognise patients involved in the outbreaks, and the typing methods confirm this relationship by assigning the outbreak isolates with identical types or profiles. To experimentally demonstrate the level of concordance between the typing data and the epidemiological data, several sets of previous outbreak isolates (i.e. confirmed epidemiologically related isolates) and a number of sporadic isolates (i.e. epidemiologically unrelated isolates) should be included (van Belkum *et al.*, 2007).

1.2.3.6 Convenience criteria

For typing methods to gain wide acceptance they should possess practical advantages relevant to the typing purposes. For example, typing methods applied in outbreak investigations should generate typing results rapidly in order to confirm that an outbreak is occurring to facilitate immediate infection control practices (Struelens, 1998). However rapidity of result generation is not as critical for global epidemiological surveillance of pathogens. In contrast, because typing data for surveillance purposes are continuously generated and compared between countries from time to time, it is preferable to use methods that generate results with a binary output (numbers or characters) to simplify data exchange and storage e.g. multi-locus sequence typing (MLST) (van Belkum *et al.*, 2001). In summary, a number

of criteria to be considered when assessing the practicality of typing methods include ease of result interpretation, ease of use, rapidity, costs and ease of result distribution (Struelens, 1998; van Belkum *et al.*, 2007).

1.2.4 Typing methods for *Salmonella*

As discussed above the typing method employed is primarily based on the purpose for the strain differentiation and is a compromise of the intrinsic properties and the provided practical benefits. The following section will provide a discussion regarding the currently available typing methods for *Salmonella*, including the advantages and disadvantages with respect to each of the methods.

1.2.4.1 Serotyping

Serotyping is a phenotypic method that differentiates *Salmonella* isolates based on the differential antigenic properties of the O (somatic lipopolysaccharide cell wall) and the H1 and H2 (phase 1 and phase 2 flagellar protein) antigens (Threlfall and Frost, 1990). Depending on the immunological variations in these two surface structures, isolates are assigned into serotypes or serovars in accordance to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). In practice, serotyping is achieved through a bacterial agglutination test using a panel of antisera prepared against the antigens (Riley, 2004). At the present time, 2610 serovars have been identified and recorded in the White-Kauffmann-Le Minor scheme, with 1547 serovars belonging to subspecies I (Guibourdenche *et al.*, 2010).

1.2.4.1.1 Epidemiological typing using serotyping

Due to the stable nature of antigenic characteristics, serotyping is the predominant method for laboratory-based surveillance of *Salmonella* infections worldwide and is performed routinely on each of the laboratory confirmed *Salmonella* isolates (Herikstad *et al.*, 2002). Although the agglutination assays are simple to perform and the typing results are generated rapidly, production of antisera is expensive, time consuming and requires experienced personnel for quality control (Seyfarth *et al.*, 2003). Consequently serotyping is generally considered as an expensive and labour-intensive procedure that is usually confined to specialist reference laboratories (Riley, 2004).

1.2.4.1.2 Molecular serotyping schemes

Recently molecular serotyping schemes has been proposed to overcome the limitations of traditional serotyping while maintaining the continuity of the historical database built by serotyping (Luk *et al.*, 1993; Echeita *et al.*, 2002; Herrera-León *et al.*, 2004; Yoshida *et al.*, 2007). To type *Salmonella* isolates using molecular serotyping methods, the gene sequences responsible for O antigenic variations are targeted which are located within the *Salmonella* gene cluster *rfb* (Luk *et al.*, 1993). Alternatively gene

sequences encoding Wzx proteins from different O-antigen clusters are targeted as they show little similarity at DNA level (Herrera-León *et al.*, 2007). By contrast, the variable regions of the *fli*C and *fli*B genes are targeted to determine the phase 1 and phase 2 flagellar antigens respectively (Echeita *et al.*, 2002; Herrera-León *et al.*, 2007). One of the significant contributions of molecular serotyping is its ability to complete serovar designations for rough strains that do not express O-antigens as well as for strains that do not express both phase 1 and 2 flagellar anitigens (Herrera-León *et al.*, 2007). Hence it was suggested that both traditional and molecular serotyping methods may be used in parallel to enhance surveillance typing of *Salmonella* (Herrera-León *et al.*, 2007).

1.2.4.2 Bacteriophage typing

While serotyping is capable of providing extensive differentiation within *Salmonella* genus into over 2500 serovars, it is of limited value for outbreak investigations as only a small number of serovars are responsible for majority of infections (Harvey *et al.*, 1993). As a consequence, subdivision of isolates within serovars is needed for organism tracing during outbreaks of *Salmonella* infections. Phage typing was traditionally employed for this purpose.

1.2.4.2.1 Epidemiological typing using phage typing

Bacteriophage or phage typing involves testing of a defined panel of typing phages on the bacterial isolates of interest. The observed differential plating efficiency of each typing phage to the tested bacterial isolates forms the basis of phage typing, and is dependent on several bacterial host factors. These include the host controlled modification systems, the bacterial cell receptors for the typing phages to bind to, the superinfection exclusion systems of the prophages residing in the bacterial cells, and the phage sequence contents at spacers of the clustered regularly interspaced short palindromic repeats (CRISPR) loci in the tested isolates (Threlfall and Frost, 1990; Schmieger, 1999; Barrangou *et al.*, 2007). A number of phage typing schemes have been established for the commonly isolated non-typhoidal *Salmonella* serovars including *S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg, *S.* Virchow and *S.* Bovismorbificans (summarised in Jones *et al.* 2000).

To demonstrate that phage typing is an effective method for outbreak epidemiological studies, it should have a high capacity to differentiate unrelated strains of the same serovars. However limited discrimination has been shown by phage typing in some important serovars, for example *S*. Enteriditis. From 1981 to 1986, 85% of the total isolated *S.* Enteritidis belonged to phage types (PT) 4 and PT 8 in England and Wales (Ward *et al.*, 1987). While in the similar timeframe, 83% of all *S.* Enteritidis isolates were PT 8 and PT 13a in the United State, and 72.6% were PT 8 in the Slovak Republic (cited in Rankin and Platt 1995; Majtánová 1997). Clearly in situations when a few phage types predominate in a

geographic area, typing methods with a higher discriminating ability are necessary for outbreak epidemiological investigations.

1.2.4.2.2 Phage type conversion

Studies of phage type conversion in the past have shown that some phage types can be unstable and may confound outbreak epidemiological investigations. This phenomenon has been most extensively demonstrated in *S.* Enteritidis. Frost *et al.* (1989) demonstrated that the conversion from PT 4 to PT 24 was due to the acquisition of an antimicrobial resistance (Inc N) plasmid, while Chart *et al.* (1989) showed that the conversion of *S.* Enteritidis PT 4 to PT 7 was due to LPS modification. Furthermore Rankin and Platt (1995) demonstrated the change of a number of phage types through lysogenisation of phages: PT 4 was converted to PT 8, PT 13 to PT 13a, PT 15 to PT 11, and PT 6a to PT 4 and PT 7. In fact phage type conversion due to lysogenisation has also been documented for other serovars. A temperate phage ST64T induced from a *S.* Typhimurium DT 64 clinical strain was able to convert *S.* Typhimurium DT 9 to DT 64, DT 135 to DT 16 and DT 41 to DT 29 (Mmolawa *et al.*, 2002). A *S.* Heidelberg phage (HIP-4) induced from a *S.* Heidelberg PT 4 strain was shown to convert PT 1 and PT 3 to PT 4 and PT 5 respectively (Harvey *et al.*, 1993).

1.2.4.2.3 Practical drawbacks of phage typing

Other drawbacks of phage typing are mainly associated with its practical disadvantages. Harvey *et al.* (1993) pointed out that interpretation of phage typing results is subjective and requires experienced operators to ensure uniformity or reproducibility between laboratories. Schmieger (1999) commented that maintenance of stocks of typing phages can be very difficult as the released phages propagated from their host strains of *S.* Typhimurium do not necessarily have identical plating properties. This is a consequence of genetic recombination between typing phages (infecting phages) and the resident prophages (Schmieger, 1999). Therefore to ensure the released phages are identical to the original typing phages, several advanced techniques such as electron microscopy and restriction enzyme digestion may be required to characterise the prepared phage stocks. Thus phage typing is considered as a technically demanding method (Tamada *et al.*, 2001).

In summary, accurate phage type assignment relies principally on use of typing phages with consistent plating properties that are difficult to maintain. Furthermore the use of phage typing alone for outbreak investigations of *Salmonella* infections can be ineffective when a small number of phage types predominate in the geographic area. As a consequence, newer genotypic methods have been developed to supplement phage typing. Methods that were applied in the past include plasmid profile typing (Threlfall *et al.*, 1994); restriction fragment length polymorphism (RFLP) of plasmids (Platt *et al.*,

1986), insertion sequence IS 200 fingerprinting (Stanley *et al.*, 1991) and ribotyping (Grimont and Grimont, 1986). However pulsed-field gel electrophoesis was widely selected as the method of choice.

1.2.4.3 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) characterises bacterial isolates on the basis of the banding patterns generated following restriction of the total genomic DNA. To perform PFGE, bacterial cells are initially embedded in molten agarose and lysed by detergent and enzymes. The released whole bacterial genomic DNA is then digested using infrequently cutting restriction enzymes to produce 10 to 30 macrorestriction DNA fragments ranging from 10 to 800 kilobases (kb) (Tenover *et al.*, 1997; Oliver and Bean, 1999). For *Salmonella*, the most commonly used restriction enzyme is *Xba*I which generates 11 to 17 DNA fragments between 40 and 800 kb in size (Tenover *et al.*, 1995; Ridley *et al.*, 1998; Tamada *et al.*, 2001). Separation of these large DNA restriction fragments is achieved using PFGE equipment where the orientation of electric field changes periodically allowing separation of DNA molecules larger than1000 kb (Maslow *et al.*, 1993).

1.2.4.3.1 PFGE the current "gold standard" typing method for *Salmonella*

As well as *Salmonella*, PFGE has been considered the "gold standard" typing method for a wide range of community-acquired and hospital-acquired bacterial pathogens including *Staphylococcus aureus*, vancomycin-resistant enterococci and *Escherichia coli* O157:H7 (Singh *et al.*, 2006). It has been well documented that PFGE is highly discriminatory in tracing outbreak strains of various *Salmonella* serovars (Threlfall *et al.*, 1996; Bennett *et al.*, 2003; Irvine *et al.*, 2009). More recently, the high discriminating ability of PFGE has been challenged by the emergence and spread of the genetically homogeneous phage types in particular the multi-resistant *S.* Typhimurium DT104 and *S.* Enteritidis PTs 4 and 8. For these phage types only a small number of PFGE profiles predominate in many geographic locations (Baggesen *et al.*, 2000; Malorny *et al.*, 2001; Liebana *et al.*, 2002; Lukinmaa *et al.*, 2006) (Gatto *et al.*, 2006). As demonstrated by Baggesen *et al.* (2000), all 125 DT 104 isolates except two generated the same *Xba*I-PFGE patterns even though they were derived from Europe and the United States. Similarly Gatto *et al.* (2006) demonstrated that over 3000 unrelated PT 4 isolates from nine European countries generated 38 PFGE patterns with most of the isolates (88%) having pattern SENTXB.001. The difficulty of PFGE in differentiating DT 104 isolates during outbreaks was clearly demonstrated by Lawson and co-workers (2004). Although all outbreak-associated isolates produced the same PFGE profile and hence confirmed as outbreak related, almost all (90%) the epidemiologically-unrelated isolates also had the same pattern resulting in inadequate discrimination by PFGE (Lawson *et al.*, 2004). Similarly PFGE could not provide adequate discrimination between the

outbreak strains and the non-outbreak strains in a *S.* Enteritidis PT 8 outbreak in Canada (Ahmed *et al.*, 2000).

1.2.4.3.2 Approaches to improve differentiating ability of PFGE

Despite the low discriminatory ability in *Salmonella*, PFGE in general is considered a valuable outbreak typing tool as it is highly adaptable to virtually any bacterial species. Furthermore the generated PFGE results have shown good concordance to epidemiological data in outbreak investigations (Barrett *et al.*, 2006). However one may argue that the good concordance observed between PFGE results and epidemiological data may be due to the poor discrimination provided by PFGE as most endemic strains including isolates in an outbreak all invariably have the same or similar PFGE profiles.

Efforts have been made to retain PFGE for use in *Salmonella*. One approach is to supplement PFGE with other molecular methods such as plasmid profiling which has been showed to provide the most discrimination among *S.* Typhimurium DT 104 strains (Malorny *et al.*, 2001; Liebana *et al.*, 2002; Lawson *et al.*, 2004). However, coupling PFGE with plasmid profiling may not be useful for subdivision of *S.* Enteritidis PT 4 as many strains carry a 38-MDa plasmid only (Threlfall *et al.*, 1994). The other suggested approach involves combining PFGE results of two or more restriction enzymes (Ridley *et al.*, 1998; Laconcha *et al.*, 2000; Zheng *et al.*, 2007). As demonstrated by Zheng *et al.* (2007), combining *Xba*I and *Bln*I digestion profiles provided high discrimination among the tested *S.* Typhimurium isolates insofar as almost every isolate generated a unique combined *Xba*I /*Bln*I pattern. While combining PFGE patterns of *Sfi*I, *Pac*I and *Not*I differentiated the *S.* Enteritidis isolates most extensively (Zheng *et al.*, 2007). However this approach may seem impractical during outbreaks due to the lengthy procedure involved and the need to run individual gels for each enzyme which differ in run conditions and times (Liebana *et al.*, 2001; Ribot *et al.*, 2006).

1.2.4.3.3 Practical drawbacks of PFGE

The replacement of PFGE is recommended by some researchers due to its practical disadvantages. PFGE is a low-throughout method involving a tedious and technically demanding procedures. Furthermore the equipment and reagents required are both expensive (Oliver and Bean, 1999; Weller, 2000; Lindstedt *et al.*, 2004; Lukinmaa *et al.*, 2004; van Belkum *et al.*, 2007).

High intra-laboratory reproducibility of PFGE may be readily achieved through use of the same PFGE procedure, reagents and electrophoresis equipment for each analysis in the laboratory; however great effort is required to achieve acceptable levels of inter-laboratory reproducibility. In the past this is relied upon the laboratories following the same testing procedure, use the same reagents and equipment, and analysing PFGE patterns according to the same interpretation guidelines (van Belkum *et al.*, 1998b;

Ribot *et al.*, 2006; van Belkum *et al.*, 2007). There are image analysis programs (e.g. BioNumerics) to assist in optimising gel images, however it can still be difficult to compare band patterns between gels and between laboratories. This is due to that position of the same bands can be readily affected by the imperfect reproducibility of running condition of each gel and hence assigned as different bands erroneously by the program (van Belkum *et al.*, 2007). Ultimately consistent band pattern assignment is highly dependent on the expertise of the operators who may also experienced in recognising any partial restriction products, doublet or triplet bands of similar sizes and faintly stained restriction bands (Tenover *et al.*, 1994).

1.2.4.4 Recently developed typing methods

A number of typing methods have been recently developed and examined for their usefulness in strain differentiation among *Salmonella*. These molecular methods, apart from being able to differentiate between *Salmonella* strains, appear to have different practical values when compared to PFGE as described below.

1.2.4.4.1 Multilocus sequence typing

Multilocus sequence typing (MLST) is a sequence-based typing method that classifies bacterial strains on the basis of the nucleotide sequence diversity present in the targeted gene loci. Originally MLST was developed as the molecular version of multilocus enzyme electrophoresis (MLEE) for *Neisserria meningitidis* (Maiden *et al.*, 1998). In MLEE, the phenotypic trait of the electrophoretic mobility of each housekeeping enzyme is determined. In contrast, MLST takes into account each nucleotide change within the targeted housekeeping genes (Maiden *et al.*, 1998). As a consequence, approximately half of the MLST loci (6-7 loci) are required to provide discrimination equivalent to MLEE (12-19 loci) indicating the typing effectiveness of MLST in comparison to MLEE (Maiden *et al.*, 1998).

1.2.4.4.1.1 Practical advantages of MLST

To carry out MLST the approximate 400 bp region within the selected gene loci are initially amplified using polymerase chain reactions (PCR), then sequenced to determine their nucleotide sequences. Since result interpretation involves direct comparisons between amplified DNA sequences, it is objective in nature and was demonstrated to be accomplished more readily than PFGE (Feavers *et al.*, 1999). More importantly, MLST sequence data are highly portable, and thus allow convenient storage and comparison of typing data between laboratories around the world (Maiden *et al.*, 1998).

1.2.4.4.1.2 Epidemiological typing using MLST

Kotetishvili *et al.* (2002) indicated that MLST using housekeeping genes could differentiate *Salmonella* strains more effectively than PFGE, and so suggested its use in outbreak investigations. However the

high discriminatory ability of MLST may be due to the use of isolates from a broad range of *Salmonella* serovars and subspecies. Therefore allelic variations are expected and these results reinforced the utility of MLST using housekeeping genes for phylogenetic study of *Salmonella* as a whole (Ross and Heuzenroeder, 2005a). Subsequently, low levels of discrimination within serovars were shown by other researchers (Fakhr *et al.*, 2005; Ross and Heuzenroeder, 2005a; Harbottle *et al.*, 2006). Using the same gene loci as Kotetishvili *et al.* (2002), none of the 85 *S.* Typhimurium isolates showed allelic variations (Fakhr *et al.* 2005). Ross and Heuzenroeder (2005b) indicated that MLST lacks the ability to discriminate between isolates of the same phage type. The study employed MLST on five housekeeping genes to differentiate *S.* Typhimurium DT 126 isolates but little discrimination was observed. Three of five loci showed identical DNA sequences, while a different allele was shown infrequently from loci *glnA* and *manB* within a group of outbreak isolates and in a single isolate respectively.

1.2.4.4.2 Amplified fragment length polymorphism

Like MLST, amplified fragment length polymorphism (AFLP) is also a PCR based typing technique. However AFLP detects non-specific genetic differences throughout the whole bacterial genome while MLST reveals genetic variations at the predetermined genomic regions (Yan *et al.*, 2003). To perform AFLP, the whole bacterial genomic DNA is digested by an infrequently-cutting restriction enzyme and a frequently-cutting restriction enzyme. For *Salmonella*, *EcoR*I (infrequently-cutting restriction enzyme) and *Mse*I (frequent restriction enzyme) are usually used (Aarts *et al.*, 1998; Lindstedt *et al.*, 2000; Hu *et al.*, 2002; Ross and Heuzenroeder, 2005b). Subsequently, all restriction DNA fragments are ligated with adapters to which the AFLP primers bind for PCR amplification. Only a subset of adapter-ligated fragments is amplified and this is based on the sequence compatibility between the adapters and the selective nucleotide(s) at the 3' extension ends of the AFLP primers. Eventually the amplified restriction fragments are presented as band patterns from which bacterial strains are differentiated (Vos *et al.*, 1995).

1.2.4.4.2.1 Comparison of AFLP with PFGE

By the PFGE and AFLP differentiate bacterial strains on the basis of the mutational changes that lead to gain and loss of restriction sites; AFLP appears to be a more sensitive method than PFGE. This is because PFGE uses one infrequent restriction enzyme (e.g. *Xba*I) that generates a small number (10 to 17 fragments) of large DNA fragments (up to 800 kbs) (Tenover *et al.*, 1997). As a consequence, small genomic differences (several kbs) derived from insertion-deletion events or chromosomal rearrangements are not likely to make detectable changes to the restriction fragment sizes (Hu *et al.*, 2002). In contrast, AFLP uses two restriction enzymes which generate approximately 50 fragments sizing between 45 to 500 bp for comparison between isolates (Vos *et al.*, 1995; Aarts *et al.*, 1998).

Therefore small genomic differences between strains can be more readily detected from the amplified DNA fragments and hence maximise the opportunity to differentiate between closely related bacterial strains (Hu *et al.*, 2002). Furthermore using of fluorescent-labelled primers and capillary electrophoresis greatly enhances accuracy in sizing amplified restriction fragments, and facilitates automation of the process (Lindstedt *et al.*, 2000; Ross and Heuzenroeder, 2005b).

1.2.4.4.2.2 Epidemiological typing using AFLP

As with PFGE, AFLP has been shown to have potentially limited discriminatory ability towards certain genetically homogenous serovars and phage types (Lindstedt *et al.*, 2000; Lan *et al.*, 2003; Giammanco *et al.*, 2007). A study by Ross and Heuzenroeder (2005) indicated that AFLP was useful in assisting outbreak investigations caused by *S*. Typhimurium DT 126 phage group as it was able to differentiate DT 126 isolates associated with two separate outbreaks. However such discriminatory ability was considered suboptimal as not all epidemiologically-unrelated DT 126 isolates were separated from the outbreak isolates (Ross and Heuzenroeder, 2005b). Furthermore, AFLP provided a limited level of discrimination within *S.* Typhimurium phage types DT 9 and DT 135. These two phage types are among the most common *S.* Typhimurium phage types in Australia and New Zealand (Hu *et al.* 2006; OzFoodNet 2006; OzFoodNet 2007; OzFoodNet 2008). In a study performed by Lan *et al.* (2003), a total of eight AFLP primer pairs were used, which is in contrast to one primer pair in the standard AFLP protocol, to achieve an acceptable level of discrimination for DT 135 (DI value = 0.867). However a very low level of discrimination within DT 9 was still observed (DI value = 0.383). Similarly, 16 primer pairs were necessary to differentiate the *S.* Typhimurium isolates in another study (Hu *et al.*, 2002). Taken together it seems that several primer pairs are usually needed to provide good discrimination for *Salmonella* and this may not be practical for routine use. This is because the band patterns (>100 bands) generated will be too complicated to analyse and compare (Lindstedt *et al.*, 2000; Boxrud *et al.*, 2007).

1.2.4.4.3 Multiple-locus variable-number tandem repeat (VNTR) analysis

Exploitation of tandem repeat DNA has led to the development of a novel PCR-based typing scheme, multiple-locus variable-number tandem repeat analysis (MLVA). Tandem repeat DNA is a class of contiguous short repetitive DNA that occurs in variable numbers in a single locus resulting in inter-individual length polymorphism (Nakamura *et al.*, 1987). The presence of variable number of tandem repeat (VNTR) sequences is thought to be caused by slipped-strand mispairing during DNA replication. Such mechanisms help bacteria to adapt to environmental changes. This suggestion was based on the frequent observations of tandem repeats within genes involved in biosynthesis of bacterial outer membrane proteins (Bichara *et al.*, 2006). By varying the number of tandem repeats within gene coding regions or promoter regions, either the translational or the transcriptional pathways of these surface

proteins are altered, thereby causing phase variation and allowing bacteria to alter their way to adhere host cells and evade host immune systems (reviewed in van Belkum *et al.* 1998).

1.2.4.4.3.1 Epidemiological typing using MLVA

MLVA differentiates bacterial strains by assessing the variability of several different VNTR containing loci (Lindstedt *et al.*, 2003). The targeted gene loci are initially amplified using multiplex PCR and then the length of each of the amplified products is examined using conventional electrophoresis or more commonly using capillary electrophoresis (Liu *et al.*, 2003; Cho *et al.*, 2007; Malorny *et al.*, 2008; Octavia and Lan, 2009). This is due to that capillary electrophoresis provides a more accurate size determination of amplified products (Call *et al.*, 2008). In additon, different dyes can be assigned to each of the targeted loci to facilitate simultaneous measurement of the amplified fragment length of multiple MLVA loci, which in turn reduces the result turnaround and hands-on time (Lindstedt, 2005). The sizes of the amplified products are expressed numerically and can be normalised readily to reveal the actual sizes and the number of contained tandem repeats through comparison with the electrophoretic data of MLVA loci of known sizes. The typing data are then be stored electronically and exchanged between laboratories (Lindstedt *et al.*, 2004).

Recently, MLVA schemes have been proposed for epidemiological typing of a variety of pathogenic bacteria (Lindstedt 2005). This is because MLVA is PCR-based; it is simple to perform, rapid, high throughput, and can be easily accessed in any molecular laboratory (Lindstedt, 2005). More importantly, using VNTR loci with high evolutionary rates enable MLVA to subdivide homogeneous bacterial species such as *Mycobacterium tuberculosis*, *Yersinia pestis* as well as *Salmonella* serovars (Cowan *et al.*, 2002; Lindstedt *et al.*, 2004; Pourcel *et al.*, 2004; Boxrud *et al.*, 2007; Davis *et al.*, 2009; Octavia and Lan, 2009).

1.2.4.4.3.2 MLVA typing for *Salmonella*

MLVA systems have been described for specific serovars including Typhimurium, Enteritidis, Typhi and Newport (Lindstedt *et al.*, 2003; Liu *et al.*, 2003; Boxrud *et al.*, 2007; Cho *et al.*, 2007; Davis *et al.*, 2009; Octavia and Lan, 2009; Ross and Heuzenroeder, 2009). Through analysing a small number of MLVA loci (ranging from 3 to 10 loci), the level of discrimination provided was observed to be higher than that of PFGE. Moreover, the MLVA systems enabled subdivision within the highly clonal *Salmonella* serovars and phage types including the multi-resistant *S.* Typhimurium DT 104, *S.* Enteritidis PT 4 and PT 8 (Lindstedt *et al.*, 2003; Malorny *et al.*, 2008; Beranek *et al.*, 2009). In addition, Boxrud and co-workers (2007) demonstrated the usefulness of MLVA in *Salmonella* outbreak epidemiological studies. In that study, not only could MLVA correctly identify the differences between the outbreak *S.* Enteritidis

PT 13a strains associated with four separate outbreaks but it provided a better discrimination between the outbreak isolates and the sporadic isolates in comparison to PFGE (Boxrud *et al.*, 2007).

1.2.4.4.3.3 MLVA the next "gold standard" typing method for *Salmonella*?

For MLVA to be widely accepted as the new 'gold standard' typing method for *Salmonella*, it should also demonstrate a high level of discrimination in clinically significant serovars other than those mentioned above. Recently, the use of previously described VNTR loci has enabled intra-serovar differentiation within *S.* Infantis (Ross and Heuzenroeder, 2008). By analysing three VNTR loci from the MLVA method for *S.* Typhimurium, the *S.* Infantis isolates were discriminated at a higher level than by PFGE (Lindstedt *et al.*, 2003; Ross and Heuzenroeder, 2008). However the inclusion of an additional 10 VNTR loci did not provide further discrimination since they were either not detected or were identical in the *S.* Infantis isolates (Ross and Heuzenroeder, 2008). Similar findings were also reported from other studies where VNTR loci displayed allelic variations or existed in some serovars but not the others, and this is thought to be due to the different genomic organisation between serovars (Ramisse *et al.*, 2004; Boxrud *et al.*, 2007; Beranek *et al.*, 2009; Davis *et al.*, 2009; Ross and Heuzenroeder, 2009). As a consequence, the most optimal MLVA typing of any serovar may only be obtained when genomes of serovars of interest are characterised so that the detection of VNTR loci can be facilitated (Beranek *et al.*, 2009; Ross and Heuzenroeder, 2009).

1.2.4.4.4 Multiple amplification of prophage locus typing

It has been shown that substantial amounts of genetic variation among closely related *Salmonella* strains originated from prophage elements (Figueroa-Bossi and Bossi, 2004; Thomson *et al.,* 2004; Cooke *et al.*, 2007; Lan *et al.*, 2007). A detailed discussion regarding how prophages contribute to genetic differences between *Salmonella* strains can be found later in sections 1.3.6.3 and 1.3.7. This finding has led to development of the first PCR-based typing method that targets prophage loci for differentiation of *S.* Typhimurium isolates (Ross and Heuzenroeder, 2005a). The authors named this typing scheme the multiple amplification of prophage locus typing (MAPLT), where strains are differentiated based on the the presence and absence of the targeted prophage loci. In the study, twenty-five prophage-related primers were designed from three temperate phage sequences (P22, ST64T and ST64B) to differentiate a group of 73 diverse *S.* Typhimurium isolates from sporadic infections. In total, twenty-seven distinct MAPLT profiles were observed in comparison to only five PFGE profiles from the isolates (Ross and Heuzenroeder, 2005a). Detection of prophage loci has also been used by other researchers suggesting that this approach is a feasible alternative option for *Salmonella* typing (Mikasova *et al.*, 2005; Lindstedt *et al.*, 2006; Drahovská *et al.*, 2007; Rychlik *et al.*, 2008; Wang *et al.*, 2008; Fang *et al.*, 2012). Wang *et al.,* (2008) furthered the assessment through comparing the method with MLVA for *Salmonella* outbreak investigations. In the study, a multiplex

PCR-based reverse line blot hybridisation (mPCR/RLB) method was developed that simultaneously detected thirty-two prophage-related loci. Of the 168 *S.* Typhimurium isolates that were tested, 102 mPCR/RLB profiles and 97 MLVA profiles were observed. The differentiating ability of mPCR/RLB was comparable to that of MLVA (DI value = 0.9866 for mPCR/RLB; DI value = 0.9865 for MLVA). More importantly, the mPCR/RLB method was shown to be able to generate the same mPCR/RLB profiles from the epidemiologically-related isolates in three separate outbreaks suggesting its potential use for outbreak epidemiological studies (Wang *et al.*, 2008).

Concurrently, Ross and Heuzenroeder (2008 and 2009) assessed the application of MAPLT for typing within other *Salmonella* serovars including *S.* Infantis and *S.* Enteritidis. In both studies, MAPLT displayed a higher differentiating ability than did PFGE (Ross and Heuzenroeder, 2008, 2009). In addition, MAPLT was shown to be more useful than MLVA in sub-dividing the predominant *S.* Enteritidis phage types 1 and 6a in Australia. These results in turn suggested MAPLT as a viable option for local epidemiological study of *S.* Enteritidis, particularly when the outbreaks are associated with these two predominating phage types (Ross and Heuzenroeder, 2009). However as with MLVA, there is no definite set of prophage primers that are useful for all *Salmonella* serovars due to the different content of prophage elements between serovars (Ross and Heuzenroeder, 2008, 2009). Nevertheless, it is not uncommon to find prophage primers showing differentiating capacity in more than one serovar. For example, primers amplifying locus int_{P22} is included in the MAPLT schemes for *S.* Typhimurium and *S.* Infantis (Ross and Heuzenroeder, 2008). Furthermore, although many bacteriophage sequences in *Salmonella* have not been deposited in a public domain such as Genbank, novel phage gene sequences can be identified alternatively in a less expensive way than whole genome sequencing by using DOP-PCR (degenerate oligonucleotide primed-PCR) methodology as previously described (Ross and Heuzenroeder, 2008). Eventually a pool of prophage primers may be established in a laboratory from which different combinations of primer pairs can be composed to facilitate establishment of MAPLT for various *Salmonella* serovars.

Having discussed the current typing methods available for *Salmonella*, the following sections will discuss further on the significance of bacteriophages in the evolution and the genetic diversification of *Salmonella*.

1.3 BACTERIOPHAGES

Bacteriophages are viruses that infect prokaryotic cells including bacteria and archeae (Ackermann 2007). Bacteriophages or "phages" for short were originally described by a British scientist F. W. Twort in 1915 and by a French scientist F.d' Hérelle in 1917 (cited in Bradley 1967). In 1915 Twort described a 'transmissible' agent that infected and killed staphylococcal bacteria causing the glassy transformation of the colonies. He proposed that the agent may be a virus (cited in Bradley 1967). Independently in 1917 d' Hérelle observed the lysis of *Shigella* cultures in broth and recognised the causative agent as being a parasitic virus and named it a bacteriophage (cited in Bradley 1967). It is estimated that the total number of phage particles on earth is approximately 10^{31} making them the most abundant "life forms" (Ashelford *et al.*, 2003; Suttle, 2005). Currently classification of phages is based on morphology and nucleic acid type (Ackermann, 2003). Over 5500 phages have been examined by electron microscopy and are described as tailed, polyhedral, filamentous or pleomorphic containing either DNA or RNA in either single- or double-stranded forms (Ackermann and Kropinski, 2007; Ackermann, 2009). Tailed-phages containing dsDNA are among the most common forms comprising around 96% of the total number of phages that have been investigated (Ackermann and Kropinski, 2007; Hatfull, 2008; Ackermann, 2009). As they are the most abundant "life form" on earth, tailed-phages have been studied more extensively in comparison to the other phage types and contribute most of the knowledge regarding bacteriophages at present.

1.3.1 Morphology of tailed-phages

One common classification of bacteriophages is according to the physical and physiological characteristics of the phages. Tailed-phages are all grouped in the order *Caudovirales* due to the similarities in their tailed virus morphology, modes of replication and assembly (Maniloff and Ackermann, 1998). Based on the variation of the phage tails, members of *Caudovirales* are further classified into three families: *Myoviridae* (phages with contractile tails consisting of a sheath and a central tube), *Siphoviridae* (phages with long and non-contractile tails), and *Podoviridae* (phages with short tails) (Ackermann and Kropinski, 2007). Structurally tailed-phages consist of heads with cubic symmetry and tails with binary symmetry (Ackermann 2003). The phage heads range from isometrical to prolate or elongated isosahedral in shape, while the phage tails are either helical or composed of stacked disks which commonly end with fixation structures such as baseplates, spikes or terminal fibers (Maniloff and Ackermann, 1998; Ackermann, 2006). These viral particles are composed simply of proteins and dsDNA only and generally do not possess lipid envelopes or lipid-containing structures (Ackermann, 2006).

1.3.2 Lytic versus lysogenic life style

Replication of tailed-phages can be achieved through lytic or lysogenic cycles. Lytic infection by tailed-phages involves absorption by a bacterial host cell that is followed by synthesis of viral components, assembly and release of progeny viral particles through the lysis of the infected cells (Miller and Day, 2008) (Fig. 1.1). Phages that can only undergo the lytic cycle are described as lytic or virulent phages (Miller and Day, 2008). In contrast, temperate phages have the ability to propagate via the lytic cycle or the lysogenic cycle. Lysogeny describes the latent infection by temperate phages where they establish a long-term stable relationship with the host bacterial cells (lysogens) (Casjens, 2003). In most cases, the phage genomes (referred as prophages) integrate and replicate as part of the host chromosome during cell division and progeny prophages are distributed to each of the daughter cells (Miller and Day, 2008) (Fig. 1.1). Alternatively, some phages such as coliphage P1 exist as circular plasmids in their lysogenic state (Ikeda and Tomizawa, 1968). Replication of coliphage P1 occurs at random in the cell cycle and the enzymes required for the process are encoded by the phages (Abe, 1974).

A number of environmental factors are known to influence whether a temperate phage will undergo lytic or lysogenic infection. For example, infection of bacterial cells growing in poor medium or low temperatures favours lysogenic infection (Little, 2005). Increasing the multiplicity of infection (MOI) that is the ratio of bacteriophage particles to bacterial cells will also favour lysogeny (Hendrix and Casjens, 2006).

1.3.3 Gene regulatory pathways for lytic and lysogenic life cycles

Genes located in the immunity region (*immC*) of the phage genome encode proteins regulating these two infecting processes. For the most studied phage λ, Cro protein directs development of lytic infection, while CII protein directs development of lysogenic infection (Ptashne, 2004). The decision of which infection pathway to go through is dependent on the relative level of Cro and CII proteins present in the bacterial cell after absorption of phage λ particles to the bacterial cell. Such a ratio is more influenced by the concentration of CII protein as it is metabolically unstable and can be degraded by the host membrane-bound protease system (Cheng *et al.*, 1988; Ho *et al.*, 1988). Unlike the Cro protein that is required to initiate and maintain lytic growth, CII protein is only required at the early stage of lysogeny to activate transcription from promotor P_{RE} for the synthesis of protein CI. Protein CI acts in opposition to Cro protein to promote and maintain lysogenic growth (Ptashne, 2004).

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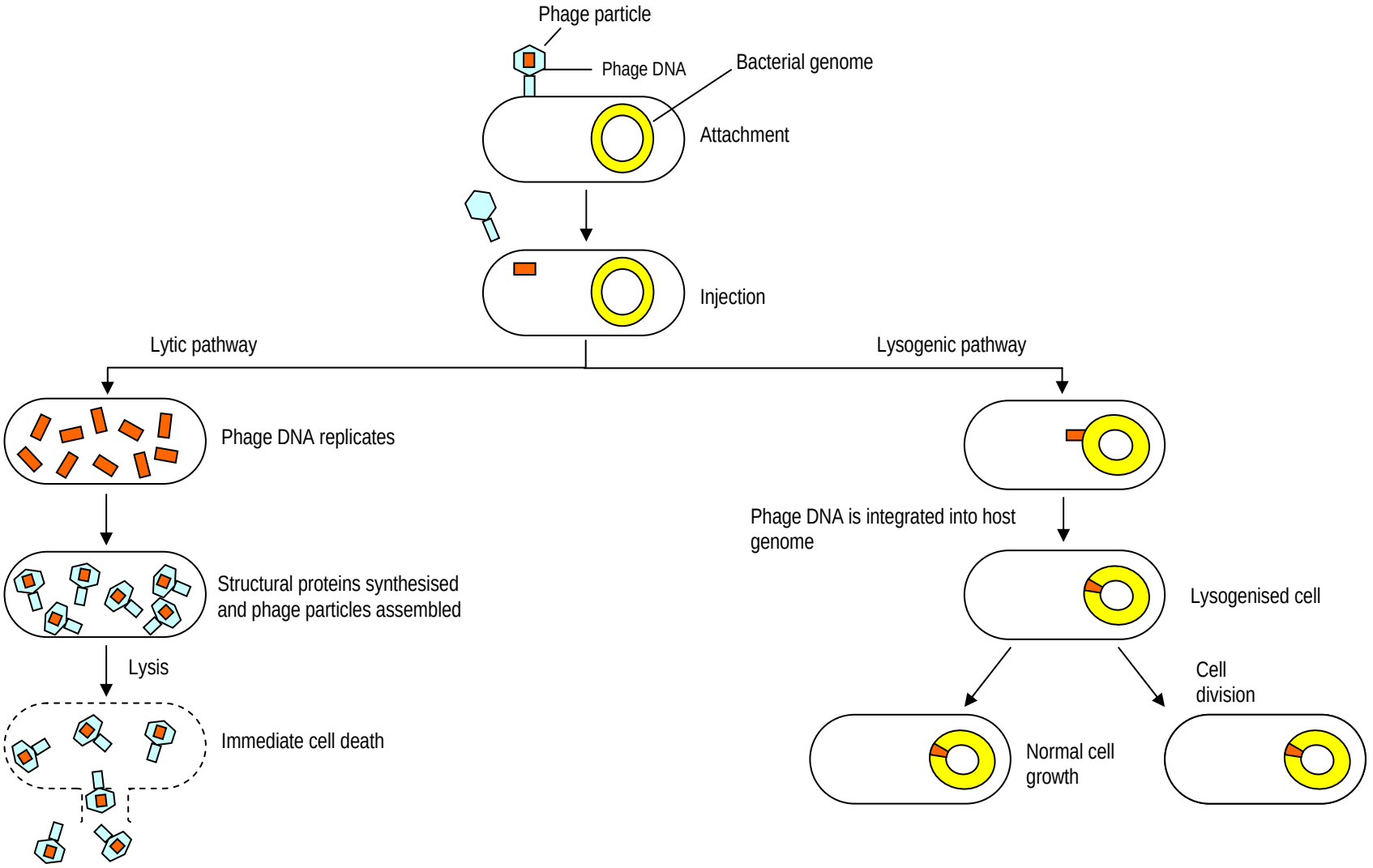
Fig. 1.1 Life cycles of bacteriophages

(Left) Lytic life cycle - Progeny phages are rapidly produced utilising the bacterial biosynthetic machinery. The infected bacterial cells are lysed in the end to release the progeny phage particles

(Right) Lysogenic life cycle - Phage DNA integrates into genome of the infected bacterial cell. Replication of phage DNA occurs along with the bacterial genome during normal cell division.


Phage particle
Phage DNA
Bacterial genome
Attachment
Injection
Lytic pathway
Lysogenic pathway
Phage DNA replicates
Structural proteins synthesised and phage particles assembled
Lysis
Immediate cell death
Phage DNA is integrated into host genome
Lysogenised cell
Cell division
Normal cell growth

Both CI and Cro proteins function as repressors to inhibit lytic and lysogenic cycles respectively (Fig. 1.2). They do so by binding to sites O_R1, O_R2 and O_R3 of the operator O_R that overlaps the promoters P_{RM} and P_R (Ptashne, 2004). The three O_R1, O_R2 and O_R3 sites are composed of non-identical but similar DNA sequences bound by CI or Cro protein with different affinities (Ptashne, 2004). CI protein shows the highest binding affinity to site O_R1 that locates in the promoter P_R and blocks RNA polymerase from binding. The binding of CI protein to site O_R1 immediately increases its affinity to site O_R2 for simultaneous binding. As a consequence, transcription of genes in P_R to the right including *cro* and the lytic genes are repressed (Ptashne, 2004). An additional function of CI protein is as an activator stimulating transcription of *cI* gene from promoter P_{RM} when both O_R1 and O_R2 are bound. As a result, the necessary level of CI proteins can be maintained throughout the lysogenic state (Ptashne, 2004). The second region CI protein binds to during the lysogenic state is the O_L region. This blocks the transcription of an early lytic gene N within the promoter P_L. Therefore no N protein, an anti-termination protein, essential for further lytic gene transcriptions is produced (Little, 2005). Conversely, Cro protein has the highest affinity and binds firstly to site O_R3 locating in the promoter P_{RM} and hence inhibiting production of CI proteins to maintain lysogeny (Ptashne, 2004). Cro proteins can also bind to O_R1 and O_R2 but with ten-fold lower strength (Ptashne, 2004). While the promoter P_R is not repressed, the *cro* and lytic genes within the promoter will be transcribed for lytic growth.

1.3.4 Prophage mediated immunity

During the lysogenic state, repressor CI protein of the integrated phage λ not only blocks transcription of lytic genes; it also protects the host cell from lysis by any incoming phage λ particles. This is due to the binding of CI proteins to the operator sites on the infecting phage particles (Campbell, 2006). The binding between repressor and operator sites is specific in nature. In other words, any phages having a similar *immC* region to phage λ will be prevented from causing lytic infection with the λ lysogens (Campbell, 2006). These phages such as HK97 (Juhala *et al.*, 2000) are said to be homoimmune to phage λ (Fig. 1.3a). In contrast, phages having different *immC* regions to phage λ will not be repressed and are able to induce lysis in a λ lysogen (Campbell, 2006). These phages are said to be heteroimmune to phage λ, such as P22 (Fig. 1.3b).

While most tailed phages utilise the same repression or immunity mechanism as phage λ, some phages such as *Salmonella* phage P22 are known to carry an additional immunity region. In addition to contain a *immC* region that produces repressor C2 protein (equivalent to CI protein of phage λ), *Salmonella* phage P22 also contains a *immI* region that produces another repressor protein Mnt that blocks transcription of an antirepressor gene *ant* in the *immI* region during lysgenic life cycle (Susskind

and Botstein, 1975). As a consequence, no antirepressor protein is present to bind and inhibit the repressor protein C2 and thus lysogenic state is maintained.

Two different situations occur when phage P22 infect lysogens of homoimmune phages with or without *immI* region. When a P22 viron infects a lysogen of a homoimmune phage (e.g. phage ES18) that does not possess an *immI* region, the Mnt repressor is inactive and hence the P22 antirepressor protein is produced to bind with the ES18 repressor protein and cause the phage ES18 to undergo a lytic cycle (Schicklmaier and Schmieger, 1995) (Fig. 1.3c). Conversely, when a P22 virion infects another lysogen of P22, the Mnt repressor protein produced from the lysogenic P22 also inhibits the transcription of the antirepressor gene of the incoming P22 viron and thus superinfection does not occur (Schicklmaier and Schmieger, 1995) (Fig. 1.3d).

Superinfection exclusion (*sie*) genes provide general protection of the integrated phages as well as the lysogens against infection of homo- or hetero- immune phages (Susskind and Botstein, 1978). Unlike gene products from *immC* or *immI* regions, the *sie* gene products are not involved in maintainance of the lysogenic state (Susskind and Botstein, 1978). Phage P22 possess two *sie* exclusion systems: *sieA* gene encodes an inner cell membrane protein that functions to prevent DNA of incoming phages from entering into the cytoplasm of the host cell (Susskind *et al.*, 1974a; Hofer *et al.*, 1995); while the *sieB* gene product aborts lytic development of incoming phages by inhibiting viral DNA, RNA and protein synthesis midway (Susskind *et al.*, 1974b; Ranade and Poteete, 1993).

1.3.5 Phage induction

A number of agents have shown experimentally to cause switching from lysogenic state to lytic state. This process is termed induction or genetic switch (Bradley, 1967). The agents widely used in laboratories for phage induction include ultraviolet light, mitomycin C and hydrogen peroxide, all of which cause damage to DNA (Bradley, 1967). As a result, phage induction occurs, and is thought to ensure survival of the phage genomes as their bacterial hosts are being threatened while the process of DNA replication is being interrupted (Miller and Day, 2008). In the absence of inducing agents, prophage induction also occurs spontaneously in a small fraction of cells (1 in 10^6 lysogenic cells) (Hendrix and Casjens, 2006), and the process is known as spontaneous induction (Little, 2005). It is not yet understood why the low rate of induction occurs spontaneously (Hendrix and Casjens, 2006). At the molecular level, phage induction occurs due to the inactivation of the phage repressor protein. When host DNA is damaged, the RecA protein is activated and cleaves the LexA repressor protein, resulting in the cellular SOS response for DNA repair. Phage repressor proteins including CI protein of phage λ can be recognised by RecA protein and thus are inactivated leading to lytic cycle development (Roberts and Devoret, 1983).

Fig. 1.2 The transcription regulations of the promoters P_{RM} and P_R of bacteriophage λ by CI and Cro (adapted from Ptashne, *et al.*, 2004).

Cro and CI are proteins encoded within the *imm*C region of λ. They are both dimeric repressors that have opposite effects on the transcriptions of genes responsible for lytic and lysogenic growth respectively.

This occurs as a result of their differing affinities for consensus binding sequences within the suboperator sites (O_{R1}, O_{R2} and O_{R3}) of the promoters.

Fig. 1.3 a-d Superinfection and immunity (adapted from Mmolawa, PhD thesis, 2002)

a: Superinfection by a homoimmune bacteriophage. Propagation is prevented because both phages possess the same *immC* region.

b: Superinfection by a heteroimmune bacteriophage. Propagation occurs because both phages possess different *immC* regions.

c: Superinfection by a homoimmune phage expressing an antirepressor. Propagation occurs because the infecting phage produces an antirepressor that inactivates the repressor of the phage.

d: Superinfection by a homoimmune phage with an *immI* region on a lysogen of a phage that also contains an *immI* region and expresses a repressor for the antirepressor gene. Propagation is prevented because both phages possess the same *immC* and *immI* regions.

1.3.6 Impact of lysogeny on bacterial evolution

1.3.6.1 Lysogenic conversion

The relationship between bacteriophages and bacteria is not as simple as parasites and hosts. Many virulence genes carried by temperate phages are expressed upon lysogeny. This process is known as lysogenic conversion (Boyd and Brussow, 2002). For examples, temperate β phage converts non-toxigenic (non-pathogenic) *Corynebacterium diphtheriae* strains into toxigenic (pathogenic) strains (Groman, 1953; Groman and Eaton, 1955). Similarly temperate phages convert non-toxigenic *Clostridium* species into botulinum neurotoxin producers (Eklund *et al.*, 1971). Since the lysogens are provided with proteins that confer a selective advantage, they have a higher chance of multiplication and sustainability in the population (Boyd and Brussow, 2002).

With regard to *Salmonella enterica*, the pathogenesis of infection is greatly dependent on two type III secretion systems (TTSS) and the delivered effector proteins. While the two TTSS are chromosomally encoded and are present in all strains of *Salmonella enterica*, the effector proteins substantially differ between strains due to the independent acquisition of phage-encoded effector proteins (Groisman and Ochman, 1996; Hensel *et al.*, 1997; Figueroa-Bossi *et al.*, 2001). For example, effector protein SopE is carried by phage SopEΦ that is widespread among the epidemic *S.* Typhimurium strains of DT 49, DT 204 and DT 204c, but is rarely found in most of other *S.* Typhimurium phage types (Mirold *et al.*, 1999). As these phage types are known to have caused major outbreaks in the 1970s and 1980s and have persisted over a longer time period than other phage types have been, it was suggested that SopE may be of selective advantage to a host cell (Mirold *et al.*, 1999). Other classes of virulence determinants have also been identified to be encoded by bacteriophages. These include periplasmic Cu/Zn superoxide dismutases SodCI and SodCIII which are encoded by phages Gifsy-2 and Fels-1 respectively to protect *Salmonella* organisms from macrophage oxidative burst during systemic infection (Farrant *et al.*, 1997; Figueroa-Bossi and Bossi, 1999; Figueroa-Bossi *et al.*, 2001). Phages ε34 and P22 contain O-antigen modification genes *rfb* and *gtr* that facilitate lysogens evading host immune response (Wright, 1971; Vander Byl and Kropinski, 2000).

1.3.6.2 Transduction

In addition to lysogenic conversion, phages influence bacterial evolution by mediating horizontal gene transfer between host cells through generalised and specialised transduction (Miller, 1998). Generalised transduction occurs at the late stage of the phage lytic life cycle when phages accidentally package fragmented bacterial chromosomal or plasmid DNA in place of the viral DNA thereby transferring the bacterial DNA to new host cells (Davison, 1999) (Fig.1.4a). Specialised transduction results from inaccurate excision of prophage genome during phage induction. The flanking bacterial

DNA is excised along with the viral genome and integrates into new host cells in subsequent phage infection (Abedon, 2008) (Fig 1.4b).

Previous studies have indicated that wild S*almonella* isolates commonly carry prophages that are released spontaneously and continuously. Among these released phages the majority (>90%) are capable of generalised transduction of chromosomal and plasmid markers (Schicklmaier and Schmieger, 1995; Schicklmaier *et al.*, 1998). Furthermore, transduction of antibiotic resistance genes has been demonstrated in *in vitro* and *in vivo* experiments using phages induced from wild *Salmonella* isolates (Schmieger and Schicklmaier, 1999; Tan, 2010). Taken together these findings suggest that generalised transduction in *Salmonella* is likely to occur frequently due to the abundance of transducers and potential hosts. The data also highlights the importance of phages in mediating spread of antibiotic resistance determinants, and potential virulence determinants for this organism.

1.3.6.3 Impact of lysogeny on bacterial genome diversification

With the availability of multiple genomic sequences of *Salmonella* strains, comparative genomic studies are largely facilitated which provide insight into the processes *Salmonella* can undergo to diversify. A pair-wise genomic comparison of *S.* Typhi Ty2 and CT18 indicated that there is at least 98% genomic similarity between these two strains (Deng *et al.*, 2003). The genomic variations between these two strains are partially due to parts of the seven prophages that are unique to the strains (Deng *et al.*, 2003). Similar findings were also illustrated from the comparative genomic studies on *S.* Typhimurium. A genomic subtractive hybridisation study performed by Hermans *et al.* (2005) indicated that the DNA sequences uniquely present in *S.* Typhimurium DT104 rather than *S.* Typhimurium LT2 are mainly prophage sequences. These sequences were found to be homologous to loci of prophages ST64B and ST104. Furthermore, a microarray study performed by Cooke *et al*. (2007) indicated that prophage sequences contribute in diversification of S. Typhimurium DT 104 phage group. In the study the DT 104 strains generated PFGE profiles different from that of the reference DT104 strain (NCTC 13348) at bands mainly corresponding to prophage sequences (Cooke *et al.*, 2007). In all, these studies indicated that not only prophage elements are abundant in *Salmonella* genomes, their presence also contribute significantly to the genetic diversity of *Salmonella*.

1.3.7 Bacteriophage evolution

It is to be expected that genetic variation between strains of a bacterial species will be increased from prophage integration. This is due to phage genomes evolving principally through horizontal gene exchange with other phages (Brüssow and Desiere, 2001). This proposal of phage evolution was first indicated from a heteroduplex analysis in which the genomes of any two lambdoid phages of *E. coli* showed regions of sequence similarity separated by non-homologous sequences (Simon *et al.*, 1971).

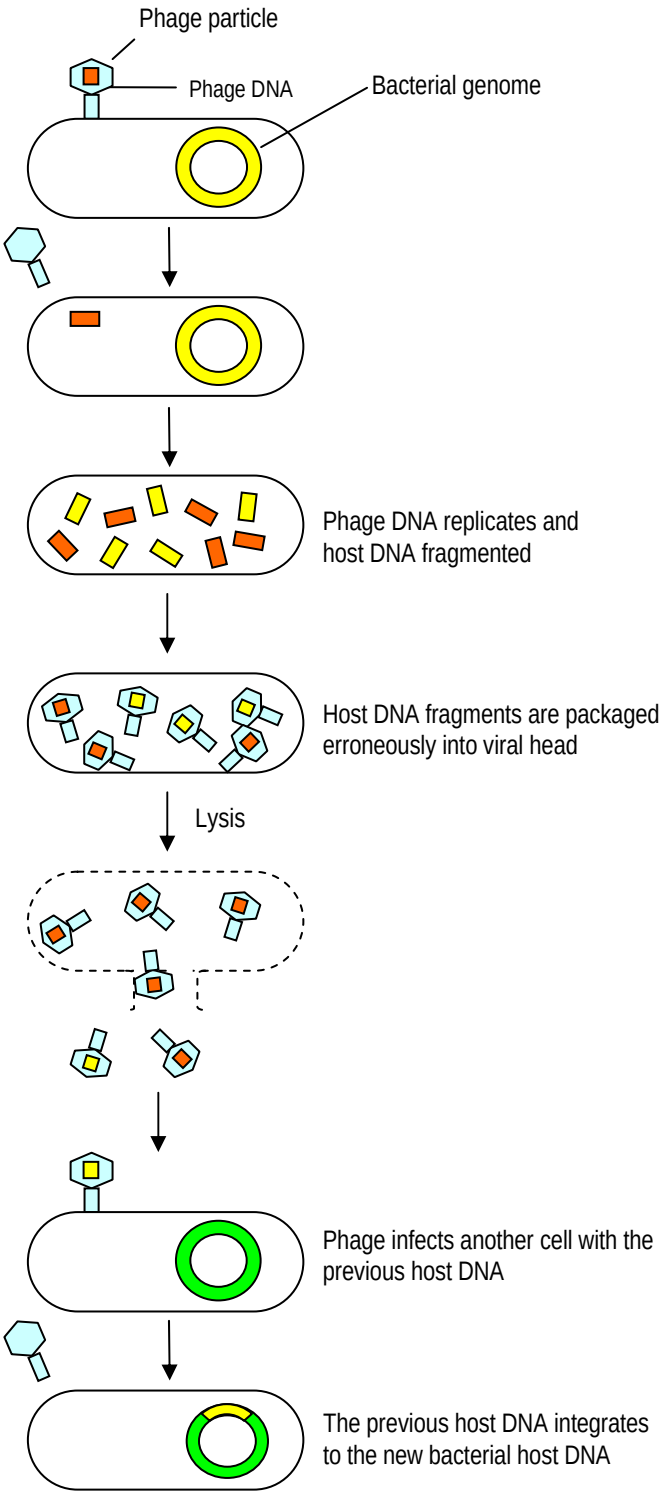


Fig. 1.4a Generalised transduction – Any part of the donor bacterial DNA has the chance to be transferred to recipient bacterial cells

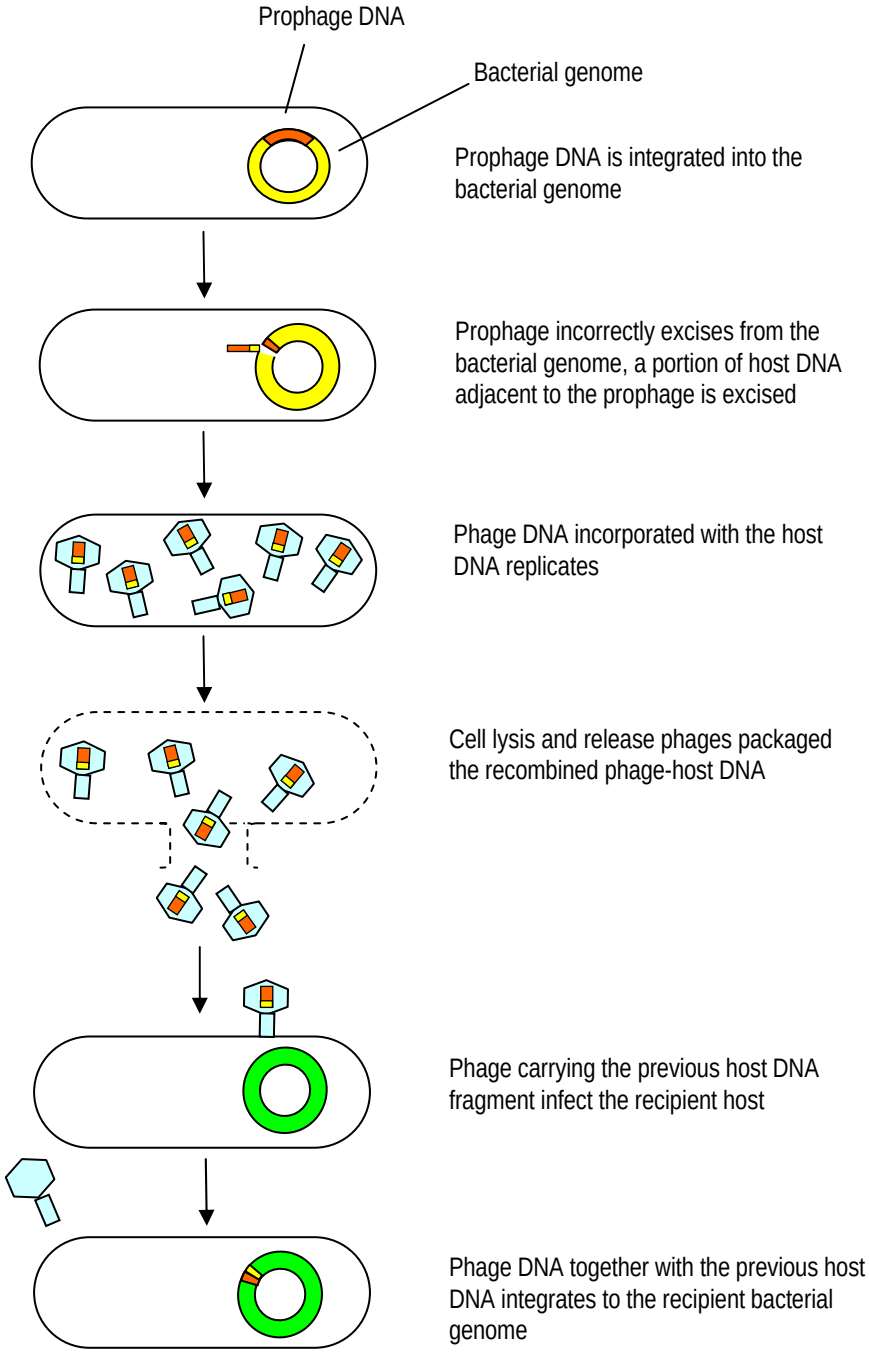


Fig. 1.4b Specialised transduction – only the DNA region of the donor bacterial cells locate adjacent to the prophage DNA has the chance to be transferred to recipient bacterial cells

Similar genetic relationships are also seen among other phage groups including mycobacteriophages and phages of lactic acid bacteria for which multiple phage sequences are available (Ford *et al.*, 1998; Brüssow and Desiere, 2001). This mechanism of phage evolution was first officially termed the modular exchange theory of phage evolution by Susskind and Botstein in 1978 (cited in Hendrix 2002).

Although the mosaic relationships are far more apparent among phages infecting related hosts (e.g. enteric hosts include *E.coli* and *Salmonella*), gene sharing between phages of distinct hosts has also been observed (Hendrix *et al.*, 1999; Juhala *et al.*, 2000; Mmolawa *et al.*, 2003). One well known example is the actinophage ϕC31 infecting *Streptomyces* species which has head proteins similar to those of lambdoid phages HK97 and HK022 (Hendrix *et al.*, 1999). Several other genes also encode proteins similar to those of mycobacteriophages L5, D29 and TM40 (Hendrix *et al.*, 1999). *Salmonella* phage ST64B is the other phage composed of genes from diverse phage groups (Mmolawa *et al.*, 2003). Overall its genome architecture is similar to that of the lambdoid phages, and most of the head genes show similarity to phage HK97 and HK022 (Mmolawa *et al.*, 2003). In contrast, most of the remaining genes encoding proteins showed similarity to that of phages infecting *Lactococcus*, *Pseudomonas*, *Caulobacter*, *Agrobacterium*, and *Streptomyces* (Mmolawa *et al.*, 2003). Taken together these observations suggest that all phage genomes are constructed with genes derived from one large common gene pool, and thus they display high genetic diversity which will be brought onto bacteria upon integration (Hendrix *et al.*, 1999; Hendrix *et al.*, 2003).

1.4 AIM OF THE STUDY

Development and evaluation of new typing approaches have mostly been carried out for the globally significant serovars namely *S.* Typhimurium, *S.* Enteritidis and *S.* Typhi. As discussed earlier, among the recently proposed typing approaches, MLVA and MAPLT have been shown to not only possess high differentiating ability, but also require simple techniques to carry out and provide objective typing data. In this study, the typing capacity of MLVA and MAPLT will be investigated for the *Salmonella* serovars that are of public health significance particularly in Australia including *S.* Virchow, *S.* Bovismorbificans and *S.* Heidelberg. The serovars are among the ten most commonly isolated serovars from humans in the recent years and have been implicated in food-borne outbreaks (ASRC Annual Reports 2000-2009). This study also seeks to further advance the level of strain differentiation through combining the use of MAPLT and MLVA.

The specific objectives involved in this study include:

- Isolation of phages residing in the clinical isolates of serovars under the study
- Identifying the phage loci that can be detected for strain differentiation in each serovar
- Examining the utility of published MLVA loci for differentiation within the serovars under investigation
- Determining the differentiating ability of the developed MAPLT, MLVA, and the combined MAPLT/MLVA through comparing with PFGE
- Genetically characterising phages of which the gene loci are frequently detected to elucidate the potential significances of these phages to *Salmonella*.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 MEDIA

2.1.1 Solid media

Solid media used to cultivate *Salmonella* isolates included Xylose-Lysine-Desoxycholate (XLD) medium (0.3% yeast extract, 0.5% L-lysine HCl, 0.38% xylose, 0.75% lactose, 0.75% sucrose, 0.1% sodium deoxycholate, 0.5% sodium chloride, 0.68% sodium thiosulphate, 0.08% ferric ammonium citrate, 0.008% phenol red, 1.25% agar, pH 7.4) and Columbia horse blood agar (HBA) medium (2.3% special peptone, 0.1% starch, 0.5% sodium chloride, 1% agar, 5% defibrinated horse blood). These media were purchased from Oxoid Australia, Thebarton, South Australia. Selection of the chemically transformed *E.coli* TOP10 cells containing plasmid pCR®2.1®-TOPO® (Invitrogen) with DOP-PCR fragments was done on Luria Bertani (LB) agars (1% trypone, 0.5% yeast, 0.5% sodium chloride, 1% agar) supplemented with 100μg/ml ampicillin, 50μg/ml kanamycin and 40mg/ml X-gal in dimethylformaide. The LB media were prepared by adding 1% No.1 bacteriological agar (Oxoid Australia, Thebarton, South Australia) to LB broth that were purchased from the Media Production Unit (MPU), SA Pathology, Adelaide, South Australia. Bacteriophage propagation, isolation and plaque assay were carried out on *Salmonella* lawns that were grown on LB media. The LB media was prepared by adding No.1 bacteriological agar (Oxoid) to LB broth purchased from MPU to the required concentration. Alternatively, the LB media was prepared by dissolving in distilled water the powdered Difco™ LB broth, Lennox (Becton, Dickinson and Company, Sparks, MD, US) according to the manufacturer's instructions, followed by adding No.1 bacteriological agar (Oxoid) to the required concentration. All inoculated plates were incubated at 37C overnight.

2.1.2 Liquid media

The two liquid media used for general bacterial cultivation were LB broth and BHI broth. LB broth was either purchased from MPU, SA Pathology, Adelaide, South Australia (1% trypone, 0.5% yeast, 0.5% sodium chloride), or prepared by dissolving the powdered Difco™ LB broth base, Lennox (Becton, Dickinson and Company, Sparks, MD, US) in distilled water as per manufacturer's instructions. BHI broth (1.25% calf brain infusion solids, 0.5% brain heart infusion solids, 1% protease peptone, 0.5% sodium chloride, 0.2% glucose, 0.25% disodium phosphate, pH 7.4) was obtained from Oxoid Australia, Thebarton, South Australia. Recovery of the chemically competent *E.coli* TOP10 cells after heat shock treatment used SOC medium (2.4% Oxoid tryptone, 2.4% Oxoid yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose). All cultures were incubated at 37C with shaking in an Orbital Mixer Incubator (Ratek Instrument, Australia). *Salmonella* isolates were stored in Snap freeze

medium (1.7% bacto tryptone, 0.3% bacto soyatone, 0.25% bacto dextrose, 0.5% NaCl, 0.25% K_2HPO_4, 10% glycerol) at -80C. This was purchased from Oxoid Australia, Thebarton, South Australia.

2.2 CHEMICALS AND REAGENTS

The following AnalR grade chemicals were used. Ethylene-diaminetretra-acetic acid (EDTA, disodium salt) was purchased from Biolab, Clayton, Australia. Glycerol and potassium chloride (KCl) were purchased from Ajax Fine Chemicals, Seven Hills, Australia. Boric acid, isopropanol, phenol (saturated with Tris-HCl, pH 8, 1mM EDTA), sodium hydroxide (NaOH), sodium lauroyl sarcosine and Tris (hydroxymethyl) aminomethane were purchased from Sigma-Aldrich, St Louis, MO, US. Chloroform, dimethylformaide, isoamyl- alcohol, gelatin, glucose, magnesium sulphate ($MgSO_4$), sodium acetate, sodium chloride, sodium deoxycholate, ethidium bromide, xylene cyanol and bromophenol blue that were used to make 2x DNA loading buffer were purchased from BDH Chemicals, Poole, England. Ethanol, hydrochloric acid, glacial acetic acid and magnesium chloride ($MgCl_2$) were obtained from Merck Chemicals, (Darmstadt, Germany), while mitomycin C and X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) were purchased from Roche Diagnostics (Mannheim, Germany). Nucleic acid dye GelRed™ in 10,000x concentration was obtained from Biotium, Hayward, CA, US.

Electrophoresis grade reagents were all obtained from BioRad Laboratories, Hercule, CA, US. These included the Certified™ molecular biology agarose powder for electrophoresis of PCR products; the low melt preparative grade agarose powder for preparation of plugs for embedding whole bacterial genomic DNA; the pulsed field certified agarose powder for pulsed-field gel electrophoresis (PFGE).

For PCR, the four deoxyribonucleotide triphosphates (dATP, dTTP, dGTP and dCTP) were purchased from GE Healthcare (Buckinghamshire, UK). The 100bp molecular weight marker used to estimate the sizes of the amplified PCR products were obtained from New England Biolabs (Buckinghamshire, UK).

2.2.1 Antibiotics

Antibiotics were added to either solid or liquid media to select for or to maintain transformants. Final concentration of 100µg/ml ampicillin (Sigma-Aldrich, St Louis, MO) and 50µg/ml kanamycin (Boehringer Mannheim, Germany) was added to LB agar plates.

2.2.2 Oligonucleotide primers

The purified synthetic oligonucleotide primers were purchased from Geneworks (Thebarton, South Australia) and Applied Biosystems (Foster City, CA, US). Primers were resuspended in sterile water to a stock concentration of 100µM that were stored in -20C. Primers used to amplify prophage loci

throughout the study are listed in Table 2.1b that were constructed based on the phage genomic sequences listed in Talbe 2.1a, unless otherwise indicated. Primers amplifying the targeted MLVA loci are listed in Table 2.2 respectively. The remaining primers used in this study were described with the context.

2.2.3 Enzymes

AmpliTaq Gold® DNA polymerase and the associated reagents including 10x buffer II (100mM Tris, 100mM KCl) and 25mM $MgCl_2$ were purchased from Roche Diagnostics, Mannheim, Germany. Restriction endonucleases *Sma*I, *Xba*I and *Hind*III were purchased from New England Biolabs, Buckinghamshire, UK. These enzymes were used with the provided appropriate 10X buffer and 1% BSA (bovine serum albumin). Lysosome, DNase I and RNase A were obtained through Boehringer Mannheim (Mannheim, Germany). Proteinase K was from Merck Chemicals (Darmstadt, Germany) and lysostaphin was from Sigma-Aldrich (St Louis, MO, US). The TA Cloning® Kit and the chemically competent *E.coli* TOP10 cells were bought from Invitrogen, Carlsbad, CA.

2.3 BACTERIAL ISOLATES

All *Salmonella* isolates were obtained from the Australian *Salmonella* Reference centre (ASRC) (SA Pathology, Adelaide, Australia), where the serotypes and the phage types of the *Salmonella* isolates were previously identified. The *Salmonella* isolates used in this thesis are listed in Appendix 1.1-1.3, unless otherwise indicated. All bacterial isolates were maintained in snap freeze medium at -80C. Fresh cultures were prepared by removing a small aliquot of the frozen stock and inoculating onto the solid medium. Plates were incubated overnight at 37C.

2.4 EXTRACTION OF BACTERIAL CHROMOSOMAL DNA

Bacterial chromosomal DNA was extracted using a simple lysis method modified from (Bell *et al.*, 2003). Single colonies of pure *Salmonella* cultures were inoculated into 2ml of BHI broth in a cell culture tray and incubated overnight at 37C with gentle shaking. One ml of each of the broth culture was then centrifuged at 16,000 *g* for 5 min. The cell pellet was resuspended in 400µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and boiled for 20 min to lyse cells and release DNA. After cooling the boiled cell suspension on ice, it was then centrifuged at 16,000g for 7 min to deposit cell debris. The supernatant containing the extracted bacterial DNA was transferred to a fresh tube and stored as DNA stock at -80C, while PCR was carried out using 1 in 50 diluted DNA that was stored at -20C.

2.5 POLYMERASE CHAIN REACTION

2.5.1 PCR cycling parameters

The same PCR cycling condition was applied for amplification of prophage loci and MLVA loci in MAPLT and MLVA respectively. Each reaction mixture contained 1X $MgCl_2$-free PCR buffer, 1.0μM each of the forward and reverse primer, 200μM of each dNTP, 1.5mM $MgCl_2$, 1.0U of AmpliTaq Gold® DNA polymerase, 2.0μl of sample DNA in a total reaction volume of 30.0μl.

In order to decrease non-specific banding, a touchdown PCR program was used (Walker and Rapley, 2000). The touchdown PCR cycling program is shown in Fig. 2.1. All PCR reactions were performed in a Corbett Research PC960G gradient thermal cycler (Sydney, Australia).

2.5.2 PCR amplification of *sucA* gene locus

Prior subjecting the extracted DNA samples to MAPLT or MLVA PCR reactions, PCR for housekeeping gene locus *sucA* was carried out using the PCR cycling condition as described above. This procedure was done to ensure that the extracted DNA is suitable for the PCR protocol and to avoid false negative results. The DNA sequence of the primers were described previously by Ross and Heuzenroeder (2005) and indicated as follow:

sucA forward (sucAF): 5' GCACCGAAGAGAAACGCTG 3'
sucA reverse (sucAR): 5' GGTTGTTGATAACGATACGTAC 3'

2.5.3 Agarose gel electrophoresis of DNA

All PCR amplified products were detected on a 1.5% (w/v) gels prepared in 1X TBE buffer (45mM Tris-borate, 1mM EDTA) (MPU, SA pathology, Adelaide, Australia). Prior to loading onto gels, aliquots of each amplified PCR products was mixed with 2x loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 20% glycerol, 2mM EDTA) in 1:1 ratio. Gels were run in 1X TBE buffer in a Gibco BRL model Horizon® 11.14 horizontal gel electrophoresis apparatus (Life Technologies, Gaithersburg, MD, US). Following electrophoresis, the gels were stained in a solution of approximately 2mg/ml ethidium bromide, or 1x GelRed™ solution and the PCR products were visualised with ultraviolet light on a model TM-36 transmitter (UVP, Inc., CA, US).

The 100bp DNA molecular weight marker was used for size estimation of the PCR products. The DNA marker contains 12 DNA fragments yielded from digestion of several restriction plasmids which are sized as follow (in kilobases, kb): 1.517, 1.200, 1.000, 0.900, 0.800, 0.700, 0.600, 0.500, 0.400, 0.300, 0.200 and 0.100.

2.6 DNA FRAGMENT SIZE ANALYSIS

Length of the amplified MLVA loci was estimated using capillary electrophoresis on an ABI 3730 DNA sequence analyser (Applied Biosystems, Foster City, CA, US) by the Division of Molecular Pathology (SA Pathology, Adelaide, Australia) or Australian Genomic Research Facility (AGRF) (University of Adelaide, Adelaide, Australia). Analysis of the electrophoresis results was performed using Peak Scanner Software version 1.0 (Applied Biosystems, Foster City, CA, US). Peaks on the electrophoregrams representing the MLVA products were identified according to dye colour and size. The actual fragment size was then ascertained by comparing electrophoresis data with those of the sequenced MLVA loci that were also subjected to capillary electrophoresis.

2.7 DNA SEQUENCING

Prior to sequencing, amplified PCR products were purified for sequencing using QIA® quick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA sequencing was undertaken using the BigDye™ Terminator version 3.1 (Applied Biosystems, Foster City, CA, US). Each 20µl sequencing reaction contained 9µl of dH_2O, 3µl of provided 5x sequencing PCR buffer (Tris-HCl pH 9.0 and $MgCl_2$), 2.0µl of BigDye™ Terminators, 2.0µl of 0.4mM forward or reverse primer and 2.0µl of the purified PCR product. The sequencing PCR protocol consisted of 25 cycles of a denaturing step for 30 sec at 96C, an annealing step for 15 sec at 50C and an extension step for 4 min at 60C.

Following amplification, the sequencing product was precipitated using isopropanol precipitation. Initially the sequencing product was added to 80µl of 75% isopropanol and 1µl of glycogen. Each tube was then vortexed vigorously and left at room temperature for one hour before pelleting by centrifugation at 16,000g for 30 min. The supernatant was removed and the DNA pellet was washed twice in 250µl of 75% isopropanol with a step of centrifugation at 16,000g for 5 min. The supernatant after each wash was discarded and in the end the DNA pellet was dried *in vacuo* to remove residual isopropanol.

Sequencing of the amplified product was performed on an ABI 3730 DNA sequence analyser (Applied Biosystems, Foster City, CA, US) by the Division of Molecular Pathology, SA Pathology, Adelaide, South Australia. Analysis of sequence data was performed with Kodon version 3.5 (Applied Maths, Kortijk, Belgium), and tested against sequences in the GeneBank database using BLASTN (Available from: http://ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990).

2.8 PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

2.8.1 Agarose plug preparation

PFGE was performed according to Maslow *et al.* (1993). Pure bacterial cultures were first grown on HBA plates and single colonies were then inoculated into 10ml BHI broths followed by overnight incubation at 37C with gentle shaking. To each of the 2.5ml of *Salmonella* broth cultures, 5ml of cold PIV buffer (10mM Tris, 1M NaCl, pH 8.0) was added and then centrifuged at 8,000 g for 25 min at 4C in a Beckmann JA2-21 centrifuge rotor to ensure integrity of bacterial DNA. For *Staphylococcus aureus* strain NCTC 8325 marker DNA, 5ml of broth culture was added to 5ml of cold PIV buffer following by centrifugation as described. The PIV buffer (supernatant) was then decanted from the cell pellet and 1ml of fresh PIV buffer was added to the cell pellet on ice.

Low melt preparative (LMP) grade agarose (1.3% w/v) was dissolved in PIV buffer by steaming for 30 min. Consequently, 1ml of molten agar was added to each fresh tube in a 50C heating block. The cell pellet was resuspended and decanted completely to the molten agar. Approximately 400µl of the agar was then loaded into a chilled plug mold and kept at 4C for solidification.

2.8.2 Cell lysis

Each agarose plug was soaked in 4ml lysis solution and incubated overnight at 37C with gentle shaking. Lysis buffer for *Salmonella* and *S. aureus* both contained 6mM Tris pH 7.6, 1M NaCl, 100mM EDTA pH7.6, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine, 1mg/ml lysosome, however lysis buffer for *Salmonella* also contained 10 mg/ml of RNAse I and the lysis buffer for *S. aureus* also contained 20 U lysostaphin.

After an overnight incubation, the lysis solution was discarded and replaced with ESP buffer (0.5M EDTA pH 8.0, 10% sodium lauroyl sarcosine and 100 µg/ml proteinase K) twice and then incubated overnight at 50C with gentle shaking.

2.8.3 Restriction endonuclease digestion

A small slice (approximately 1mm thick) of each of the agarose plugs was washed twice in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) for 2 hours followed by 2 more washes for 1 hour each at 37C with gentle shaking. Restriction enzyme mix (100µl) containing 20 U *Sma*I restriction enzyme, 1x restriction enzyme buffer and 100µg/ml of bovine serum albumin was then added to each agarose plug and incubated overnight. Agarose-embedded DNA of all *Salmonella* isolates were digested with *Xba*I at 37C, while *S. aureus* NCTC 8325 marker DNA was digested with *Sma*I at 30C.

2.8.4 Pulsed-field gel electrophoresis

Pulsed-field gels (1% w/v) were prepared by dissolving pulsed field certified agarose (BioRad laboratories, Hercules, CA, US) in 0.5x TBE (45mM Tris-borate, 1mM EDTA) and soaked overnight at 4C in the same buffer to improve performance of the gels. The restriction enzyme mix was discarded and the agarose plugs were loaded into the wells of the gels. Agarose plugs containing the reference organisms were loaded evenly throughout the gel. Melted low melt preparative grade agarose (1% w/v) prepared in 0.5x TBE (45mM Tris-borate, 1mM EDTA) was then pipetted onto each well and allowed to solidify before running the gels.

Electrophoresis was performed on CHEF (Clamped Homogeneous Electric Field) - DRIII apparatus from BioRad Laboratories according to the manufacturer's instructions in recirculating 0.5x TBE buffer cooled to 14C. The electrophoresis was set with initial pulse times with 1 second, final pulse times with 50 sec, included angle at 120^{o}, voltage at 6 V/cm for 21 hours.

2.8.5 Visualisation

Pulsed-field gels were stained with ethidium bromide solution (0.5μg/ml in water) for 30 min and visualised under UV illumination on a Model TM-36 transilluminator (UVP, Inc., San Gabriel, CA, US) and photographed.

2.9 TYPING DATA ANALYSIS

Data generated from MAPLT, MLVA and PFGE were analysed using BioNumerics version 4.6.1 software (Applied Maths, Kortrijk, Belgium). Dendrograms were constructed according to the typing data of each of the methods to display the relative genetic relationship between isolates. For MAPLT, the typing data were entered into the software as "1" or "0" to indicate the presence or absence of the amplified phage loci. Dendrograms were constructed using the multi-state coefficient with zero tolerance and clustering by UPGMA (Drahovská *et al.*, 2007).

Typing data of MLVA were entered into BioNumerics as numerical values indicating fragment lengths of the amplified MLVA loci to construct dendrograms as described above. When an amplified MLVA locus was not detected in a test isolate, a value of zero was entered. As the annealing temperature of several published MLVA primers are not optimal for the PCR cycling condition used in this study; alternative primer pairs were designed and used. Table 2.3 lists the formula deducing the expected fragment lengths that would be generated from the published primers based on the MLVA loci amplified in this study. The number of repeat units in each amplified MLVA locus was determined based on the size of the amplified MLVA loci and the size of the franking regions using the formula in Table 2.2.

Dendrograms based on the composite data set from MAPLT and MLVA were constructed using the averaging experiment-related similarity matrices and UPGMA clustering. For PFGE, gel images were normalised following by band assignments to each of the restriction patterns. Dendrograms showing degree of similarity between banding patterns were generated using the Dice coefficient and unweighted-pair group method using arithmetic averages (UPGMA) clustering.

Comparison of the discriminatory power between each of the typing methods was undertaken using Simpson's index of diversity (DI) that indicates the probability of a typing method in differentiating two unrelated bacterial isolates from the test population (Hunter and Gaston, 1988). The 95% confidence interval of surrounding each DI value was calculated as described by Grundmann *et al.,* (2001). The Simpson's indexes and the corresponding 95% confidence intervals were calculated using online tool available at http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool.

Fig. 2.1 Touchdown PCR program

Initial denaturation	94C	10 min	
Denaturation	94C	30 sec	1 cycle
Annealing	59C	30 sec	
Extension	72C	1 min	
Denaturation	94C	30 sec	1 cycle
Annealing	58C	30 sec	
Extension	72C	1 min	
Denaturation	94C	30 sec	1 cycle
Annealing	57C	30 sec	
Extension	72C	1 min	
Denaturation	94C	30 sec	3 cycles
Annealing	56C	30 sec	
Extension	72C	1 min	
Denaturation	94C	30 sec	5 cycles
Annealing	55C	30 sec	
Extension	72C	1 min	
Denaturation	94C	30 sec	9 cycles
Annealing	54C	30 sec	
Extension	72C	1 min	
Denaturation	94C	30 sec	10 cycles
Annealing	53C	30 sec	
Extension	72C	1 min	
Denaturation	94C	30 sec	10 cycles
Annealing	52C	30 sec	
Extension	72C	1 min	
Final extension	72C	5 min	
Store	4C	∞	

Table 2.1a Table of genomic sequences applied to construct primers amplifying the prophage loci in Table 2.1b. All genomic sequences can be found in the NCBI database using the stated accession numbers.

Genomic sequences	Genbank accession number	Genomic sequences	Genbank accession number
P22	AF217253	ST64T	AY052766
ST64B	AY055382	186	U32222.1
Fels-2	AE006468 (from 2844427 to 2879233)	Gifsy-1	AE006468 (from 2728552 to 2777042)
Gifsy-2	AE006468 (from 1098187 to 1144026)	ES18	AY736146
SfV	U82619.2	P1	AF035607
P7	AF503408	*S.* Newport str 254	CP001113
sopE locus	AF043239		

Table 2.1b Table of primers amplifying the prophage loci in chapters 3 to 5

Phages	Gene loci	Gene functions	Primers (5'→ 3')	Location in genomes	Fragment sizes (bp)
P22	*int*	Integrase	PintF: CATTTCCTGCAATACCGAAATCGG	3257-3717	461
			PintR: GCTGGCTTGAGCCTCACG		
	*sie*B	Superinfection exclusion	sieBF: CGATGAACAACTCATGGTGGC	11507-12117	611
		protein	sieBR: AGCGAGGTAAGGTATTTGTCG		
	*nin*B	NinB protein	ninBF1: AACCTTTGAAATTCGATCTCCAGC	16512-16891	380
			ninBR1: CTTCGTCTGACCACTTAACGC		
ST64T/P22	*gtr*C	O-antigen conversion	gtrCF: CTACTACTCGCTATTCTTTGCGC	152-689	538
			gtrCR: CATTAACACCTCTGACCACATCC		
	*g*17	Put. superinfection	PT17F: GGCTGTTGTTTCTTCTTTCAGGC	10609-10921	313
		exclusion protein	PT17R AGGAAATATGAAATTACGTGTCTGGC		
	*c*1	CI protein	PTc1F: CTTTACCAATCTGAACCGCCG	13901-14332	432
			PTc1R: CTGAGTTGTTTTGGCATAATTACTCC		
	c2	CII protein	PTc2F: GGAATTGTTAGAGGCCTTGCC	12886-13287	402
			PTc2R: GATTTCCCCTGATTAGCTGGG		
ST64B	SB04	Portal protein	SB04F: TGTCATACGACACCTATACCG	2513-3298	786
			SB04R: TGTTCTGCACCATGTGCAATG		
	SB06	Major capsid protein	SB06F1: ACGACAAGCGCGTTGAGGC	4394-4943	550
			SB06R1: GCTTCCACGTTGAAGAAGGC		
	SB21	Put. head assembly	SB21F1: CTGTATGGTTATATCGATTATCTGG	15411-15888	478
		protein	SB21R1: GATTTCCTTTGCCCAGATGACG		

Phages	Gene loci	Gene functions	Primers (5'→ 3')	Location in genomes	Fragment sizes (bp)
ST64B	SB28	Integrase	SB28F1: TGCAGTCAAGAGGACGTCC	21366-21994	629
			SB28R1: TGCCGATATGCTGATCTGGC		
	SB38	CI protein	BIM1F: ATGGTGGCCTTGTCGACGC	28028-28502	474
			BIM1R: GCTAACGTGAAGGATTTGTTCCG		
186	*gene P*	Endolysin	P186PF: TCACCGATTACAGCGACCAC	7877-8200	324
			P186PR: TGGTGACCAGCTTTTCGAGAC		
	gene O	Baseplate protein	P18BPF: GTGCGATTCGTAACCTCATCC	9763-10215	453
			P18BPR: GTTTGTTGGTGCATACCACCT		
	gene G	Tail protein	P186GF: GACGTTCTCTCGATGACGATG	17385-18198	814
			P186GR: CAATGGCACTACCGGTAACAG		
	cII	CII protein	P186cIIF: GACATAGCGGGATTAGTCTGC	23499-23892	394
			P186cIIR: GTCACAACATGGCGAAGCTG		
Fels-2	STM2695	Putative late control	Fels2lateF: GCCGGATAAACAGCAGGTTC	2845247-2845744	497
		protein D	Fels2lateR: AAGACGTGACGACGGTGATG		
	STM2697	Tail protein	Fels2GF: CTTCATTGAGCGCAAGACGC	2848716-2849082	366
			Fels2GR: CGTCTGCAGGTGATTCTTAATGC		
	STM2714	Lysis regulatory protein	Fels2lysBF: TGACCTTTCCAGACGGCACT	2859042-2859437	395
			Fels2lysBR: TGGTTCTGGCGCTGGTACTT		
	STM2719	Small terminase subunit	Fels2TerF: TGTCATTGCGCAGTCTGCG	2861311-2861864	553
			Fels2TerR: CTATGACCTGATGCTGCTGC		

Phages	Gene loci	Gene functions	Primers (5'→ 3')	Location in genomes	Fragment sizes (bp)
Fels-2	STM2723	Portal protein	Fels2portF: ATCACAGCTCACCGATAGCAG	2866018-2866646	628
			Fels2portR: CATCATGTCATCACGGCTTACG		
	STM2736	CII protein	Fels2cIIF: TGTATGGAAACGGCAGCCAG	2875360-2875723	363
			Fels2cIIR: GTCACAACATGGCGAAGCTG		
	STM2738	CI protein	Fels2cIF: TAAGGCTTACAATTTCAGCTCACG	2876238-2876726	488
			Fels2cIR: ACTACAATTTTGTTGCCTGGAACG		
	STM2739	Integrase	Fels2intF: AGATGGCGTGCTTATGTCGC	2877177-2877771	594
			Fels2intR: GTGAGCGTAAACCATCGTCA		
Gifsy-1	STM2584	Type III secretive protein	Gifsy1gogBF: ACTGCTCATCATGTTACCTCTATTC	2729177-2729779	603
		(GogB)	Gifsy1gogBR: GCAGGTTGGTATTTCCCATGC		
	STM2608	Terminase large subunit	Gifsy1AF: GATCACGCATCCATTATGTTCAC	2756675-2757449	775
			Gifsy1AR: TATTCCCGTACCGCTTACCAC		
Gifsy-2	STM1005	Integrase	Gifsy2intF: CGCTCTTCTCTGACATCCAC	1098308-1098911	604
			Gifsy2intR: ACGACAGACGAGTTCATCAGG		
	STM1032	Putative capsid protein	Gifsy2CapF: GTGACGCAATGGTACAGGATG	1120027-1120674	648
			Gifsy2CapR: TGTCTTCACTTCCGGCATTATC		
	STM1048	Host specificity protein J	Gifsy2JF: CGTAAGGCGTATGACGTTCTG	1133788-1134393	606
			Gifsy2JR: CTGCGACTGAATCTCCGTACT		

Phages	Gene loci	Gene functions	Primers (5'→ 3')	Location in genomes	Fragment sizes (bp)
Gifsy-1/2	STM2619/ STM1021	Unknown (NinG)	GifsyninGF: ACACTGAAGATGGATGTTGAAGC	^2763906-2764306	401
			GifsyninGR: GCCGTAAGTGCGCAAACAAAG		
	STM2632/ STM1008	Exodeoxyribonuclease	GifsyRecF: CTAAAGACAATATGACCTGGACG	^2771241-2771795	555
			GifsyRecR: GAATAGCCGATACACGGTTGC		
	STM2594/ STM1041	Tail protein	GifsyHF: TACAGCAGCATCGGCATACTG	^2744517-2745177	661
			GifsyHR: GCAGGCAATGGCAATGGTATC		
SopEϕ	*sopE*	Type III secretion protein	SopEF: CGAGTAAAGACCCCGCATAC	1622-1984	363
		(SopE)	SopER: GAGTCGGCATAGCACACTCA		
ES18	*gene 9*	putative coat protein	ES18PCPF: TGGAACGCACAGCATGATGC	6368-6853	486
			ES18PCPR: GGACTGCACCTGAATATTCGG		
SfV	*orf5*	Putative capsid protein	SfV05F: TCCTGGCGAAAGTTGTTGAG	4688-5342	655
			SfV05R: ATCGCACACGACGGATAATG		
	orf11	Tail sheath protein	SfV11F: CGAATACTGCACAGGACAGC	7587-8255	669
			SfV11R: CGCTGGTATCGTTCATCTCG		
	orf16	Putative tail protein	SfV16F: ACGATCGCTGGTACAGATTGC	13327-14019	693
			SfV16R: TCGGGTTCAGGCAGATAAGC		
	orf26	Integrase	SfV26F: ATGCAGTATCATTCAGCGCAAC	21050-21802	753
			SfV26R: GCAGAGGTGAAATATGGTACG		
	orf34	CI protein	SfV34F: ACGCTTTGAGATAACGCTGTG	25793-26251	459
			SfV34R: GCAGGTTTATGGGAGATGGTG		

^ Locations of amplified sequences in phage Gifsy-1 genomes

Phages	Gene loci	Gene functions	Primers (5'→ 3')	Location in genomes	Fragment sizes (bp)
P1	*orf47*	Structural lytic transglycosylase	P1sitF: TGACCTTGATCGCGTACTCAC	2380-3079	700
			P1sitR: GAATCCGATGCCAAACAGAG		
P7	*sit*	Putative structural	P7sitF: TGACCTTGATCGCGTACTCAC	44668-45368	701
		injection transglyosylase	P7sitR: TAGCCACCAGGAGACATCTG		
Prophage in *S.*	A2929	Tail sheath	SL254tailF: AGGCGGATTACCTGAAACGTC	2861996-2862590	595
Newport SL254			SL254tailR: ATATCCACCGCCTTCTTGCTC		
Prophage	*DOP13.7	Possible tail assembly	DOP13.7F: CGGTTAGCTCCGTGGTTAAG	Not applicable	442
from V16		protein	DOP13.7R: GCAGCGTTGTTGAGTTACCAG		
Prophage	*M13-222	unknown	M13-222F: TGAACGCCGACGCTAGTAAG	Not applicable	364
from B33			M13-222R: TGATTGCCGGGGTATTTAGC		

Primers amplifying gene loci of phages P22, ST64T, ST64B were previously described by Ross and Heuzenroeder (2005). Primers amplifying locus *sopE* was described by Drahovská *et al.,* (2007). *Primers amplifying loci DOP13.7 and M13-222 were constructed based on the DOP-PCR amplified prophage loci from isolate *S.* Virchow V16 and *S.* Bovismorbificans B33 respectively.

Table 2.2 Table of primer sequences amplifying the MLVA loci in the study and the formulae used to calculate the number of repeats from the amplified fragment sizes for all the MLVA loci except STTR-3. Locus STTR-3 is known to contain tandem repeats of two different lengths that are 27 bp and 33 bp in size (Lindstedt *et al.*, 2003). These two types of tandem repeats can occur variably. Therefore the amplified STTR-3 loci with different sizes were subjected to nucleotide sequencing to determine the number of tandem repeats.

Loci	Primer sequences	Repeat unit (bp)	Size of flanking region (bp)	No. of tandem repeats
STTR-2	STTR2F: GTTCCCTTCCAGATTACGG	60	134	(X-134)/60
	STTR2R: CAGGTCTTACCACCTTGCC			
STTR-3	STTR3F: CGTTGAAAATAACGGTGGC	27 or 33	129	Not applicable
	STTR3R: CCTTTATCGATGGTGACGC			
STTR-5	STTR5F: GCTGCAGTTAATTTCTGCG	6	284	(X-284)/6
	STTR5R: TCAGTAAAACGGTGATCGC			
STTR-6	STTR6F: CAGATTTTTCACCATCTGCGC	6	345	(X-345)/6
	STTR6R: AGTTGCTTCAGGATATCTGGC			
STTR-7	STTR7F: GCAGCCGTTCTCACTGG	39	261	(X-261)/39
	STTR7R: TCTACCGGTTCAACTTCGC			
STTR-9	STTR9F: ATGATCGACCACGATCTTGCC	9	217	(X-217)/9
	STTR9R: CAAACGACCGCTATTCGTCG			
STTR-10	STTR10F: CCATTCCTGATGCATTCTGCC	6	135	(X-135)/6
	STTR10R: CTGTCAGGGAATATCAGCAGC			
Sal02	Sal02F: CGTCAGACAGCCCATGATAC	6	435	(X-435)/6
	Sal02R: ATTGGCCTGGTGCTGCTTAG			

Loci	Primer sequences	Repeat unit (bp)	Size of flanking region (bp)	No. of tandem repeats
Sal04	Sal04F2: CGCCAGTTTATCTGGAAACC	20	344	(X-344)/20
	Sal04R2: CCGGCTTGTTGTTTGTGAAC			
Sal10	Sal10F: AGTGGCAGCGCGTTATTGC	12	640	(X-640)/12
	Sal10R: TTCGTGAAAACGGCGTACC			
Sal15	Sal15F: CAGTTATTGGCGTACCGGATG	12	545	(X-545)/12
	Sal15R: TGCACGGTTCTTACGTCACTG			
Sal20	Sal20F2: AGCAGCCGACACAACTTAACG	3	334	(X-334)/3
	Sal20R2: ACCATCCAGCGACGTTCATC			
Sal23	Sal23F: CCCGCACACTAAGGAGAGAC	12	214	(X-214)/12
	Sal23R: ACCGCGTTAGTGGCTAACAT			
TR1	TR1F: CTCACCAGCTTACGTTGCG	7	329	(X-329)/7
	TR1R: TTGCCATGACATGTGTTTAGCC			
2628542	2628542F: CTGCCATCGGCATTACGATAC	36	301	(X-301)/36
	2628542R: ATGGAGCACAGACCACTAACG			
SE01	SE01F: AGACGTGGCAAGGAACAGTAG	7	233	(X-233)/7
	SE01R: CCAGCCATCCATACCAAGAC			
SE02	SE02F: CTTCGGATTATACCTGGATTG	7	168	(X-168)/7
	SE02R: TGGACGGAGGCGATAG			

Loci	Primer sequences	Repeat unit (bp)	Size of flanking region (bp)	No. of tandem repeats
SHTR-1	SHTR-1F: CTGAGCCTGTAAAACGGATGG	7	444	(X-444)/7
	SHTR-1R: GCTTTCCAGGCAAGAGAGTC			
SHTR-2	SHTR-2F: AGTGACAACCTTTGCCAGTGC	5	242	(X-242)/5
	SHTR-2R: TCCTGGGTATCAATGGTGTCC			
SHTR-3	SHTR-3F: CACCCATGCAACAGAACGAG	10	333	(X-333)/10
	SHTR-3R: CAAGCACTGGCAAAGGTTGTC			
SHTR-4	SHTR-4F: CCGCTTAATCCTGAACTCCTC	9	392	(X-410)/9
	SHTR-4R: TCCTTTGTGGTCTACGCGTTC			
SHTR-5	SHTR-5F: CTGGCATACGCAAAAACAGC	9	400	(X-400)/9
	SHTR-5R: CGGGAATCGTATTCGGTCTCT			
SHTR-6	SHTR-6F: GTGTTCCCGAATCTCATCTGC	9	442	(X-442)/9
	SHTR-6R: TGGCTGGCTCAGGTTAAGAG			
SHTR-7	SHTR-7F: ACGGTCTTAAAGCCGGAACAC	18	347	(X-347)/18
	SHTR-7R: GCGCTCAATCACTTTCACCAC			
SHTR-8	SHTR-8F: TCGCAGAAGCGAAAAGAAGG	15	292	(X-292)/15
	SHTR-8R: TCTAAGCCTTTCCTCGTCCAAG			
SHTR-9	SHTR-9F: TGAGAACGCTGTGTACCAACC	15	319	(X-319)/15
	SHTR-9R: CAGATGTGCGTGTTTCTGGTC			

Table 2.3 Table of formulae converting the sizes of the amplified MLVA loci in this study to the fragment sizes expected from the priming sites as referenced below.

MLVA Loci	Conversion to published amplified fragment length	References
STTR-2	FL-23	Lindstedt *et al.,* (2003)
STTR-3	FL-56	Lindstedt *et al.,* (2003)
STTR-5	FL-103	Lindstedt *et al.,* (2003)
STTR-6	FL-81	Lindstedt *et al.,* (2003)
STTR-7	FL+21	Lindstedt *et al.,* (2003)
STTR-9	FL-82	Lindstedt *et al.,* (2004)
STTR-10	FL+176	Lindstedt *et al.,* (2004)
Sal02	FL-340	Ramisse *et al.,* (2004)
Sal04	FL-190	Ramisse *et al.,* (2004)
Sal10	FL-469	Ramisse *et al.,* (2004)
Sal15	FL-368	Ramisse *et al.,* (2004)
Sal20	FL-189	Ramisse *et al.,* (2004)
Sal23	Published primer sequences used	Ramisse *et al.,* (2004)
TR1	FL-152	Liu *et al.,* (2003)
SE01	Published primer sequences used	Boxrud *et al.,* (2007)
SE02	Published primer sequences used	Boxrud *et al.,* (2007)
2628542	FL-82	Witonski *et al.,* (2006)

FL = Amplified fragment length of the MLVA loci

CHAPTER 3 MOLECULAR TYPING OF *SALMONELLA* VIRCHOW

3.1 INTRODUCTION

Non-typhoidal salmonellosis frequently manifests as an acute, self-limiting gastroenteritis that is among the most common food-borne infection throughout the world. The causative agents are usually serovars of *Salmonella enterica* subspecies I with *Salmonella* Typhimurium and *Salmonella* Enteritidis implicated in the majority of food-borne *Salmonella* infections worldwide including Australia. In contrast, *Salmonella* serovar Virchow is a less common serovar worldwide but is prevalent in certain geographic regions. Recently *S.* Virchow was reported among the ten most common serovars isolated from humans in Asia, Europe and the Oceania regions between 2001 and 2007 (Hendriksen *et al.*, 2011). In Australia, *S.* Virchow has always been endemic, particularly in the Australian state of Queensland and has ranked among the 10 most common serovars from the human sources from as early as 1991 (ASRC Annual Reports 1991-2009). However between 1996 and 2006, the isolation rate of *S.* Virchow declined from 7.2% to 0.02%, and it was no longer ranked in the top ten of disease causing serovars from 2003 to 2006 (ASRC Annual Reports 1996-2006). However in 2007, *S.* Virchow reappeared as one of the most common serovars isolated from humans and its relative isolation rate increased from 2.7% in 2007 to 5.4% in 2009 (ASRC Annual Reports 2007-2009). In the European region, *S.* Virchow has caused noticeably higher numbers of human salmonellosis cases in England and Wales since 1977 (Chamber *et al.*, 1987). During the period of 1981 to 1990 *S.* Virchow was the third most common serovar isolated from humans at 6% (Torre *et al.*, 1993). *S.* Virchow has emerged recently in other countries such as Israel where it has been ranked as the second most commonly isolated serovar from humans since 2000 and accounted for 16 to 20% of all human non-typhoidal salmonellosis (Weinberger *et al.*, 2006).

As with most non-typhoidal *Salmonella* serovars, *S.* Virchow is a ubiquitous organism that can be detected in various food animals and environmental sources such as chickens, pigs, horses and sewage sludge (ASRC Annual Reports 1986-2009). However poultry and related products were reported to be the most prevalent reservoir in endemic countries. Recently (2000-2009), almost all Australian *S.* Virchow isolates received by the Australian *Salmonella* Reference Centre (ASRC) were from poultry and eggs (ASRC Annual Reports 2000-2009). Likewise *S.* Virchow is routinely associated with chickens and their products and its control is deemed important in the United Kingdom (Willcocks *et al.*, 1996; Threlfall, 2002).

Globally, most reported *S.* Virchow outbreaks were poultry-associated; this is expected as poultry are the main reservoir of this serovar (Semple *et al.*, 1968; Maguire *et al.*, 2000; Adak and Threlfall, 2005). Other food sources are also implicated in *S.* Virchow outbreaks such as sun-dried tomatoes and processed milk products (Usera *et al.*, 1998; Taormina *et al.*, 1999; Bennett *et al.*, 2003). Severe clinical outcomes have been reported from *S.* Virchow outbreaks where infants developed meningitis and bacteraemia through the consumption of contaminated powdered infant formula (Ruiz *et al.*, 1995; Usera *et al.*, 1998). Systemic *S.* Virchow infections in young children have also been reported in Australia and the United Kingdom (Messer *et al.*, 1997; Ispahani and Slack, 2000). A recent study from Israel showed that *S.* Virchow exhibits a higher invasiveness in children less than 2 years old and causes more life-threatening extra-intestinal infections than other common *Salmonella* serovars including *S.* Typhimurium and *S.* Enteritidis (Weinberger *et al.*, 2004). Therefore it is necessary to establish typing methods for both routine surveillance and outbreak investigations of *S.* Virchow infections.

Bacteriophage (phage) typing is one widely used phenotypic method for differentiation of clinically significant *Salmonella* serovars such as *S.* Virchow. The current international phage typing scheme for *S.* Virchow was developed in 1987 and comprises 13 typing phages (Chamber *et al.*, 1987). Fifty-seven lysis patterns (phage types) have been identified (Torre *et al.*, 1993). Phage types (PTs) 8 and 26 are the most predominant phage types in UK, representing 50% of the UK isolates (Torre *et al.*, 1993). Australia and Spain are two countries routinely applying phage typing as well. In Spain, the most frequent *S.* Virchow phage types detected were PTs 8, 19, and 31 in years 1990-1996 (Martín *et al.*, 2001), whereas in Australia the most commonly detected phage types were PTs 8, 31 and 34 during a similar time period (ASRC Annual Reports 1986-1996). These results demonstrate the role phage typing plays in global surveillance of the *S.* Virchow population. It is possible that PT 8 may be a global phage type predominating in endemic countries, while PT 26 and PT 34 were considered geographically specific to the UK and Australia respectively (Sullivan *et al.*, 1998). In addition, phage typing has indicated changes in *S.* Virchow populations within a particular source. With respect to *S.* Virchow in Australia, no significant change of the incidence of predominant phage types from human sources was observed in recent years (2000-2009) where PT 8 is the most prevalent phage type (>50%) in most years (ASRC Annual Reports 2000-2009). In contrast, there were noticeable changes in the *S.* Virchow phage type populations in chickens and eggs, based upon the *S.* Virchow isolates received by ASRC. Even though PT 8 was the most common phage type from chickens and eggs during 2000-2009, the proportion of PT 8 has decreased from 81.9% of the total *S.* Virchow derived from these sources in 2000 to 35.5% in 2009 and is no longer as predominant (ASRC Annual Reports 2000-2009). This observation suggests that non-poultry sources such as fresh produce could be sources of infections and outbreaks.

A number of *S.* Virchow food-borne outbreaks have been reported that were caused by the predominating phage types PTs 8 and 26. In these instances, an additional typing method with further differentiating ability is required to identify the outbreak-related isolates from sporadic ones as phage typing alone may not support the link between illness and source as suggested by epidemiological information. A number of molecular methods have been applied including ribotyping and plasmid profiling (Usera *et al.*, 1998; Maguire *et al.*, 2000; Martín *et al.*, 2001). Currently, pulsed-field gel electrophoresis (PFGE) is the 'gold standard' method for *S.* Virchow as with other *Salmonella* serovars. As demonstrated by Bennett *et al.* (2003), PFGE demonstrated an epidemiological link between the affected people and the food source (semi-dried tomatoes) by showing that all the PT 8 isolates had an undistinguishable PFGE pattern. PFGE has also been used together with plasmid profiling and has shown that cases of antibiotic-resistant PT 8 infection in England between September 2004 and February 2005 were due to the consumption of imported frozen chicken from a single supplier as well as identifying an outbreak in Northern Ireland in September 2004 (Adak and Threlfall, 2005).

In a separate outbreak incident in Spain, PFGE was used in accordance with the interpretation guideline developed by Tenover *et al.* (1995) to demonstrate the probable close genetic relationships (less than 3 band differences) between the outbreak *S.* Virchow isolates from the patients and the contaminated infant formula that were otherwise separated by phage typing into phage types 4, 4a and 2 (Usera *et al.*, 1998). However applying PFGE and the interpretation guideline as above was ineffective in investigating *S.* Virchow outbreaks in Israel since the Israeli *S.* Virchow isolates produced PFGE band patterns differing by one to three bands only (Weinberger *et al.*, 2006). Likewise, inadequate discrimination by PFGE was also observed for the multi-resistant *S.* Typhimurium DT 104 and *S.* Enteritidis PT 4 where there was only a small number of PFGE profiles generated from isolates of these phage types in the endemic countries (Malorny *et al.*, 2001; Liebana *et al.*, 2002; Lindstedt *et al.*, 2003; Lukinmaa *et al.*, 2006; Beranek *et al.*, 2009). All these findings have necessitated the development of alternative high-resolution typing methods that are able to provide robust and objective strain differentiation.

Multiple-locus variable-number tandem repeat analysis (MLVA) is one new typing methodology that is being increasingly applied for epidemiological typing of *Salmonella*. The discriminatory power of MLVA has shown to be superior to PFGE for the homogeneous phage type groups including multi-resistant *S.* Typhimurium DT 104 and *S.* Enteritidis PT 4 as mentioned previously (Lindstedt *et al.*, 2003; Malorny *et al.*, 2008; Beranek *et al.*, 2009). Multiple amplification of prophage locus typing (MAPLT) is another PCR-based typing approach offering high level of intra-serovar differentiation. For the *Salmonella* serovars that have been subjected to MAPLT including *S.* Typhimurium, *S.* Enteritidis and *S.* Infantis,

improved strain separation was often demonstrated in comparison to PFGE (Ross and Heuzenroeder, 2005a, 2008, 2009).

This chapter describes the development of MAPLT, MLVA, and a composite MAPLT / MLVA assay for *S.* Virchow. The differentiating ability of each of the methods was determined by comparison with PFGE, with emphasis on the predominating PT 8 group. Subsequently, the method providing the highest resolution was further examined for its suitability for outbreak epidemiological typing. This study also illustrates any long-term genetic relationship between the PT 8 populations in the past and the present time.

3.2 MATERIALS AND METHODS

Bacterial genomic DNA extraction, PCR procedures for MAPLT and MLVA, and the PFGE protocol were described in Chapter 2. The prophage loci examined in this chapter were either previously described or identified in this study as described below. The primers constructed to amplify the targeted prophage loci can be found in Table 2.1b. All tested MLVA loci were previously published and the primers amplifying these MLVA loci are listed in Table 2.2. The typing data were analysed as described in Chapter 2.

3.2.1 Bacterial isolates

A total of sixty-two *S.* Virchow isolates were used in this study (Appendix 1.1). Among these isolates forty-three of which were isolated between 2005 and 2008 from various sources and geographic locations within Australia. The remaining isolates were collected in 1998 and eleven were implicated in the *S.* Virchow PT 8 sun-dried tomatoes outbreak (Bennett *et al.,* 2003) (Appendix 1.1). All isolates were obtained from the Australian *Salmonella* Reference Centre (ASRC), SA Pathology, Adelaide, Australia. The serotypes and the phage types of these isolates were determined previously by ASRC.

3.2.2 Detection and propagation of inducible bacteriophages

Inducible phages integrated in the *S.* Virchow isolates were detected and genetically characterised in order to design MAPLT primers. The procedure involved two broad tasks that began with detecting and propagating temperate phages that were integrated in the isolates, then carrying out sequencing of the extracted phage genomic DNA.

3.2.2.1 Bacteriophage induction

Small-scale induction: Bacteriophages were induced from isolates as described by Gemski *et al.* (1978) and Yee *et al.* (1993). Briefly 10ml of an overnight bacterial culture was diluted 1 in 100 in LB broth and incubated until OD_{600} of the culture reached 0.3 that took approximately 3 hours. Mitomycin C was then added at a final concentration of 1µg/ml and the culture was incubated for a further 20 hours with vigorous shaking. Bacteriophages were harvested by centrifugation at 11,000 g for 20 min at room temperature. The supernatant containing bacteriophages was filtered using a 0.45µm filter (Satorius Stedim Biotech, Hanover, Germany). The crude lysate was stored at 4C until used for plaque assay and phage propagation.

Large-scale induction: Large-scale (100 to 500ml) of phage induction was carried out using the following protocol modified from that of Brown *et al.* (1994). This method concentrated phages from lysate by centrifugation (Brown *et al.*, 1994). To the filtered lysate as prepared above using a 0.45µm filter (Millipore Corporation, Bedford, MA, US), 5µg/ml of DNase and 5µg/ml of RNase were added followed by incubation at 37C for 30 min with gentle shaking to remove bacterial nucleic acids. The lysate was then centrifuged at 10,000g for one hour at 4C and the supernatant was discarded. The phage pellet was resuspended in 4ml of SM buffer (0.58% NaCl, 0.2% $MgSO_4$.7H2O, 5% 1M Tris pH7.5, 0.01% gelatin solution) for storage at 4C till use for DNA extraction.

3.2.2.2 Plaque assay

Spot-test of the mitomycin C lysates onto lawns of indicator bacterial strains was performed to detect any induced putative phages. A lawn of indicator strain was prepared by adding 500µl of overnight broth culture of indicator strain was added to 9ml of molten 0.5% LB agar (top agar layer). After vortexed briefly this was poured into a 150mm (diameter) MH plate. When the top agar layer was set, 10µl of each of the mitomycin C lysates was spotted onto the bacterial lawn, allowed to dry and incubated overnight at 37C. Any phages that were present and infective to the indicator strains were detected by plaque formation (An example can be seen in Fig. 3.1-step 2).

3.2.2.3 Bacteriophage propagation

Bacteriophages were propagated to increase yield of DNA that would be extracted in the next step. Phage stocks were prepared using the plate lysate method on 150mm (diameter) plates. The indicator strain was prepared by growing overnight cultures in LB broth that was supplemented with 5mM calcium chloride in a 1 in 100 dilution until OD_{600} of the culture reached 0.3.

For each phage plate, a total of 1ml of phage and bacteria mixture was prepared by adding 300µl of mitomycin C phage lysate to 600µl of the bacterial culture (Tan, 2010). The phage-bacteria mixture was incubated at 37C for 15 min to allow phage absorption. Subsequently the mixture was added to 9ml of molten 0.5% LB agar, mixed gently and poured onto a MH plate and allowed to solidify. All plates were incubated for 8 to 12 hours at 37C. After incubation, 5ml of SM buffer was added onto each plate and all plates were left on bench for 2 hours to allow phage elution. To maximise the yield of phages, top agar layer and phages eluted in the SM buffer were both transferred into sterile centrifuge tubes. Phages were then recovered by centrifugation at 3220 *x g* for 20 min following by filtration of the supernatant using 0.45µm filters. Phage titres were determined as described below and in most cases the final titre was around 10^6 to 10^8 pfu/ml.

3.2.2.4 Determination of bacteriophage titres

Serial dilutions of each phage lysate were made to a dilution factor of 10^{-10} on 96 well microtitre trays (BD Falcon, Franklin Lakes, NJ, US). In each well, 10µl of bacteriophage suspension was added to 90µl of SM buffer then mixed well by pipetting. The lawns of bacterial indicator strains were prepared on 90mm (diameter) MH plates. Procedure involved was the same as above but used a reduced amount of 200µl of bacterial broth culture and 3ml of molten 0.5% LB agar. An aliquot of 10µl of each dilution was transferred on a lawn of indicator strain, starting from the most diluted sample. Spots were allowed to dry at room temperature then plates were incubated overnight at 37C. The number of plaques was counted at spots where individual plaques could be observed, and the number of plaque forming units (pfu) per ml was calculated. For example: if there were 5 plaques seen on the spot of a 10^{-6} dilution, the pfu/ml in that well would be 500 pfu/ml and hence the titre of the initial bacteriophage suspension would be determined as 500 x 10^6 pfu/ml or 5 x 10^8 pfu/ml.

3.2.3 Genetic characterisation of induced bacteriophages

3.2.3.1 Bacteriophage DNA extraction

Prior to DNA extraction, phage suspensions were further concentrated by centrifugation at 37,548 *x g* for 2 hours at 4C in a Beckman Optima™ TLX Ultracentrifuge with a TLA 100.4 rotor. Supernatant was discarded and a glassy pellet of phage particles was visible at the bottom of the tube. To the phage pellet, 500µl of SM buffer was added and the tube was kept overnight at 4C to resuspend the phage particles.

To release DNA from phage particles, the phage suspension was extracted once with an equal volume of phenol, then twice with chloroform. For each extraction, the suspension was mixed vigorously for 30 sec and centrifuged at 16,000 *x g* for 5 min in order to separate the phases. Phage DNA in the aqueous phase was precipitated using 0.1 volume of 3M sodium acetate (pH 7) and 100% ethanol overnight at -

20C. The phage DNA was pelleted by centrifugation at 16,000 *x g* for 30 min the next day. After the supernatant was discarded the phage DNA pellet was washed twice in 70% alcohol with centrifugation at 16,000 *x g* for 5 min to remove salt. The phage DNA pellet was air-dried for 10 min and re-dissolved in sterile water.

3.2.3.2 DNA quantification

The concentration of extracted phage DNA dissolved in dH_2O was determined through absorption measurement at 260nm using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, US) according to the manufacturer's instructions. An absorption at 1.0 at A_{260} is equal to 50µg of double stranded DNA in 1ml.

3.2.3.3 Degenerate-oligonucleotide primed (DOP)-PCR

Degenerate-oligonucleotide primed (DOP)-PCR was carried out initially to increase the DNA yield for sequencing (Telenius *et al.*, 1992). DOP-PCR achieves universal DNA amplification through using a DOP-PCR primer that is an oligonucleotide with a region of degenerate sequence, and a low stringency PCR cycling condition at the initial PCR cycles that allow random binding and amplifications along DNA templates (Telenius *et al.*, 1992; Kuukasjarvi *et al.*, 1997). The latter PCR cycles used a higher annealing temperature to amplify specifically the DOP-PCR products generated from the initial cycles.

DOP-PCR in this study was carried out as previously described (Ross and Heuzenroeder, 2008). For each 50µl PCR reaction mixture there were 1X $MgCl_2$-free PCR buffer, 1.6µM of DOP-PCR primer, 200µM dNTPs, 3.0mM $MgCl_2$, 1.0 U of AmpliTaq Gold® DNA polymerase and 5.0µl of phage DNA suspension. The DOP-PCR primers used are listed as follow:

DOP2: 5' ACTGTCCAGTGCGACCAGNNNNNNGTGG 3'
DOPJ: 5' AGCAGCGACTGTCGACACNNNNNNGTGG 3'
DOP4: 5' ACTGTCCAGTGCGACCAGNNNNNNCAGC 3'

PCR reaction was performed on a Bio-Rad iCycler™ with the cycling condition as follow: an initial denaturation step at 94C for 10 min; 10 cycles of 94C for 30 sec, 30C for 1 min and 72C for 5 min; 30 cycles of 94C for 30 sec, 50C for 1 minute and 72C for 5 min, then a final extension step at 72C for 10 min. DOP-PCR products were visualised using gel electrophoresis and the procedure is described in section 2.5.3.

3.2.3.4 *in vitro* cloning of DOP-PCR products

Prior to cloning, the mixture of DOP-PCR products amplified from a phage genome were purified using QIA® quick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An aliquot of 2.0µl of the PCR products was ligated with 1µl of the vector pCR®-2.1-TOPO (Invitrogen, Carlsbad, CA, US) in the presence of 1µl of the 10X ligation buffer, 1µl of the T4 DNA ligase and 8µl of sterile distilled water. Ligation reaction was incubated overnight at 4C. On the next day 2µl of the reaction mixture was transferred into a vial of 25µl of the chemically competent *E.coli* TOP10 cells (Invitrogen, Carlsbad, CA, US), and the mixture was incubated on ice for 30 min. The cells were then heat shocked at 42C for 1 minute and transferred back to ice. SOC broth (250µl) was added to the cells that were incubated at 37C with vigorous shaking for 1 hour. Two LB plates containing 100µg/ml ampicillin, 50µg/ml kanamycin and 40µl of 40mg/ml X-gal were spread with 50µl and 200µl of the incubated culture and incubated overnight at 37C.

White colonies were selected that presumably contained cells with ligated plasmids. Each of the selected white colonies was inoculated into 2ml of LB broth containing 100µg/ml ampicillin, 50µg/ml kanamycin. All broth cultures were incubated overnight at 37C with gentle shaking.

3.2.3.5 M13 PCR and sequencing

M13 PCR was carried out to detect the cloned DOP-PCR product. The PCR reaction mixture was prepared as described in section 2.5.1 and an aliquot of 2µl of each broth culture was added as PCR template. The PCR cycling conditions were as follows: 1 cycle of 94C for 10 min, 30 cycles of 94C for 30 sec, 56C for 30 sec, and 72C for 1min, and 1 cycle of 72C 10 min. The M13 primer sequences used were the same as described by the manufacturer:

M13 forward (-20) (M13F): 5' GTAAAACGACGGCCAG 3'
M13 reverse (M13R): 5' CAGGAAACAGCTATGAC 3'

The M13 PCR products were detected using gel electrophoresis following the procedure described in section 2.5.3. Prophage sequences that were amplified as DOP-PCR products were determined through sequencing of the M13 PCR products. The sequencing procedure used is described in section 2.7.

The complete procedure involved in detection and characterisation of phage sequences was illustrated in Fig. 3.1.

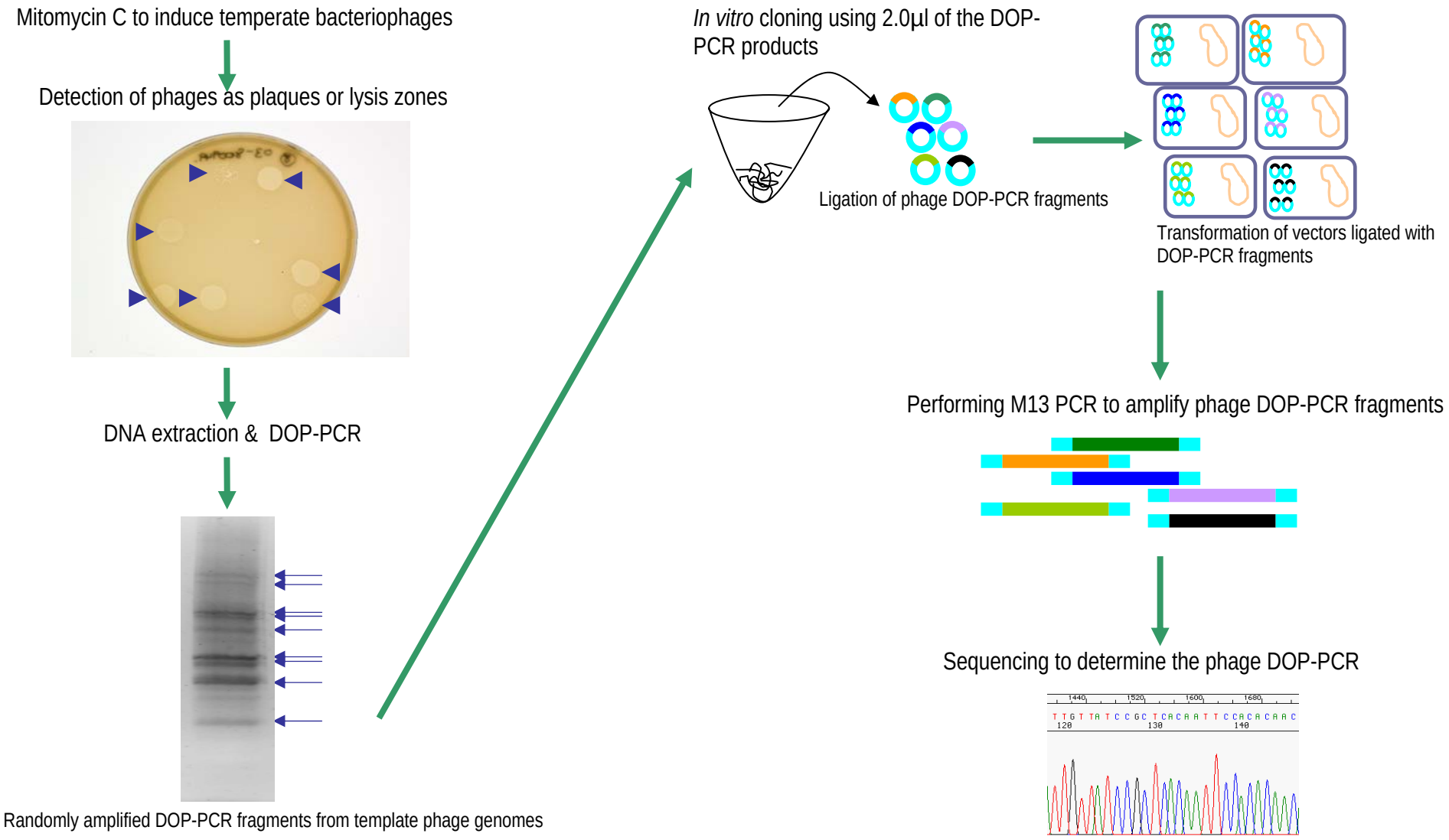


Fig. 3.1 A flowchart showing the steps involved for determination of genomic sequences of the induced bacteriophages

3.3 RESULTS

3.3.1 MAPLT

3.3.1.1 Identification of prophage loci useful for strain differentiation

DOP-PCR and nucleotide sequencing demonstrated that *S.* Virchow isolates harboured a range of different phage elements as indicated in Table 3.1. MAPLT primers were constructed based on to the sequenced genomes of phages Fels-2 (McClelland *et al.*, 2001), Gifsy-1 (McClelland *et al.*, 2001), Gifsy-2 (McClelland *et al.*, 2001), 186 (Finnegan and Egan, 1979), ES18 (Casjens *et al.*, 2005), SfV (Allison *et al.*, 2002), and P7 (NCBI accession number AF503408) (Table 2.1a). A novel gene locus (DOP 13.7) was also detected from the lysate PV16 along with loci from coliphage 186. The predicted amino acid sequence encoded by the gene locus DOP 13.7 (689 bp) was found to show similarity to predicted phage tail fibre proteins in putative prophages of various sequenced *Salmonella* strains thereby confirming this locus as being phage related.

In total twenty-eight targeted prophage loci were present in at least one of the *S.* Virchow isolates (n = 43) (Table 3.2). Generally it was observed that the presence of gene loci between the aforementioned phages varied greatly among the isolates but gene loci from the same phages were detected at a similar rate regardless the different functional modules they were derived from. For example, all seven Fels-2 gene loci targeted were either amplified in the same five or seven isolates though they were located in different modules including immunity (STM2736, STM2738), lysogeny (STM2739); tail (STM2695, STM2697); head (STM2719), and lysis modules (STM2714) (McClelland *et al.*, 2001) (Appendix 2.1). The only exception was observed from the two Gifsy-2 loci where the integrase gene locus was detected in almost all isolates (n = 42), but the gene locus encoding a putative capsid protein was detected in isolates V11 and V12 only (Appendix 2.1).

3.3.1.2 MAPLT differentiation of *S.* Virchow isolates

Based on the observations described above, nine specific prophage loci could be utilised to separate the isolates equally well as utilising the full twenty-eight loci (Table 3.3). Therefore the MAPLT typing results presented in this chapter are based on the targeted nine prophage loci listed in Table 3.3. There were fourteen MAPLT profiles observed, six of which were generated by more than one isolate and eight were uniquely generated by single isolates (Fig. 3.2). There was a large proportion of PT 8 isolates (n = 24) included in this study due to its epidemiological significance to Australia and elsewhere. The PT 8 isolates were subdivided into seven profiles in which 60% (n = 15) generated MAPLT profile 4 (Fig. 3.2).

With regard to the non-PT 8 isolates (n = 19), these were separated into eleven MAPLT profiles (Table 3.4). It was shown that MAPLT did not classify these isolates in concordance with the phage types. Among the eleven MAPLT profiles, four (MAPLT profiles 1, 4, 7, 11) were found to contain more than one isolate of different phage types. At the same time, isolates of the same or similar phage types included in this study generated different MAPLT profiles. For example, the PT 31 isolate V16 was separated from the other PT 31 isolate V07 by additionally amplifying locus DOP13.7. Isolates V05 and V10 were typed as PT 25 and PT 25a respectively. The lysis patterns of these two phages types were highly similar but MAPLT separated isolates V05 and V10 by showing that isolate V05 (MAPLT profile 11) contained the targeted Fels-2 gene locus STM2736 that was otherwise absent in isolate V10 (MAPLT profile 1). Simultaneously, isolate V10 contained locus DOP 13.7 that was not detected in V05.

3.3.2 PFGE

The *S.* Virchow isolates were also subjected to PFGE. Seventeen PFGE profiles were observed, with eight profiles generated by multiple isolates and nine profiles generated by single isolates. In general, PFGE did not classify the isolates in the same way as MAPLT apart from the PT 8 isolates (Fig. 3.3). Seven PFGE profiles were generated from the twenty-four PT 8 isolates with sixteen of which generated PFGE profile 4. It was noted that all of the PT 8 isolates with the PFGE profile 4 had the MAPLT profile 4 (Fig. 3.3). In contrast, the remaining eight PT 8 isolates were linked differently by PFGE and MAPLT. For example, while PFGE clustered PT 8 isolates V29 and V57 in PFGE profile 1, MAPLT clustered isolate V29 with a different PT 8 isolate V24 in MAPLT profile 2, and clustered isolate V57 with V30 in MAPLT profile 11. With regard to the non-PT 8 isolates, there were fourteen PFGE profiles generated. Similarly to MAPLT, PFGE differentiated the non-PT 8 isolates despite that they have the same or similar phage types (Fig. 3.3).

3.3.3 MLVA

A number of MLVA loci showing discriminative ability in the published studies were also examined in this chapter for differentiation of *S.* Virchow isolates. In total sixteen published MLVA loci were analysed by selecting from the MLVA schemes for *S.* Typhimurium (Lindstedt *et al.*, 2003; Lindstedt *et al.*, 2004), *S*. Enteritidis (Boxrud *et al.*, 2007), *S.* Typhi (Liu *et al.*, 2003), and *Salmonella* species (Ramisse *et al.*, 2004) (Table 3.5). Only locus STTR-5 displayed allelic variation with eight alleles observed. Six of the eight STTR-5 alleles were observed in the PT 8 isolates, while five alleles were observed in the non-PT 8 isolates. The remaining sixteen loci either generated fragments of identical size or were not detected in any of the isolates.

Table 3.1 DOP-PCR fragments that showed similarity to the published phage gene loci

Published phages	Lysates	Indicator strains	DOP-PCR fragments	Phage gene loci & encoded proteins	Matches in the published genomes	E-value
Fels-2	PV08	*S.* Enteritidis 1727	8-1	STM2697; putative tail protein	2848024-2847417	0.0
			8-2	STM2719; small terminase subunit protein	2861228-2861965	0.0
			8-3	STM2733; unknown function	2874021-2874411	7e-139
	PV05	*S.* Enteritidis 1727	5-1	Intergenic; between STM2699 & STM2700	2849243-2849951	0.0
	PV07	*S.* Enteritidis 1727	7-1	STM2697; putative tail protein	2848845-2849740	0.0
			7-2	STM2695; late control protein D	2844803-2845423	0.0
Gifsy-1	PV10	*S.* Enteritidis 1727	10-1	STM2594; *gene H* minor tail protein	2745850-2746489	0.0
			10-2	STM2594; *gene H* minor tail protein	2744440-2744852	0.0
Gifsy-2	PV15	[b]n/a	15-1	intergenic; between STM1048 & STM1049	1135598-1136022	0.0
186	PV16	*S.* Enteritidis 1727	16-1	*gene M*; baseplate protein	10201-10715	0.0
			16-2	*gene O*; baseplate protein	9492-9909	2e-170
			16-3	*orf2*; capsid portal protein	121-1179	0.0
			16-4	*gene G*; tail protein	17076-17961	0.0
			16-5	*gene W*; terminase subunit protein	2877-3274	7e-177

Published phages	[a]Lysates	Indicator strains	DOP-PCR fragments	Phage gene loci & encoded proteins	Locations in the published genomes	E-value
P7	PV01	[b]n/a	1-1	*darB*; structural head protein	19079-19398	9e-162
			1-2	*gene 25*; putative tail stability determining protein	79837-80298	0.0
			1-3	*sit*; putative structural injection transglycosylase	44197-44602	6e-146
ES18	PV14	*S.* Enteritidis 1727	14-1	*gene 9*; putative coat protein	6334-6938	0.0
			14-2	*gene 16*; putative tail shaft protein	9756-10049	4e-134
SfV	PV58	[b]n/a	58-1	*orf16*; tail protein	12698-13667	0.0
			58-2	*orf11*; tail sheath protein	8112-8465	6e-165
ST64B	PV58	[b]n/a	58-3	SB06; major capsid protein	4263-4838	0.0
			58-4	SB04; portal head protein	2962-3936	0.0

[a]Phage lysates induced from the test isolates. For example, PV08 = phage lysate of isolate V08

[b]n/a = not applicable

Indicator strain S. Enteritidis 1727 was sourced from Tan (2010)

Table 3.2 Positive amplifications of prophage gene loci from the 43 *S.* Virchow isolates

Prophages	Gene loci	Gene functions	No. of positive isolates
P22	*ninB*	ninB protein	2
ST64B	SB04	Portal protein	1
186	*gene P*	Endolysin	14
	gene O	Baseplate protein	14
	gene G	Tail protein	11
	cII	CII protein	14
Fels-2	STM2695	Putative control protein D	7
	STM2697	Tail protein	7
	STM2714	Lysis regulatory protein	5
	STM2719	Small terminase subunit	7
	STM2736	CII protein	7

Prophages	Gene loci	Gene functions	No. of positive isolates
Fels-2	STM2738	CI protein	5
	STM2739	Integrase	7
Gifsy-1	STM2594	Tail protein	40
	STM2608	Terminase large subunit	39
Gifsy-1/2	STM2619/ STM1021	Unknown (NinG)	43
Gifsy-2	STM1005	Integrase	42
	STM1032	Putative capsid protein	2
SopEϕ	*sop*E	Type III secretion protein (SopE)	41
ES18	*gene 9*	Putative coat protein	1

Prophages	Gene loci	Gene functions	No. of positive isolates
SfV	*orf 5*	Capsid protein	1
	orf 11	Tail sheath protein	3
	orf 16	Tail protein	1
	orf 26	Integrase	1
	orf 34	CI protein	1
P7	*sit*	Putative structural injection transglyosylase	3
P1	*sit*	Structural lytic transglycosylase	1
A prophage in isolate V16	DOP13.7	Possible tail fibre assembly protein	2

All prophage loci were amplified using primers listed in Table 2.1b

Table 3.3 Primers of the MAPLT scheme for *Salmonella* Virchow

Prophages	Gene loci	Encoded proteins	Primer sequences (5' → 3')
P22	*nin*B	ninB protein	[a] ninBF1: AACCTTTGAAATTCGATCTCCAGC
			[a] ninBR1: CTTCGTCTGACCACTTAACGC
ST64B	SB04	Put. portal protein	SB04F: TGTCATACGACACCTATACCG
			SB04R: TGTTCTGCACCATGTGCAATG
ES18	*gene 9*	putative coat protein	PCPF: TGGAACGCACAGCATGATGC
			PCPR: GGACTGCACCTGAATATTCGG
186	*cII*	CII protein	P186cIIF: GACATAGCGGGATTAGTCTGC
			Fels2cIIR: GTCACAACATGGCGAAGCTG
186	*gene P*	Endolysin	P186PF: TCACCGATTACAGCGACCAC
			P186PR: TGGTGACCAGCTTTTCGAGAC
Fels2	STM2736	CII protein	Fels2cIIF: TGTATGGAAACGGCAGCCAG
			Fels2cIIR: GTCACAACATGGCGAAGCTG
Gifsy-1	STM2608	Terminase large subunit	Gifsy1AF: GATCACGCATCCATTATGTTCAC
			Gifsy1AR: TATTCCCGTACCGCTTACCAC
P7	*sit*	Put. structural injection transglyosylase	P7sitF: TGACCTTGATCGCGTACTCAC
			P7sitR: TAGCCACCAGGAGACATCTG
Prophage in isolate V16	DOP13.7	Possible tail fibre protein	DOP13.7F: CGGTTAGCTCCGTGGTTAAG
			DOP13.7R: TAGCCACCAGGAGACATCTG

[a] Primer sequences described by Ross and Heuzenroeder (2005)

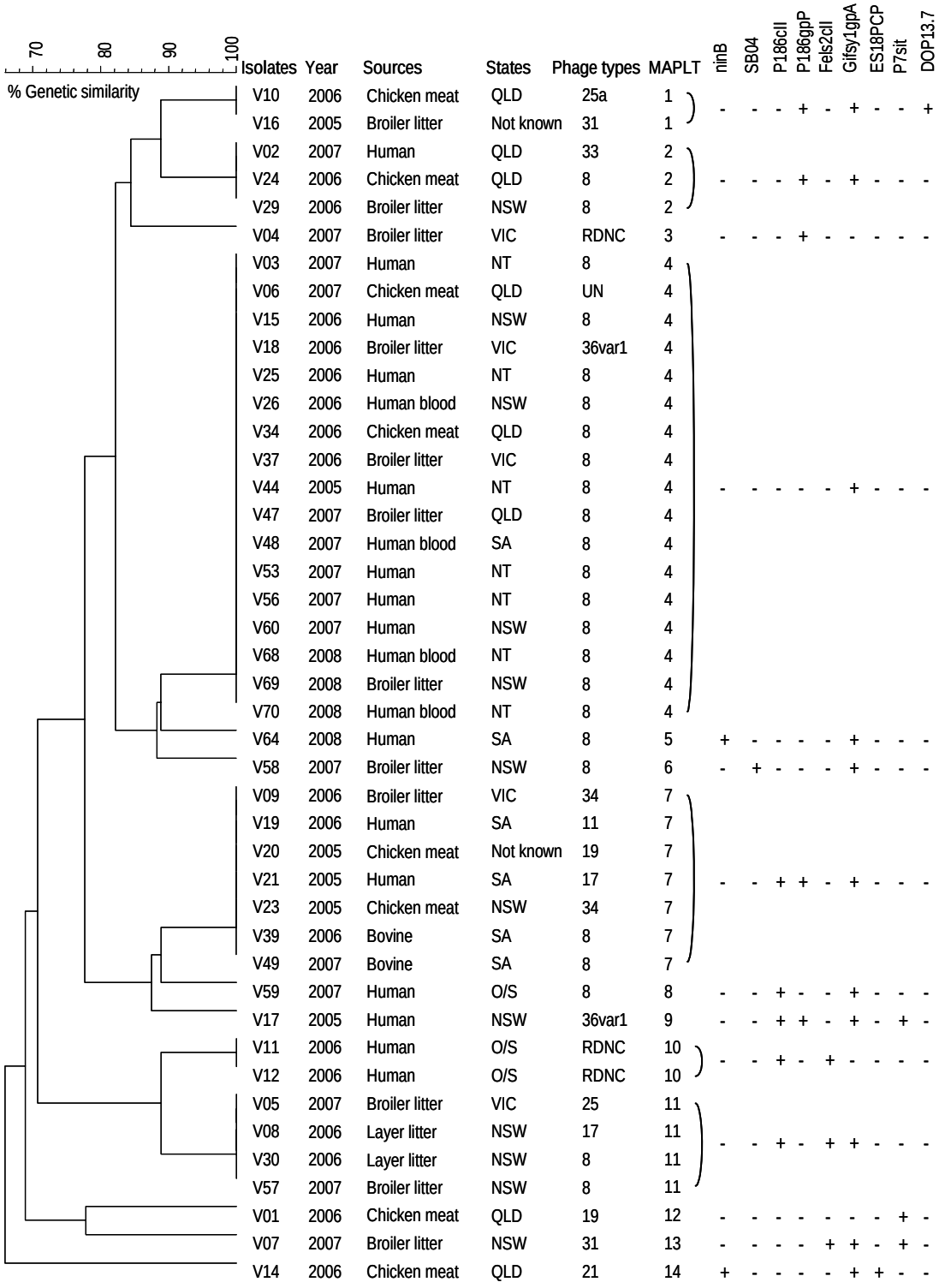


Fig. 3.2 Dendrogram showing the genetic relationship between the 43 *S.* Virchow isolates as generated by MAPLT results. Symbol + indicated positive amplification of the prophage loci; symbol – indicated negative PCR amplification of the prophage loci.

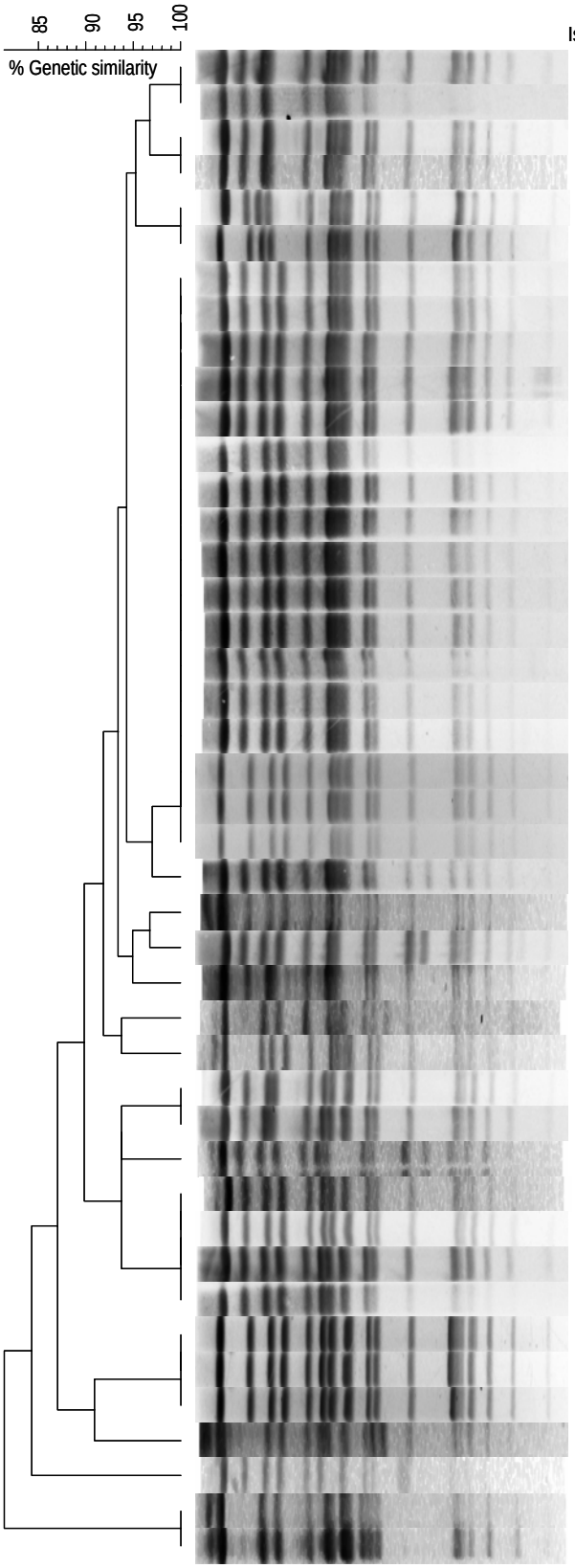
UN = untypable; RDNC = reacts does not conform.

Abbreviations for Australian states: NSW = New South Wales; NT = Northern Territory; QLD = Queensland; SA = South Australia; WA = Western Australia; O/S = overseas travel

Table 3.4 MAPLT profiles generated by the 19 non-PT 8 isolates

MAPLT profiles	Phage types	Isolates	Year	Sources	States
1	25a	V10	2006	Chicken meat	QLD
	31	V16	2005	Chicken litter	Not known
2	33	V02	2007	Human	QLD
3	RDNC	V04	2007	Chicken litter	VIC
4	[b]UN	V06	2007	Chicken meat	QLD
	36var1	V18	2006	Chicken litter	VIC
7	34	V09	2006	Chicken litter	VIC
	34	V23	2005	Chicken meat	NSW
	11	V19	2006	Human	SA
	19	V20	2005	Chicken meat	Not known
	17	V21	2005	Human	SA
9	36var1	V17	2005	Human	NSW
10	[a]RDNC	V11	2006	Human	SA
	[a]RDNC	V12	2006	Human	SA
11	17	V08	2006	Chicken layer	NSW
	25	V05	2007	Chicken litter	VIC
12	19	V01	2006	Chicken meat	QLD
13	31	V07	2007	Chicken litter	NSW
14	21	V14	2006	Chicken meat	QLD

[a] RDNC = Reacts does not conform; [b] UN = untypable



Isolates	Year	Sources	States	Phage types	PFGE	MAPLT
V29	2006	Broiler litter	NSW	8	1	2
V57	2007	Broiler litter	NSW	8	1	11
V07	2007	Broiler litter	NSW	31	2	13
V24	2006	Chicken meat	QLD	8	2	2
V10	2006	Chicken meat	QLD	25a	3	1
V18	2006	Broiler litter	VIC	36var1	3	4
V25	2006	Human	NT	8	4	4
V26	2006	Human blood	NSW	8	4	4
V37	2006	Broiler litter	VIC	8	4	4
V44	2005	Human	NT	8	4	4
V47	2007	Broiler litter	QLD	8	4	4
V48	2007	Human blood	SA	8	4	4
V53	2007	Human	NT	8	4	4
V56	2007	Human	NT	8	4	4
V58	2007	Broiler litter	NSW	8	4	6
V60	2007	Human	NSW	8	4	4
V64	2008	Human	SA	8	4	5
V68	2008	Human blood	NT	8	4	4
V69	2008	Broiler litter	NSW	8	4	4
V70	2008	Human blood	NT	8	4	4
V03	2007	Human	NT	8	4	4
V06	2007	Chicken meat	QLD	UN	4	4
V15	2006	Human	NSW	8	4	4
V59	2007	Human	O/S	8	5	8
V16	2005	Broiler litter	Not known	31	6	1
V34	2006	Chicken meat	QLD	8	7	4
V05	2007	Broiler litter	VIC	25	8	11
V01	2006	Chicken meat	QLD	19	9	12
V14	2006	Chicken meat	QLD	21	10	14
V08	2006	Layer litter	NSW	17	11	11
V30	2006	Layer litter	NSW	8	11	11
V04	2007	Broiler litter	VIC	RDNC	12	3
V02	2007	Human	QLD	33	13	2
V09	2006	Broiler litter	VIC	34	13	7
V39	2006	Bovine	SA	8	13	7
V49	2007	Bovine	SA	8	13	7
V19	2006	Human	SA	11	14	7
V23	2005	Chicken meat	NSW	34	14	7
V21	2005	Human	SA	17	14	7
V17	2005	Human	NSW	36var1	15	9
V20	2005	Chicken meat	Not known	19	16	7
V11	2006	Human	O/S	RDNC	17	10
V12	2006	Human	O/S	RDNC	17	10

Fig. 3.3 Dendrogram constructed based on the PFGE patterns of the 43 *S.* Virchow isolates. The PFGE patterns were related by the BioNumerics program with the band tolerance level and optimisation both set at 1.3%.

Table 3.5 MLVA loci used in differentiating the 43 *S.* Virchow isolates

MLVA loci	No. of alleles	[a]No. of tandem repeats ([b]no. of isolates)	References
STTR-2	1	10 (43)	Lindstedt *et al.,* (2003)
STTR-3	1	15 (43)	Lindstedt *et al.,* (2003)
STTR-5	8	6 (1), 8 (1), 10 (9), 11 (15), 12 (10),13 (3), 14 (1), 15 (3)	Lindstedt *et al.,* (2003)
STTR-6	Not appliable	Not amplified (43)	Lindstedt *et al.,* (2003)
STTR-7	1	9 (43)	Lindstedt *et al.,* (2003)
STTR-9	1	2 (43)	Lindstedt *et al.,* (2004)
STTR-10	Not applicable	Not amplified (43)	Lindstedt *et al.,* (2003)
Sal02	1	2 (43)	Ramisse *et al.,* (2004)
Sal04	1	0 (43)	Ramisse *et al.,* (2004)
Sal10	1	2 (43)	Ramisse *et al.,* (2004)
Sal15	1	2 (43)	Ramisse *et al.,* (2004)
Sal20	1	9 (43)	Ramisse *et al.,* (2004)
Sal23	1	3 (43)	Ramisse *et al.,* (2004)
SE01	Not applicable	Not amplified (43)	Boxrud *et al.,* (2007)
SE02	Not applicable	Not amplified (43)	Boxrud *et al.,* (2007)
TR1	1	2 (43)	Liu *et al.,* (2003)

All MLVA loci were amplified using primers listed in Table 2.2.
[a] The number of tandem repeats contained within the amplified MLVA loci; [b] Number of isolates amplified with the MLVA alleles.

3.3.4 Composite assay of MAPLT / STTR-5

Locus STTR-5 was the only MLVA locus assessed that showed allelic variation and was incorporated into the 9-loci MAPLT scheme. In total twenty-three profiles were seen from the forty-three *S.* Virchow isolates (Fig. 3.4). There were eight profiles generated by more than one isolate and profile 3 contained the highest number of isolates which were all PT 8 but one isolate (V06) that was untypable by phage typing. For the remaining five of the seven profiles generated by multiple isolates (profiles 4, 8, 9, 12, 18), each was generated by isolates of different phage types. The twenty-four PT 8 isolates were differentiated into twelve profiles, while the nineteen non-PT 8 isolates generated fifteen profiles.

3.3.5 Comparison of differentiating abilities between MAPLT, PFGE and composite MAPLT / STTR-5

As summarised in Table 3.6, the composite MAPLT / STTR-5 method was found to be the most discriminative method in this study trialled, having a Simpson's index of diversity (DI) value over 0.9. By contrast, PFGE and MAPLT both had the lower DI values of approximately 0.8. All three methods differentiated the non-PT 8 isolates equally well giving DI values above 0.9 (Table 3.7). Therefore the overall improved differentiating ability of the MAPLT / STTR-5 method would be due to the enhanced differentiation between the PT 8 isolates. As illustrated in Table 3.7, the MAPLT / STTR-5 method differentiated the PT 8 isolate most extensively into twelve profiles and gave a DI value of 0.87. In contrast, much lower DI values at 0.56 and 0.61 were calculated for PFGE and MAPLT respectively, and there were eight profiles observed from either method.

3.3.6 Outbreak investigations using MAPLT / STTR-5 typing method and PFGE

A set of eleven *S.* Virchow PT 8 isolates involved in the sun-dried tomatoes outbreak in 1998 (Bennett *et al.*, 2003) together with eight epidemiologically-unrelated PT 8 isolates collected between 1996 and 1998 were tested with both the composite MAPLT / STTR-5 method and PFGE. The suitability of the composite MAPLT / STTR-5 method for outbreak epidemiological typing was assessed by comparing this data with that of PFGE.

Using PFGE, the nineteen isolates were differentiated into three profiles with a maximum of two PFGE band differences. All the eleven PT 8 cases from the described case-control study 1998 (Bennett *et al.*, 2003) generated the same PFGE profile 3 (Fig. 3.5). It was noted that three epidemiologically-unrelated isolates also generated this PFGE profile including isolate V98-14, which was obtained from a patient during the course of the outbreak. In regard to the remaining five epidemiologically-unrelated isolates, little differentiation was observed as four isolates generated an indistinguishable PFGE profile 1. In comparison, the MAPLT / STTR-5 typing scheme subdivided the same nineteen isolates into six profiles (Fig. 3.6). Similar to PFGE, all eleven outbreak isolates and the epidemiologically-unrelated isolate

V98-14 that had PFGE profile 3 (Fig. 3.5) generated MAPLT / STTR-5 profile 1. In contrast, the four isolates generating PFGE profile 1 were separated into three profiles based on the allelic differences observed at locus STTR-5.

3.3.7 Comparison of typing profiles between all PT 8 isolates

A comparison of typing profiles from all the PT 8 isolates (n = 43) obtained for this study was also undertaken. It was observed that the PFGE profile of the outbreak isolates was observed in two recent PT 8 isolates obtained between 2005 and 2008 in PFGE cluster A (Fig. 3.7). For the remaining PT 8 isolates from the 1990s, four isolates (V98-01, V98-07, V97-17 and V97-19) clustered with the majority of the recent PT 8 isolates in PFGE cluster B and isolate V98-15 generated a unique PFGE profile. By using MAPLT / STTR-5, the eleven outbreak isolates clustered with one recent PT 8 isolate (V24) in MAPLT / STTR-5 cluster I, while three isolates V98-01, V98-07 and V98-17 were clustered with the recent PT 8 isolates in MAPLT / STTR-5 clusters II and III (Fig. 3.8). The remaining two isolates from the 1990s including V98-15 and V97-19 generated individual MAPLT / STTR-5 profiles (Fig. 3.8).

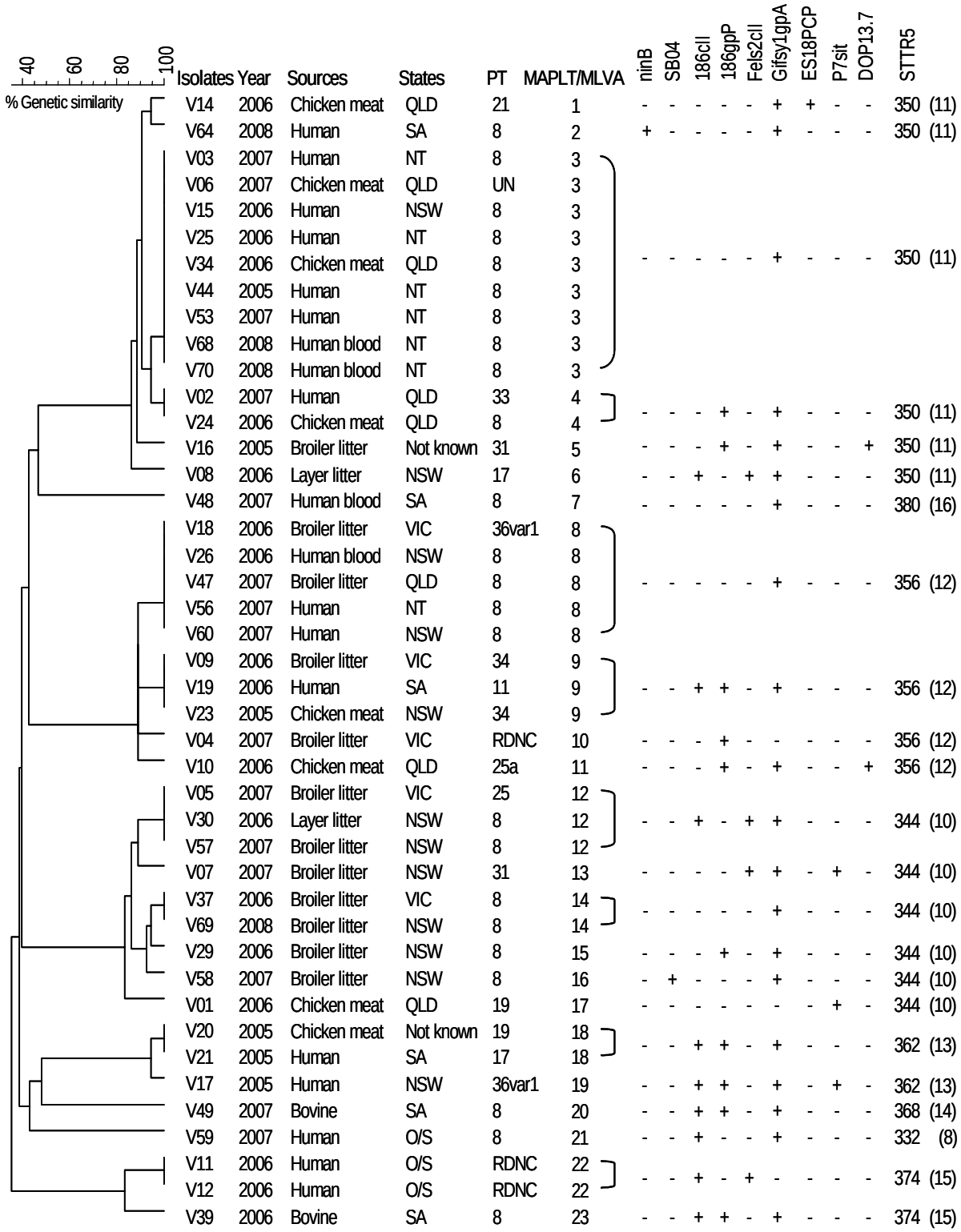


Fig. 3.4 Dendrogram depicting genetic relationship between the 43 *S.* Virchow isolates based on 9 MAPLT loci and MLVA locus STTR-5.

MAPLT results are presented as + (positive amplification) and – (negative amplification). MLVA results were presented as the amplified fragment length (in bp). Numbers of tandem repeats were indicated in the brackets.

Table 3.6 Comparison of the differentiating abilities between the 3 methodologies based on the calculated Simpson's index of diversity for the 43 *S.* Virchow isolates

	No. of primers used	No. of profiles	Simpson's index of diversity (95% confidence interval)
PFGE	na[1]	17	0.83 (0.73 to 0.94)
MAPLT	9	14	0.81 (0.72 to 0.91)
MAPLT / STTR-5	10	23	0.94 (0.90 to 0.98)

[1]na = not applicable

Table 3.7 Differentiating ability of PFGE, MAPLT and MAPLT / STTR-5 for the 24 PT 8 isolates and the 18 non-PT 8 isolates

	Simpson's index of diversity (no. of profiles)	
	PT 8 (n = 24)	Non- PT 8 isolates (n = 19)
PFGE	0.56 (7)	0.97 (14)
MAPLT	0.61 (7)	0.92 (11)
MAPLT / STTR-5	0.87 (12)	0.97 (15)

All values illustrated in this table were derived from Fig. 3.1 (MAPLT), Fig. 3.2 (PFGE), and Fig. 3.3 (MAPLT / STTR-5)

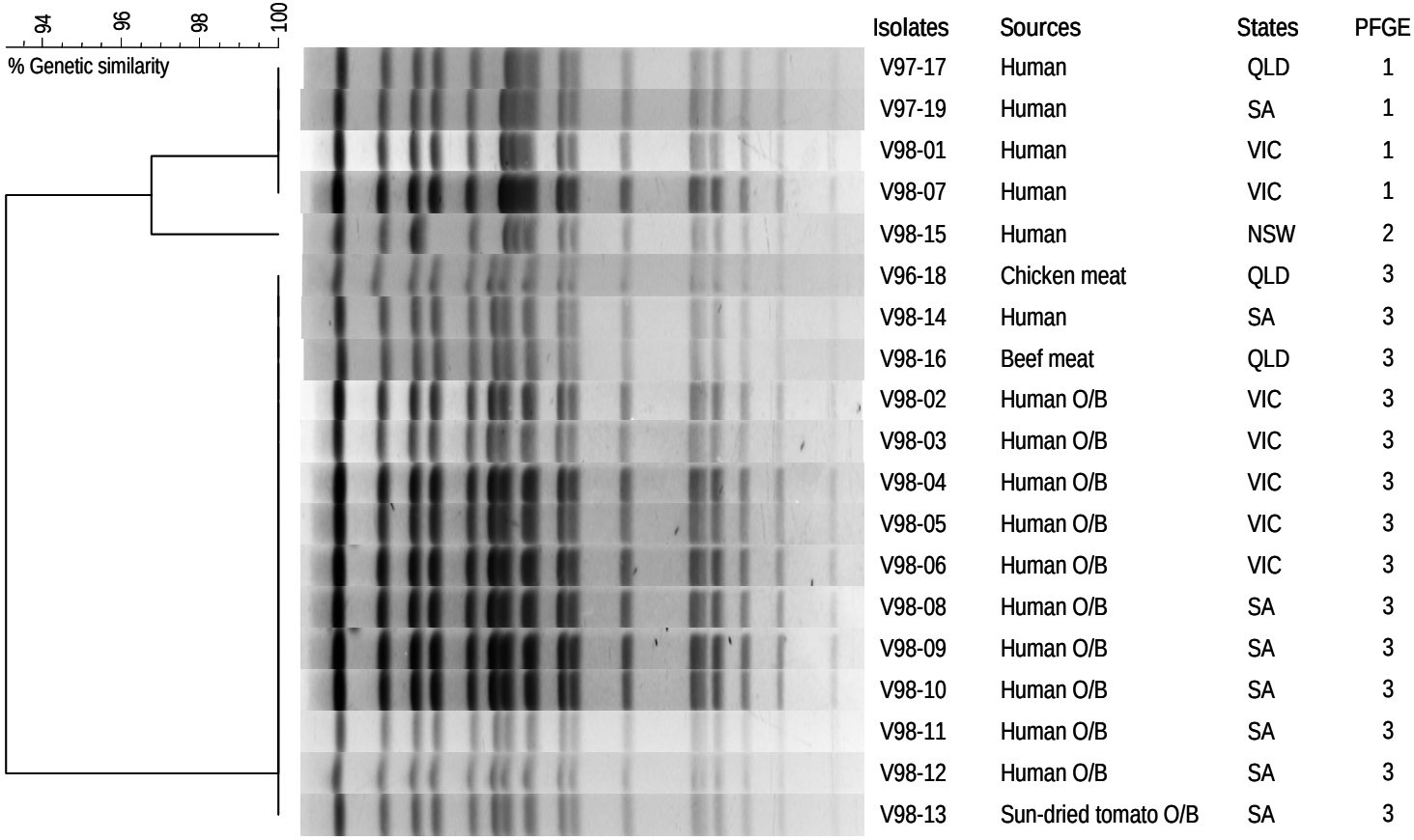
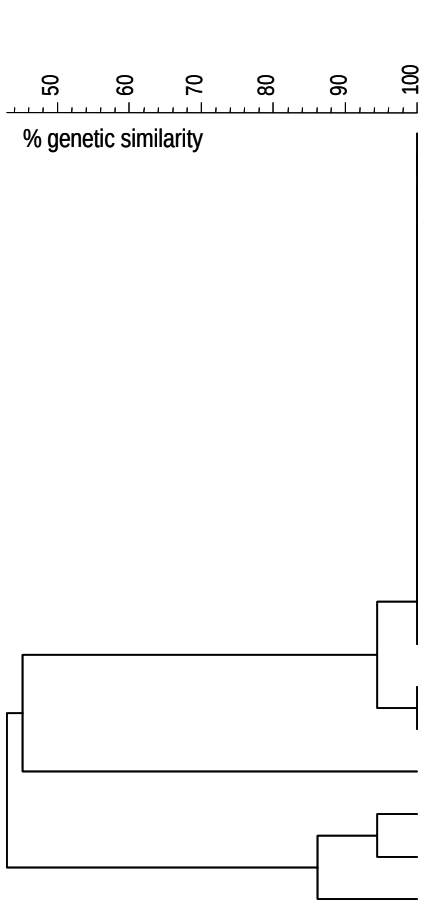


Fig. 3.5 Dendrogram showing the genetic similarity between the 19 PT 8 isolates collected in late 1990s as determined by PFGE. The band patterns were analysed by BioNumerics program with the band tolerance level and optimisation at 0.9% and 1.1% respectively. The isolates associated with the 1998 outbreak were designated with O/B.



ninB	SB04	P186cII	P186 gpP	Fels2cII	Gifsy1gpA	ES18PCP	P7sit	DOP13.7	STTR-5	Isolates	Sources	States	PFGE	MAPLT/STTR-5
-	-	-	+	-	+	-	-	-	247	V98-02	Human O/B	VIC	3	1
-	-	-	+	-	+	-	-	-	247	V98-03	Human O/B	VIC	3	1
-	-	-	+	-	+	-	-	-	247	V98-04	Human O/B	VIC	3	1
-	-	-	+	-	+	-	-	-	247	V98-05	Human O/B	VIC	3	1
-	-	-	+	-	+	-	-	-	247	V98-06	Human O/B	VIC	3	1
-	-	-	+	-	+	-	-	-	247	V98-08	Human O/B	SA	3	1
-	-	-	+	-	+	-	-	-	247	V98-09	Human O/B	SA	3	1
-	-	-	+	-	+	-	-	-	247	V98-10	Human O/B	SA	3	1
-	-	-	+	-	+	-	-	-	247	V98-11	Human O/B	SA	3	1
-	-	-	+	-	+	-	-	-	247	V98-12	Human O/B	SA	3	1
-	-	-	+	-	+	-	-	-	247	V98-13	Sun-dried tomato O/B	SA	3	1
-	-	-	+	-	+	-	-	-	247	V98-14	Human	SA	3	1
-	-	-	+	-	+	-	-	-	247	V98-16	Beef meat	QLD	3	1
-	-	-	-	-	+	-	-	-	247	V98-01	Human	VIC	1	2
-	-	-	-	-	+	-	-	-	247	V98-07	Human	VIC	1	2
-	-	-	-	-	+	-	-	-	223	V97-19	Human	SA	1	3
-	-	-	-	-	+	-	-	-	241	V97-17	Human	QLD	1	4
-	-	-	-	+	+	-	-	-	241	V98-15	Human	SA	2	5
-	-	+	+	-	+	-	-	-	241	V96-18	Chicken meat	QLD	3	6

Fig. 3.6 Dendrogram constructed based on the MAPLT / STTR-5 typing results showing the genetic similarity between the 19 PT 8 isolates collected in late1990s. Isolates associated with the 1998 outbreak were designated with O/B.

This dendrogram also showed that using the 9-loci MAPLT scheme would have already differentiated between the outbreak isolates and the non-outbreak isolates the same way as PFGE.

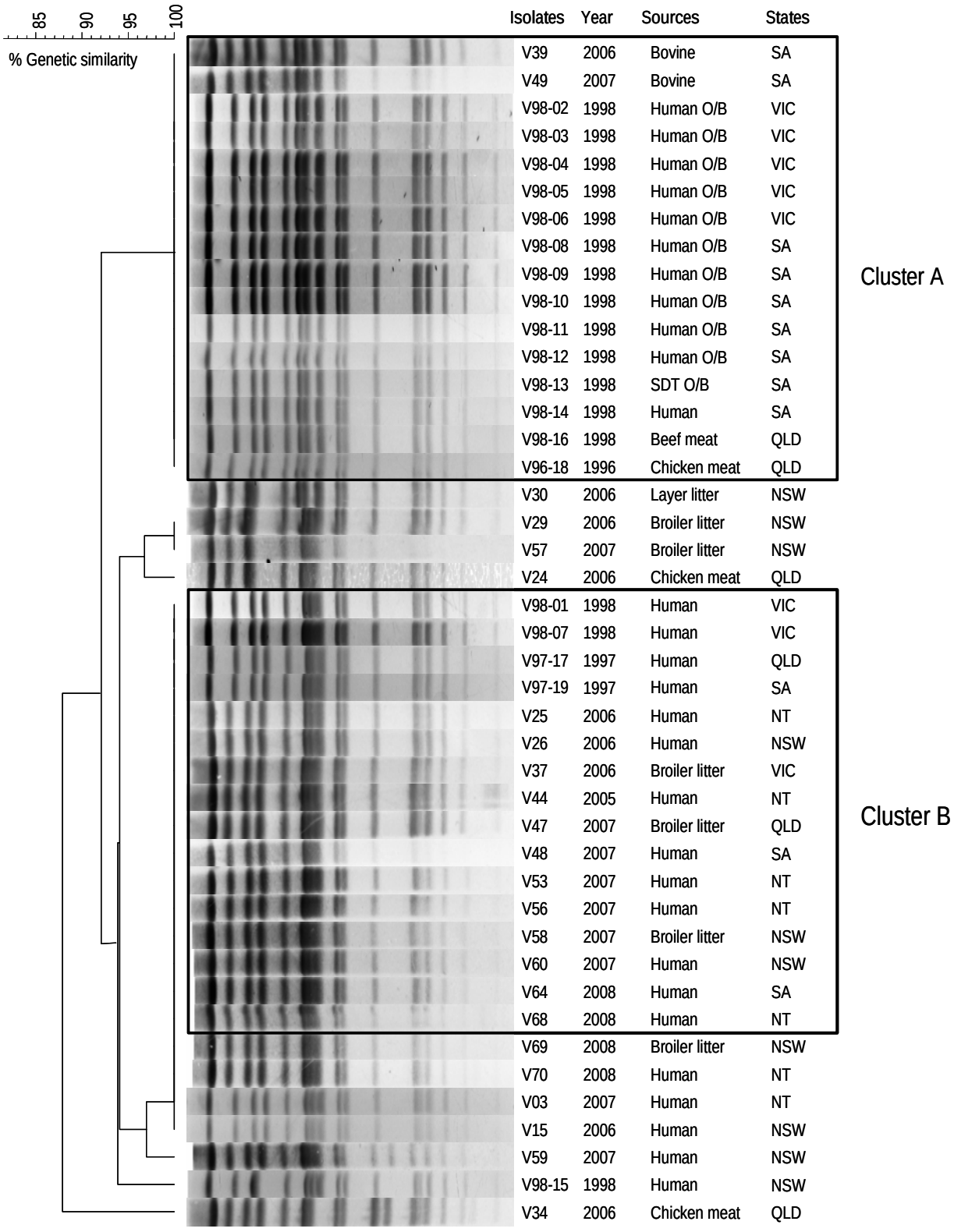
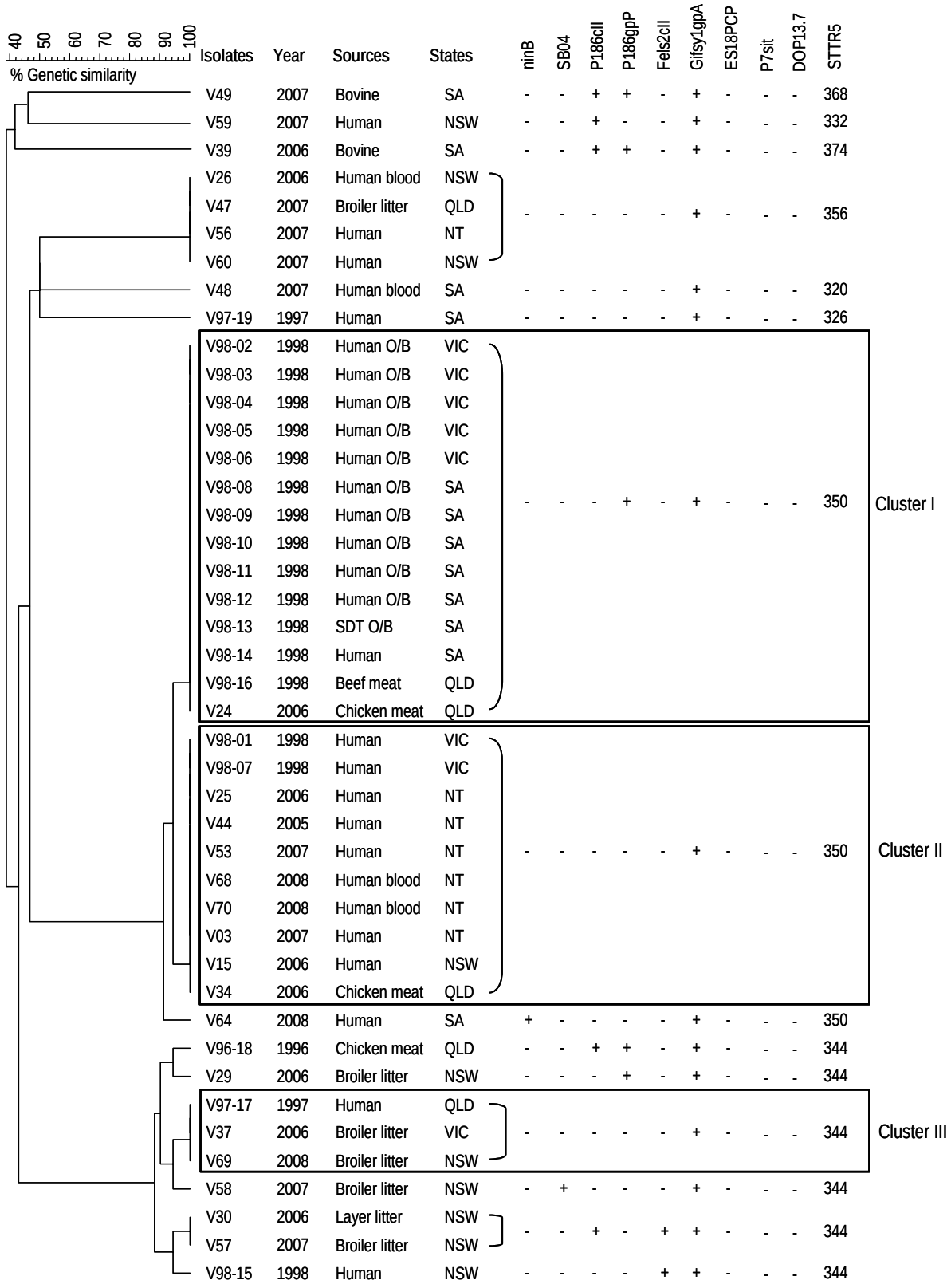


Fig. 3.7 Construction of the dendrogram is based on the PFGE patterns of the 43 *S*. Virchow PT 8 isolates. The PFGE patterns were analysed by the BioNumerics program with the band tolerance level set at 1.4% and optimisation set at 1.3%.

PFGE illustrates the genetic difference between the 1998 outbreak isolates (in cluster A) with the majority of the recently collected PT 8 isolates which are in cluster B.

SDT = sun-dried tomatoes; O/B indicated isolates related to the PT 8 outbreak in 1998



Isolates	Year	Sources	States	ninB	SB04	P186cII	P186gpP	Fels2cII	Gifsy1gpA	ES18PCP	P7sit	DOP13.7	STTR5	
V49	2007	Bovine	SA	-	-	+	+	-	+	-	-	-	368	
V59	2007	Human	NSW	-	-	+	-	-	+	-	-	-	332	
V39	2006	Bovine	SA	-	-	+	+	-	+	-	-	-	374	
V26	2006	Human blood	NSW											
V47	2007	Broiler litter	QLD	-	-	-	-	-	+	-	-	-	356	
V56	2007	Human	NT											
V60	2007	Human	NSW											
V48	2007	Human blood	SA	-	-	-	-	-	+	-	-	-	320	
V97-19	1997	Human	SA	-	-	-	-	-	+	-	-	-	326	
V98-02	1998	Human O/B	VIC											
V98-03	1998	Human O/B	VIC											
V98-04	1998	Human O/B	VIC											
V98-05	1998	Human O/B	VIC											
V98-06	1998	Human O/B	VIC											
V98-08	1998	Human O/B	SA											
V98-09	1998	Human O/B	SA	-	-	-	+	-	+	-	-	-	350	Cluster I
V98-10	1998	Human O/B	SA											
V98-11	1998	Human O/B	SA											
V98-12	1998	Human O/B	SA											
V98-13	1998	SDT O/B	SA											
V98-14	1998	Human	SA											
V98-16	1998	Beef meat	QLD											
V24	2006	Chicken meat	QLD											
V98-01	1998	Human	VIC											
V98-07	1998	Human	VIC											
V25	2006	Human	NT											
V44	2005	Human	NT											
V53	2007	Human	NT	-	-	-	-	-	+	-	-	-	350	Cluster II
V68	2008	Human blood	NT											
V70	2008	Human blood	NT											
V03	2007	Human	NT											
V15	2006	Human	NSW											
V34	2006	Chicken meat	QLD											
V64	2008	Human	SA	+	-	-	-	-	+	-	-	-	350	
V96-18	1996	Chicken meat	QLD	-	-	+	+	-	+	-	-	-	344	
V29	2006	Broiler litter	NSW	-	-	-	+	-	+	-	-	-	344	
V97-17	1997	Human	QLD											
V37	2006	Broiler litter	VIC	-	-	-	-	-	+	-	-	-	344	Cluster III
V69	2008	Broiler litter	NSW											
V58	2007	Broiler litter	NSW	-	+	-	-	-	+	-	-	-	344	
V30	2006	Layer litter	NSW	-	-	+	-	+	+	-	-	-	344	
V57	2007	Broiler litter	NSW											
V98-15	1998	Human	NSW	-	-	-	-	+	+	-	-	-	344	

Fig. 3.8 Dendrogram showing genetic relationship between all the PT 8 isolates collected in this study based on the MAPLT / STTR-5 typing results. All the outbreak isolates were in cluster I that also contained a 2006 PT 8 isolate. Two MAPLT / STTR-5 profiles were shared between isolates collected from the recent years and from the 1990s (clusters II and III).

SDT = sun-dried tomatoes ; O/B indicated isolates related to the PT 8 outbreak in 1998

3.4 DISCUSSION

Salmonella enterica serovar Virchow is one of the more public health significant *Salmonella* serovars that is endemic in Australia. Food-borne outbreaks associated with *S.* Virchow have been reported and frequently associated with the predominating phage type 8. As with many other *Salmonella* serovars, PFGE has been routinely employed for further differentiating within phage type groups to discriminate between the outbreak-associated isolates and the sporadic isolates. While PFGE has been shown to be helpful in assisting in *S.* Virchow PT 8 outbreak epidemiological studies in endemically affected countries, it may be of limited use in countries such as Israel as *S.* Virchow has only recently emerged as a problem and that PFGE suggested close genetic similarities between isolates (Weinberger *et al.*, 2006). Furthermore it is worthwhile to adopt new typing procedures that are comparatively simple and more rapid to perform than PFGE, and also provide objective typing data.

In this study, although four typing approaches were assessed to differentiate forty-three epidemiologically-unrelated *S.* Virchow isolates, ultimately three methods, PFGE, MAPLT and composite assay of MAPLT / STTR-5 were examined. As indicated from the DI values (Table 3.6), the 9-loci MAPLT scheme could provide an equivalent level of strain differentiation as PFGE for *S.* Virchow. Furthermore, high DI values were obtained from both methods when separating the non-PT 8 isolates (Table 3.7), and that both PFGE and MAPLT showed the ability to detect genetic differences between isolates of same non-PT 8 phage types as described in sections 3.3.1.2 and 3.3.2. These results suggested the high level of genetic diversity among the non-PT 8 *S.* Virchow isolates in general and the possibility of using MAPLT for routine surveillance of *S.* Virchow.

Both PFGE and MAPLT clustered most of the PT 8 isolates together and that the isolates were derived from human and chicken-related samples. The results suggested a possible close genetic similarity between PT 8 isolates from humans and chicken-related samples supporting the view that chickens are likely to be a common source of *S.* Virchow for humans in Australia as with other countries (Semple *et al.*, 1968; Maguire *et al.*, 2000; Adak and Threlfall, 2005). As stated in section 3.3.1.2, one human PT 8 isolate V59 generated the PFGE and MAPLT profiles different to the remaining human PT 8 isolates. The epidemiological information indicated that this patient had a history of overseas travel. This result indicated that this case of PT 8 infection may not have been acquired in Australia, while providing evidence of genetic diversity in prophage populations between PT 8 isolates from different countries.

It was noted that isolate V06 from chicken meat was not typable by phage typing and was clustered with the other chicken-related PT 8 isolates in PFGE profile 4 and MAPLT profile 4. This result suggested a close genetic relationship between the untypable V06 isolate with the PT 8 chicken-related isolates.

This conclusion is supported by the strong association between PFGE profile 4 and MAPLT profile 4 with the chicken-related PT 8 isolates, and that none of the non-PT 8 isolates from chicken-related samples generated PFGE profile 4. One possible reason explaining the different phage typing result of the chicken meat isolate V06 is that the isolate has undergone phage type conversion from PT 8 to untypable, for instance through lysogenic infection by a phage not detected by MAPLT. Phage type conversion have been demonstrated in a number of *Salmonella* serovars including Typhimurium (Mmolawa *et al.*, 2002), Enteritidis (Chart *et al.*, 1989; Rankin and Platt, 1995; Baggesen *et al.*, 1997) and Heidelberg (Harvey *et al.*, 1993). Therefore it is also possible that this occurs in *S.* Virchow as though the changes are not reflected by PFGE or MAPLT as with a previous observation where the phage-type converted isolates and their parental isolates produced indistinguishable PFGE profiles (Mmolawa *et al.*, 2002).

Both PFGE and MAPLT showed similar discrimination between the *S.* Virchow PT 8 isolates, combining MAPLT with MLVA locus STTR-5 showed comparatively higher differentiating ability within this phage type and give the highest DI value at 0.87. A more important observation of this study was that the 10-loci MAPLT / STTR-5 scheme was able to confirm the identical genetic relationship between the outbreak isolates from 1998 (Fig. 3.6). However as demonstrated in Figs. 3.7 and 3.8, some of the PT 8 isolates collected in the 1990s generated the same PFGE profile and MAPLT profile as the majority of the recently collected PT 8 isolates. This observation suggests there may be a persistent *S.* Virchow clone harboured in Australia that may hamper the effectiveness of MAPLT / STTR-5 method for local epidemiological typing. Improved discrimination may be achieved through identifying additional MAPLT loci. As suggested from this study, a diverse range of prophage elements was detected in PT 8 isolates including some elements that were infrequent in the *S.* Virchow population. For example, isolate V64 contained locus $ninB_{P22}$ while isolate V58 contained locus $SB04_{ST64B}$, and various loci of phage SfV targeted in the study. All these prophage loci were rarely detected among *S.* Virchow isolates but were shown useful for subdivision within PT 8 (Appendix 2.1).

This study suggested that development of a MLVA scheme for *S.* Virchow was not effective using the previous described MLVA loci. In agreement with previous studies, allelic diversity of MLVA loci varied between serovars (Ramisse *et al.*, 2004; Ross and Heuzenroeder, 2008, 2009). One exception to this was locus STTR-5, which has displayed allelic variation in all the serovars including *S.* Virchow (Lindstedt *et al.*, 2003; Ramisse *et al.*, 2004; Witonski *et al.*, 2006; Boxrud *et al.*, 2007; Ross and Heuzenroeder, 2008).

While determining the typing values of the published MLVA loci, a search of useful loci was also conducted based on the draft genome of *S.* Virchow strain SL 491 (contig 1: NCBI reference number ABFH02000001; contig 2: ABFH02000002, and contig 3: ABFH02000003) (Tables 3.8a and b). In total, seventy-nine regions containing tandem repeats were found and they were from contigs 1 and 2. The program did not find any MLVA loci in the contig 3. Several loci has been previously described that include loci STTR-2, -3, -5, -7 that were described by Lindstedt *et al.,* (2003); and locus 2628542 that was described by Witonski *et al.,* (2006). Additionally, some of the tested MLVA loci were not recognised by the TRF program even though DNA sequences of these loci were found within the contigs, for example STTR-9, Sal02 and Sal04. Three MLVA loci including SE01, SE02 that were derived from *S.* Enteritidis (Boxrud *et al.*, 2007) and STTR-10 that were derived from *S.* Typhimurium (Lindstedt *et al.*, 2004) were not recognised by the TRF program, nor were they amplified from any *S.* Virchow isolates in this study. It was noted that loci SE01 and SE02 were also not found in *S.* Infantis (Ross and Heuzenroeder, 2008), while STTR-10 was not detected in *S.* Enteritidis (Ross and Heuzenroeder, 2009). The results suggested that these loci are serovar specific and illustrate the necessity to obtain at least one sequenced genome of a serovar to effectively ascertain the MLVA loci that would be contained within it. Furthermore, the availability of multiple genomes of a serovar would further increase the chance of identifying discriminative MLVA loci considering some MLVA loci such as STTR-10 may not be detected in all *S.* Typhimurium isolates (Ross *et al.*, 2011) and would have been missed if the sequenced *S.* Typhimurium strain did not contain it.

Table 3.8a Locations of twenty-two selected tandem repeat sequences in the *S.* Virchow strain SL 491 contig 1 (NCBI accession no ABFH02000001)

Locations in genome	Repeat length (bp)	No. of repeats	Total SNPs (bp)	Previously described MLVA loci
99301 - 99358	15	3	7	
293101 - 293133	15	2	1	
315027 - 315057	10	3	0	
389064 - 389088	12	2	0	
659631 - 659673	21	2	3	
730952 - 731008	23	2	1	
1022767 - 1022803	15	5	2	
1221814 - 1221848	15	2	1	
1263077 - 1263110	17	2	0	
1290802 - 1290885	42	2	5	
1293145 - 1293241	42	2	2	
1541080 - 1541537	33	13	23	STTR-3
1541515 - 1541587	27	2	5	
1713043 - 1713103	24	2	4	
1722551 - 1722642	33	2	8	
1743316 - 1743352	18	2	2	
1781426 - 1781469	21	2	3	
1849158 - 1849198	21	2	3	
1995037 - 1995104	6	11	0	STTR-5
2439992 - 2440016	12	2	0	
2502511 - 2502649	36	2	8	2628542
2785474 - 2785506	15	2	0	
2788007 - 2788622	60	10	104	STTR-2
2851908 - 2851963	28	2	0	
2865250 - 2865279	15	2	1	
3014534 - 3014565	12	7	0	

Table 3.8b Locations of twenty-two selected tandem repeat sequences in the *S.* Virchow strain SL 491 contig 2 (NCBI accession no ABFH02000002).

Locations in genome	Repeat length (bp)	No. of repeats	Total SNPs (bp)	Previously described MLVA loci
568333 - 568838	93	5	30	
725821 - 725849	11	2	0	
725884 - 725915	11	2	0	
953806 - 953841	17	2	2	
985959 - 985983	6	4	0	
1109062 - 1109432	39	9	21	STTR-7
1110271 - 1110320	18	2	5	
1275259 - 1275294	18	2	2	
1322886 - 1323244	45	8	27	
1588388 - 1588555	58	2	9	
1804952 - 1805000	22	2	2	

These loci were identified by Tandem Repeat Finder (Benson 1999) and contain direct tandem repeats (<100bp). Number of repeat units was adjusted to disregard the additional bases being considered as "partial repeat unit" by the program. SNP = single nucleotide polymorphism detected in the repeat units.

3.5 CHAPTER SUMMARY

Two alternative methods were developed in this study for differentiation of *S.* Virchow isolates. One method was a 9-loci MAPLT scheme that was demonstrated to have the similar differentiating ability with the benchmark 'gold-standard' method PFGE. Furthermore, both PFGE and the 9-loci MAPLT scheme showed similar ability in sub-dividing the predominating PT 8 isolates. MLVA typing was performed using previously described MLVA loci but only locus STTR-5 showed allelic variation. This led to the development of a combined MAPLT / STTR-5 typing scheme comprising all nine prophage primers of the MAPLT scheme and MLVA locus STTR-5. This method demonstrated an enhanced differentiation of the PT 8 isolates and the capacity to detect genetic linkages between outbreak isolates. Future investigation should be carried out to further improve the typing ability of the current composite assay by examining the MLVA loci identified from the sequenced *S.* Virchow strain SL 491 in conjunction with identifying additional discriminative MAPLT primers.

CHAPTER 4 MOLECULAR TYPING OF *SALMONELLA* BOVISMORBIFICANS

4.1 INTRODUCTION

Salmonella enterica serovar Bovismorbificans or *S.* Bovismorbificans is among the less commonly isolated serovars on a worldwide basis (Hendriksen *et al.*, 2011). With the exception of the Oceania region, *S.* Bovismorbificans was reported from the European region as 14th most commonly isolated serovars in recent years (Hendriksen *et al.*, 2011). For the two countries in the Oceania region, *S.* Bovismorbificans was not detected in New Zealand between 2001 and 2007 (Hendriksen *et al.*, 2011), while it has always been endemic in Australia and has ranked among the top 10 most common serovars from humans as early as 1987 (ASRC 1986-2009; Murray 1994). In recent years, between 1999 and 2009, the incidence rate of *S.* Bovismorbificans in humans has been generally below 10% however this figure reached 11.3% in 2001 and 15% in 2006 due to the occurrence of two outbreaks (ASRC 1986-2009). An outbreak in 2001 was confined to the Australian state of Queensland where lettuce was the source (Stafford *et al.*, 2002). A second outbreak in 2006 was caused by a nationally distributed processed meat product (salami) that impacted two states, Victoria and South Australia (OzFoodNet, 2006). In other parts of the world, a nationwide outbreak of *S.* Bovismorbificans has occurred in Germany, where raw pork mince was identified as the source disseminating the *Salmonella* organisms through various processed pork products (Gilsdorf *et al.*, 2005). The most serious outbreaks of *S.* Bovismorbificans occurred in Finland. This is because food products are traded internationally, and *S.* Bovismorbificans has crossed national borders from Australia to Finland causing two large sprout-borne outbreaks (Pouhiniemi *et al.*, 1997). In more recent years, another nationwide outbreak of *S.* Bovismorbificans occurred in Finland due to the consumption of contaminated sprouted alfalfa seeds (Rimhanen-Finne *et al.*, 2011).

Before the introduction of molecular methods, bacteriophage (phage) typing was used to differentiate outbreak-related isolates from the unrelated ones during *Salmonella* outbreaks. The first phage typing scheme known for *S.* Bovismorbificans was established by the Australian *Salmonella* Reference Centre (ASRC) which is able to distinguish 36 phage types using a panel of 10 phages (Liesegang *et al.*, 2002). From this study, it was noted that a few (five) phage types were predominant in the countries where *S.* Bovismorbificans is commonly isolated. As mentioned in section 1.2.4.2.3, determination of phage types is subjective in nature and is highly reliant on the expertise of the operators in particular to distinguish between phage types with similar reaction patterns. One example is the *S.* Bovismorbificans phage types 12 and 14, where both patterns consist of lysis patterns to the panel phages but are distinguished due to the weaker reactions to the phages 1 to 5 for phage type 12 (Liesegang *et al.*,

2002). Ideally an additional typing method providing supplementary data to confirm possible epidemiological links between isolates of the same phage types or phenotypically closely related phage types involved in outbreaks is required.

A number of molecular methods have been applied to *S.* Bovismorbificans isolates and their usefulness in epidemiological typing of this serovar have been measured. These include ribotyping and IS*200* typing, both of which showed subdividing ability within *S.* Bovismorbificans, but only to the extent of determining relationships for long-term or global epidemiological studies (Ezquerra *et al.*, 1993; Nastasi *et al.*, 1994). Plasmid profiling had a comparatively higher differentiating ability (Ezquerra *et al.*, 1993; Liesegang *et al.*, 2002). However the typing capacity of plasmid profiling as applied to *S.* Bovismorbificans may be restricted as a number of isolates do not contain plasmids for differentiation (Ezquerra *et al.*, 1993; Liesegang *et al.*, 2002). In contrast, pulsed-field gel electrophoresis (PFGE) achieved total typability and displayed a high differentiating ability. As demonstrated by Liesegang and co-workers (2002), application of PFGE alone generated 28 PFGE patterns from 162 *S.* Bovismorbificans isolates compared to 17 plasmid profiles and 10 ribotypes. By combining PFGE with phage typing a total of 50 combined types were seen. Therefore it was suggested to apply PFGE with phage typing for local or outbreak epidemiological studies (Liesegang *et al.*, 2002).

However as stated in section 1.2.4.3.3, while PFGE appears to be the most effective molecular typing method for *S.* Bovismorbificans, using PFGE may further increase subjectivity of the typing data as the band patterns are also interpreted based upon personal judgement, though to a lesser extent than phage typing (Tenover *et al.*, 1994; Ross and Heuzenroeder, 2005a). Furthermore the methodology is time-consuming, tedious, and band patterns from different gels can be difficult to compare even with computer software (Lindstedt *et al.*, 2003). Consequently, newer molecular typing approaches that are fast, easy to perform, reproducible, and enable objective data generation and exchange are being developed and evaluated for typing various significant *Salmonella* serovars.

A high level of strain differentiation within *Salmonella* serovars has been shown using some recently developed typing methods including the PCR-based typing methods, multiple-locus variable-tandem repeat analysis (MLVA) and multiple amplification of prophage locus typing (MAPLT). With MLVA, allelic variation at targeted loci containing tandem repeats are detected as amplified fragment length variations for strain differentiation (Lindstedt *et al.*, 2003). Similar to MLVA, MAPLT also detects micro-variations at bacterial genomes, from which prophage loci in the *Salmonella* genomes are targeted (Ross and Heuzenroeder, 2005a). As indicated from previous studies, genetic elements related to phages are widespread in *Salmonella* and are frequently found to vary even between genetically closely related strains (Thomson *et al.*, 2004; Hermans *et al.*, 2005; Cooke *et al.*, 2007). This is due to phages

evolving through horizontal gene exchange between bacteria leading to groups of genetically mosaic-related phages infecting the same hosts.

Currently, PFGE is the 'gold standard' typing method for epidemiological typing of *S.* Bovismorbificans. While being discriminative in this serovar, PFGE lacks the practical advantages of the recently proposed PCR-based methods such as MLVA and MAPLT that generate objective typing data and are comparatively fast and simple to perform. Therefore this chapter aimed to assess the typing capacity of these two methodologies with respect to *S.* Bovismorbificans to indicate whether they can be used as the alternatives to PFGE. Furthermore, the typing ability of a composite assay of MAPLT / MLVA was assessed and compared with that of MAPLT and MLVA when they were performed individually. Finally, capacity of the composite assay of MAPLT and MLVA for outbreak epidemiological typing was determined.

4.2 MATERIALS AND METHODS

This chapter described the development of MAPLT, MLVA and a composite assay of MAPLT / MLVA for typing *S.* Bovismorbificans. Genomic DNA of the *S.* Bovismorbificans isolates, the procedures involved in MAPLT, MLVA and PFGE, and the subsequent data analysis were carried out as in Chapter 2, unless otherwise indicated. The MAPLT and MLVA primers used in this chapter are listed in Table 2.1b and Table 2.2 respectively. The following sections advise the bacterial isolates and the methods specific to this chapter.

4.2.1 Bacterial Isolates

A total of seventy-three *S.* Bovismorbificans isolates were used in the study that were collected from the Australian *Salmonella* Reference Centre (ASRC), SA Pathology, Adelaide, Australia. Sixty of the isolates were from humans, animals or the related food products in Australia and collected between 2005 and 2008. These isolates were not epidemiologically-related. The remaining thirteen *S.* Bovismorbificans isolates were collected from a PT 11 food-borne outbreak that occurred in 2006 (OzFoodNet 2006). All isolates were previously serotyped and phage-typed by ASRC.

4.2.2 Direct DNA extraction from non-viable PT 11 outbreak isolates

In 2006, a food-borne outbreak caused by *S.* Bovismorbificans PT 11 was reported that affected a total fifteen patients across two Australian states (OzFoodNet 2006). The outbreak source was confirmed to be the nationally distributed processed meat product capocollo (salami) from which the *S.* Bovismorbificans PT 11 organisms were isolated (OzFoodNet 2006). A total of sixteen isolates associated with the outbreak investigation were obtained to determine the ability of the composite assay

of MAPLT / MLVA in outbreak epidemiological typing. However none of the isolates could be revived from the storage media (semi-solid media) although a number of culture media and the supplement Ferrioxamine E (Reissbrodt *et al.*, 2000) were used. Therefore DNA extraction was carried out directly from the storage media.

QIAamp® DNA mini kit (Qiagen, Hilden, Germany) was used for this purpose. The procedure was performed based on the blood and fluid protocol as per the manufacturer's instructions with modifications at steps involving lysis of bacterial cells. Each tube containing the stored cells was vortexed vigorously to the agar media into fine pieces. Then 300µl of the agar was transferred into a fresh 2ml tube using a sterile wide-bore pipette. An equal amount of sterile water was added and the tubes were incubated at 65C until the agar was melted (approximately 30 min). The tubes were left in room temperature to cool down then 300µl of AL buffer and 30µl of proteinase K both provided by kit were added and mixed. The mixture was then incubated at 56C for 20 min for cell lysis. Tubes were cooled down at room temperature and centrifuged briefly to bring the liquid down before an addition of 300µl of 100% alcohol. After tubes were again vortexed and centrifuged briefly, the contents in each tube was transferred to the provided column. The subsequent steps involving DNA wash were performed as described in the kit manual. The extracted DNA was eluted in 50µl EB buffer.

PCR amplification of the house keeping gene locus *sucA* was carried out to ensure that the extracted bacterial DNA was suitable for PCR reactions as described in section 2.5.2. Using the online sequence search engine (BlastN, http://blast.ncbi.nlm.nih.gov/Blast.cgi), it showed that locus STTR-5 is specific to *Salmonella*. Therefore PCR amplification of locus STTR-5 was also carried out to ensure that the extracted DNA was of *Salmonella* origin. The PCR reaction mix was prepared as described in section 2.5.1. The PCR cycling condition included one cycle of denaturing at 94C for 10 min; 50 cycles of amplification each with a step of 94C for 30 sec, 54C for 30 sec, and 72C for 50 sec; following by one cycle of final extension at 72C for 7 min. Gel electrophoresis was applied to detect PCR products as described in section 2.5.3.

4.3 RESULTS

4.3.1 PFGE

The sixty non-outbreak-related *S.* Bovismorbificans isolates generated 26 PFGE profiles. Sixteen unique profiles were generated by single isolates with ten profiles seen from multiple isolates (Fig. 4.1). The results indicated that PFGE classified isolates independently to the phage types of the isolates. Seven of the 10 PFGE profiles containing multiple isolates included isolates of various phage types. Likewise, isolates with the same phage type often produced different PFGE profiles. For example the

PT 24 isolates (n = 24), yielded 12 PFGE profiles that were widely distributed in the dendrogram (Fig. 4.1). Similar observations were seen for PT 13 isolates (n = 8, 3 PFGE profiles) and PT 14 isolates (n = 9, 6 PFGE profiles).

4.3.2 MAPLT

Primers from the previously developed MAPLT schemes for *S.* Typhimurium (Ross and Heuzenroeder 2005) and *S.* Virchow (Chapter 3) were evaluated for their usefulness in differentiating *S.* Bovismorbificans isolates in this study. It was found that several established primers amplifying gene loci of phages P22, ST64T, ST64B, Gifsy-1, 186, Fels-2 and SopEϕ were differentially detected in the isolates and therefore incorporated in this assay (Table 4.1). Two additional primers were developed based on the phage DNA sequences identified using DOP-PCR (section 3.2.2.3). One primer set amplified a prophage tail sheath locus from the sequenced *S.* Newport strain SL 254 (NCBI accession no CP001113), while the other primer set amplified a novel phage locus showing similarity to part of the phage 186 *cII* gene. These two prophage gene sequences were both identified from the mitomycin-C lysate of isolate B33.

In total, twenty-six prophage loci were targeted that could be detected from at least one isolate (Table 4.1). Similarly, all isolates amplified at least one selected prophage locus. Primers amplifying Gifsy-related gene loci STM2594 and STM1005 gave the highest numbers of positive amplification results. These two loci were amplified from 58 and 59 of 60 isolates respectively. However it was noted that the least amplified phage loci were also Gifsy-1 and -2 related, each was amplified by one isolate only. While there was a high degree of variability in the presence of the targeted gene loci of phages Gifsy-1 and Gifsy-2, a consistently low rate of amplification was observed from the five selected ST64B phage loci. Locus $SB04_{ST64B}$ was amplified from 4 isolates and was the most frequently detected among the five ST64B phage loci targeted. All the selected ST64B gene loci are from different functional modules, with the exceptions of loci $SB04_{ST64B}$ and $SB06_{ST64B}$ both of which locate in the head assembly module (Mmolawa *et al.*, 2003).

In most cases when multiple gene loci of the same sequenced phages were targeted, they were detected from the same isolates. For example, five gene loci of phage Fels-2 were examined in this study; three including STM2695, STM2714 and STM2738 were detected from the same 21 isolates. The other two Fels-2 gene loci STM2697 and STM2739 were detected from the same 44 isolates. Results also showed that genes locating in close proximity in the same functional modules showed a low degree of variability in presence in the isolates. For example the two gene loci of cro_{ST64T} and $c2_{ST64T}$ located at the immunity module of phage ST64T showed no variation in distribution and were detected from the same 28 isolates. In contrast the distribution of the putative gene loci encoding Fels-

2 CI protein (STM2738) and integrase protein (STM2739) located in the immunity and lysogeny modules respectively showed marked variation between isolates. Overall none of the isolates contained all the targeted gene loci of the sequenced phages indicating a genetically mosaic relationship between the integrated prophages and the sequenced phages.

Similar to PFGE, MAPLT classified the isolates with no correlation to the sources, time and geographical locations of the isolates. A total of 22 MAPLT profiles were observed using a set of eleven MAPLT primers (Table 4.2). Among these 22 MAPLT profiles, 13 were generated by more than one isolate and MAPLT profile 9 was seen in the highest number of isolates (n = 9). Nine MAPLT profiles were uniquely seen from single isolates with three produced by isolates of phage types uniquely included in this study. These included MAPLT profiles 8, 9 and 17 which were produced by isolates B02 (PT40), B03 (PT11) and B24 (PT39) respectively. It was noted that the result was similar to that of PFGE where only isolates B02 (PT40) and B03 (PT11) generated unique PFGE profiles (Fig. 4.2).

Both MAPLT and PFGE demonstrated the diverse genetic relationship between isolates that were untypable by phage typing, where all six PT untypable isolates included were separated by both methods. Moreover, four of the six PT untypable isolates were assigned in the same way by both MAPLT and PFGE although none of these isolates were related epidemiologically. For example, isolate B09 generated the identical PFGE profile 5 and MAPLT profile 3 as isolate B39, and isolate B07 had the identical PFGE profile 22 and MAPLT profile 4 as isolates B12 and B36. With regard to the two PT untypable isolates B06 and B08, isolate B06 generated a unique PFGE profile 23; while MAPLT grouped B06 with B48 (PFGE profile 20) into MAPLT profile 5. Similarly the PT untypable isolate B08 had a unique PFGE profile 13 but was grouped by MAPLT with the 3 other isolates that had PFGE profile 12. It was observed that although PFGE separated these PT untypable isolates from the isolates that were otherwise clustered by MAPLT, only minor differences between their PFGE profiles were observed (Fig. 4.3). Between PFGE profiles 12 and 13, there was a three-fragment difference, while between PFGE profiles 20 and 23 there were a two-fragment difference. According to Tenover *et al.* (1995), one random genetic event could produce PFGE profiles differing up to three fragments and the isolates were considered genetically closely related.

Intra-phage type differentiating ability of MAPLT was examined in particular for the *S.* Bovismorbificans phage types that are commonly seen in Australia. These phage types included PT 24, 13 and 14. MAPLT differentiated the PT 24 isolates (n = 23) into ten MAPLT profiles and most isolates (n = 8, 35%) generated the MAPLT profile 6. The nine PT 14 isolates generated five MAPLT profiles and most PT 14 isolates (n = 3, 33%) had the MAPLT profile 20. In contrast, MAPLT only differentiated the eight PT 13 isolates into 3 MAPLT profiles, with MAPLT profile 14 containing the most PT 13 isolates (n = 5, 62%).

Similarly PFGE grouped the eight PT 24 isolates having MAPLT profile 6 into PFGE profile 19, however most of the remaining PT 24 isolates were grouped differently by MAPLT and PFGE. The PT 14 isolates generating MAPLT profiles 20 (n = 3) and 21 (n = 2) produced PFGE profiles 16 and 12 respectively. However the two PT 14 isolates with MAPLT profile 18 generated two distinct PFGE profiles 8 and 17. For the PT 13 isolates, the five isolates with MAPLT profile 14 were grouped into PFGE profile 1. The remaining 3 isolates were grouped differently by the two methods.

4.3.3 MLVA

MLVA typing was carried out using the previously published MLVA loci that were from the MLVA schemes for *S.* Typhimurium (Lindstedt *et al.*, 2003) and *Salmonella* species (Ramisse *et al.*, 2004). In total fourteen MLVA loci were examined (Table 4.3). The highest number of alleles (9 alleles) was observed at locus STTR-5, where a high proportion of isolates (68.3%) amplified the allele (338 bp) containing nine tandem repeats. Similarly while seven alleles were seen at locus STTR-6, this locus was not detected in most isolates (65%). There were six alleles observed for locus STTR-9, where 40% of the isolates amplified this locus (253 bp) containing four repeats. The remaining STTR loci show little or no allelic variation, while locus STTR-10 was not detected from any isolate. The six MLVA loci described by Ramisse *et al.* (2004) showed no allelic variation (Table 4.3).

It was noted that the amplified products of loci STTR-3 and STTR-7 contained tandem repeat units different to those that have been reported for *S.* Typhimurium with regard to the truncations. In this study the amplified STTR-3 loci contained tandem repeats that were all 33 bp long, where the STTR-3 locus seen in *S.* Typhimurium has variable truncations at the tandem repeat units at the 5' end (Lindstedt *et al.*, 2003; Ross *et al.*, 2011). Conversely, the STTR-7 loci observed in this study had a 9-bp truncation at the first tandem repeat unit, but the STTR-7 locus observed from the examined *S.* Typhimurium isolates did not have any truncated tandem repeat units (personal communication, Dr. Ian Ross, SA Pathology, Adelaide).

Like PFGE and MAPLT, MLVA also did not group isolates in the same manner as phage typing (Fig. 4.4). Among the 21 MLVA profiles, 7 MLVA profiles were observed from more than one isolate but only 2 profiles (MLVA profiles 1 and 15) were generated by isolates of the same phage type. Likewise, isolates of the same phage type often generated a number of MLVA profiles. For example, the twenty-three PT 24 isolates generated nine MLVA profiles, while the nine PT 14 isolates generated four MLVA profiles. The only exception was PT 13 where all the PT 13 isolates had MLVA profile 17. It was also observed that there was little concordance in grouping between the three molecular methods and that isolates having different PFGE and MAPLT profiles often generated identical MLVA profiles. The two

exceptions were seen for MLVA profiles 1 and 15 that were generated by isolates of the same phage type, PFGE and MAPLT profiles for each MLVA profile respectively.

4.3.4 Composite assay of MAPLT / MLVA

A set of six MAPLT and three MLVA loci (Table 4.4) could be used at minimum to achieve the maximum differentiation of isolates into 35 profiles (Fig. 4.5). It was noted that isolates clustered in several MAPLT or MLVA profiles were further subdivided. For example the nine isolates generating MAPLT profile 6 were further differentiated into four MAPLT / MLVA profiles due to the variations of the number of tandem repeats at MLVA locus STTR-6. Similarly the four isolates with MAPLT profile 2 were separated into four MAPLT / MLVA profiles as the isolates showed differences in the number of tandem repeats at MLVA loci STTR-5 and STTR-9. On the other hand, the largest MLVA cluster (MLVA profile 17) was subdivided into seven MAPLT / MLVA profiles as the targeted prophage loci were differentially amplified from the seventeen isolates.

4.3.5 Comparison of the differentiating abilities between the four molecular typing approaches

The typing ability of each molecular method was measured by the Simpson's index of diversity (DI) (Table 4.5). Three typing methods, PFGE, MAPLT and the composite MAPLT / MLVA showed similar DI values. Apart from achieving the highest DI value, the composite assay of MAPLT / MLVA subdivided the 60 *S.* Bovismorbificans isolates into the most number of profiles. The DI value calculated for PFGE was almost the same to the MAPLT / MLVA system, however there were nine less PFGE profiles. In contrast, the DI value calculated for MLVA and the number of generated MLVA profiles were both the lowest among the four molecular methods.

The differentiating capacity of each molecular method within the common *S.* Bovismorbificans phage types was examined (Table 4.6). Based on the calculated DI values, the composite assay of MAPLT / MLVA was shown to be the most discriminative for PT 24, while the remaining three methods demonstrated similar but lower differentiating abilities. However in the case of PT 14, the combined MAPLT / MLVA was as discriminative as PFGE and MAPLT as indicated by the similar DI values. In comparison, MLVA showed a much lower discriminative ability for PT 14 with a DI value of 0.75. Likewise the three methods including the composite assay of MAPLT / MLVA, MAPLT and PFGE demonstrated the same discriminative ability for PT 13 while MLVA failed to differentiate any of the included PT 13 isolates.

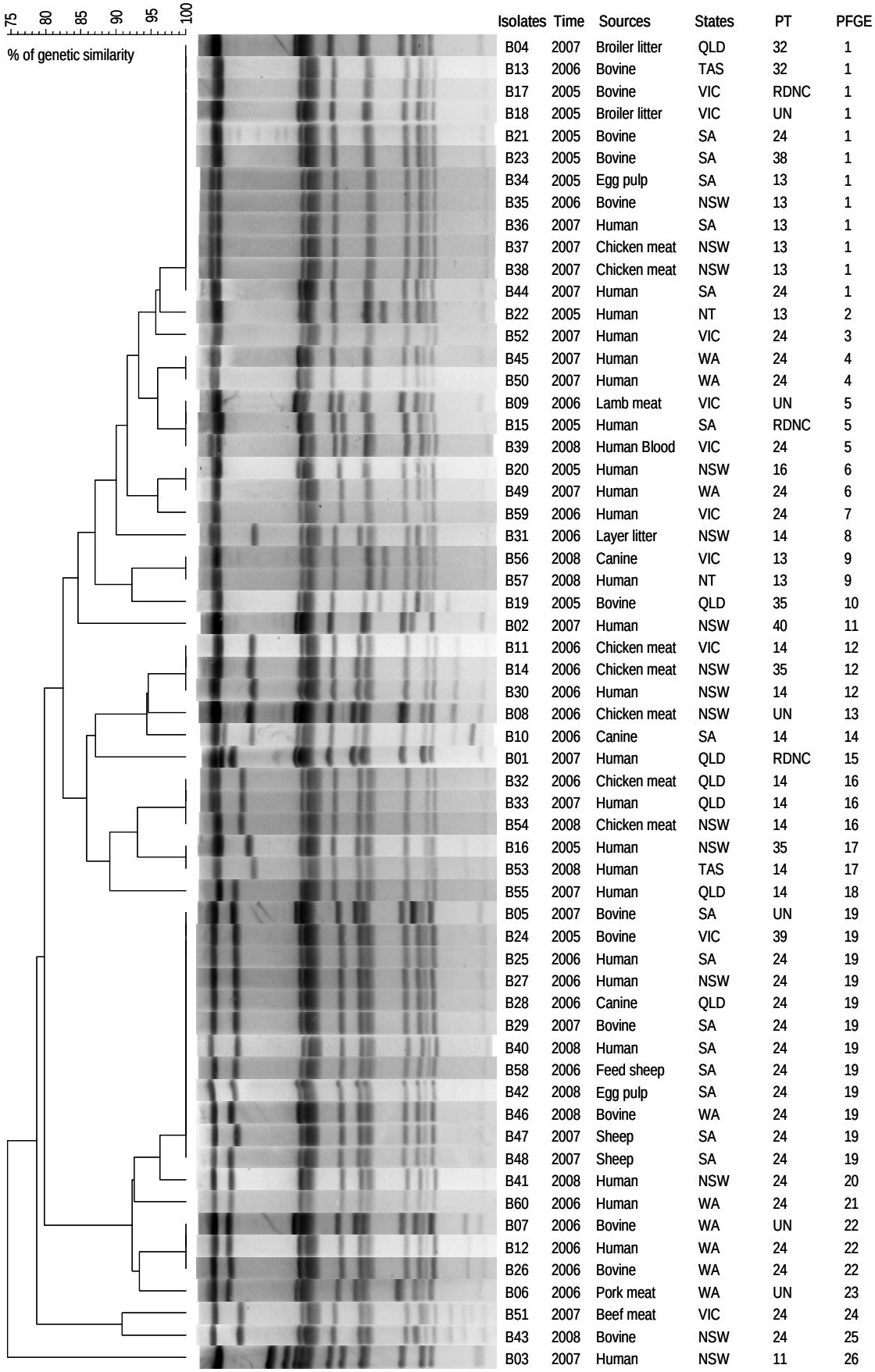


Fig. 4.1 Dendrogram constructed based on the PFGE patterns of the 60 *S.* Bovismorbificans isolates. The PFGE patterns were related by the BioNumerics program with the band tolerance level and optimisation set at 1.7% and 0.7% respectively. UN = untypable; RDNC = reacts but does not conform. Abbreviations for locations: NSW = New South Wales; NT = New Territory; QLD = Queensland; SA = South Australia; WA = Western Australia

Table 4.1 Positive amplifications of prophage gene loci from the 60 *S.* Bovismorbificans isolates

Prophages	Gene loci	Gene functions	No. of positive isolates
P22/ST64T	*cI*	CI protein	26
	cII	CII protein	28
P22	*ninB*	ninB protein	30
ST64B	SB04	Portal protein	4
	SB06	Major capsid protein	3
	SB21	Putative head assembly protein	2
	SB28	integrase	3
	SB38	CI protein	3
186	*gene G*	Tail protein	15
	gene O	Baseplate protein	22
	cII	CII protein	23

Prophages	Gene loci	Gene functions	No. of positive isolates
Fels-2	STM2695	Put. late control protein D	21
	STM2697	Tail protein	44
	STM2714	Lysis regulatory protein	21
	STM2739	Integrase	44
	STM2738	CI protein	21
Gifsy-1	STM2584	Type III secretive protein (GogB)	22
	STM2608	Terminase large subunit	22
Gifsy-2	STM1005	Integrase	59
	STM1032	Putative capsid protein	1

Prophages	Gene loci	Gene functions	No. of positive isolates
Gifsy-1/2	STM2594/ STM1041	Tail protein	58
Gifsy-1/2	STM2619/ STM1021	Unknown (NinG)	33
Gifsy-1/2	STM2632/ STM1008	Exodeoxyribonuclease	1
SopEϕ	*sop*E	Type III secretion protein (SopE)	7
Prophage in *S.* Newport SL254	A2929	Tail sheath protein	31
Prophage in isolate B33	Not applicable	Unknown	11

All prophage loci were amplified using primers listed in Table 2.1b

Table 4.2 Primers for the MAPLT scheme for *Salmonella* Bovismorbificans

Prophages	Gene loci	Gene functions	Primer sequences (5'→ 3')
P22/ST64T	*cI*	CI protein	[a] PTc1F: CTTTACCAATCTGAACCGCCG
			[a] PTc1R: CTGAGTTGTTTTGGCATAATTACTCC
P22	*ninB*	ninB protein	[a] ninBF1: AACCTTTGAAATTCGATCTCCAGC
			[a] ninBR1: CTTCGTCTGACCACTTAACGC
ST64B	SB04	Portal protein	SB04F: TGTCATACGACACCTATACCG
			SB04R: TGTTCTGCACCATGTGCAATG
	SB38	CI protein	[a] BIM1F: ATGGTGGCCTTGTCGACGC
			[a] BIM1R: GCTAACGTGAAGGATTTGTTCCG
186	*gene G*	Tail protein	P186GF: GACGTTCTCTCGATGACGATG
			P186GR: CAATGGCACTACCGGTAACAG
	gene O	Baseplate protein	P18BPF: GTGCGATTCGTAACCTCATCC
			P18BPR: GTTTGTTGGTGCATACCACCT
	cII	CII protein	P186cIIF: GACATAGCGGGATTAGTCTGC
			P186cIIR: GTCACAACATGGCGAAGCTG

prophages	Gene loci	Encoded proteins	Primer sequences (5'→ 3')
Fels-2	STM2697	Tail protein	Fels2GF: CTTCATTGAGCGCAAGACGC
			Fels2GR: CGTCTGCAGGTGATTCTTAATGC
	STM2714	Lysis regulatory protein	Fels2lysBF: TGACCTTTCCAGACGGCACT
			Fels2lysBR: TGGTTCTGGCGCTGGTACTT
SopEϕ	*sopE*	Type III secretion protein	[b] SopEF: CGAGTAAAGACCCCGCATAC
			[b] SopER: GAGTCGGCATAGCACACTCA
Prophage in *S.* Newport SL254	A2929	Tail sheath protein	SL254tailF: AGGCGGATTACCTGAAACGTC
			SL254tailR: ATATCCACCGCCTTCTTGCTC

[a] Primer sequences previously described by Ross and Heuzenroeder (2005)

[b] Primer sequences previously described by Drahovská *et al.,* (2007)

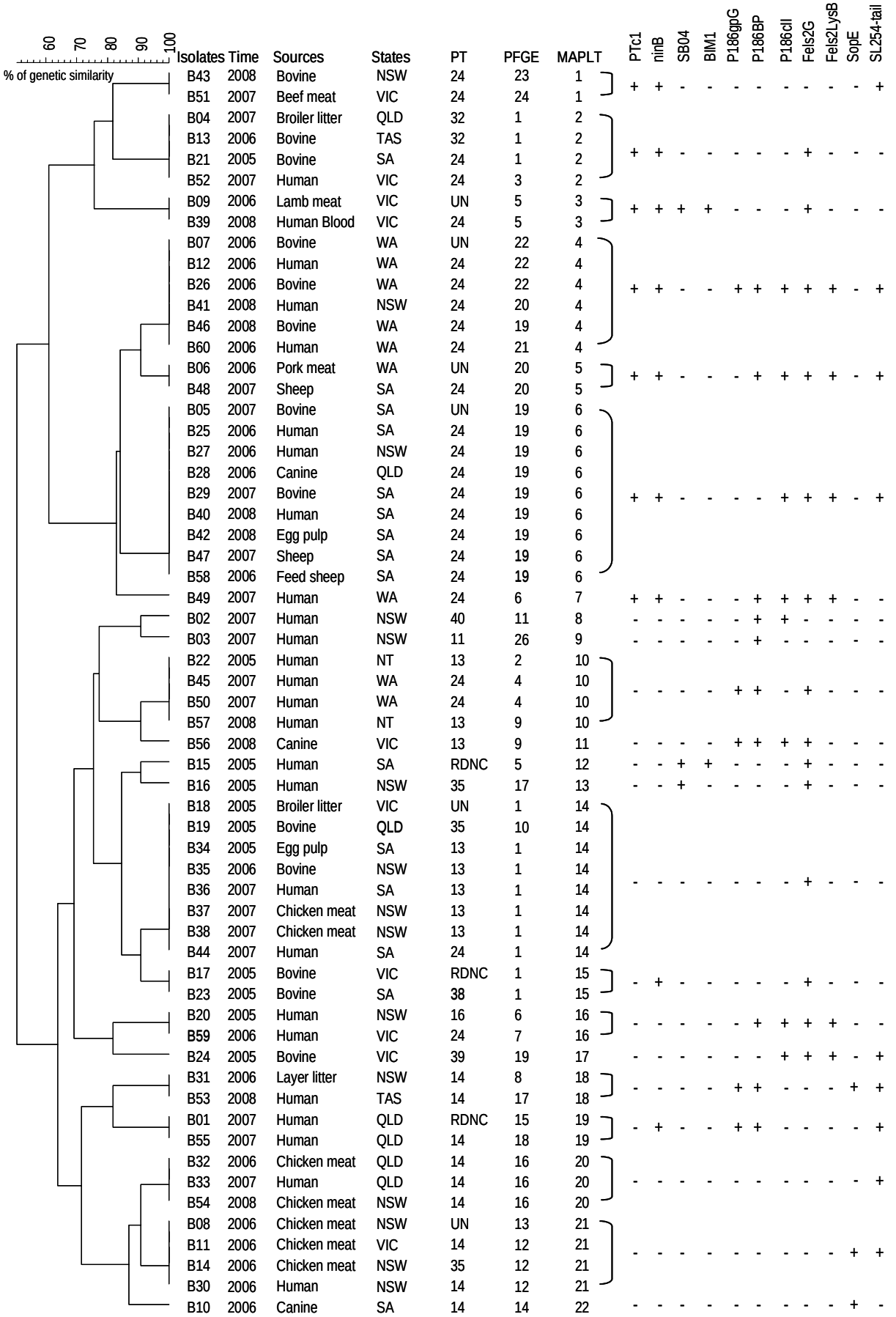
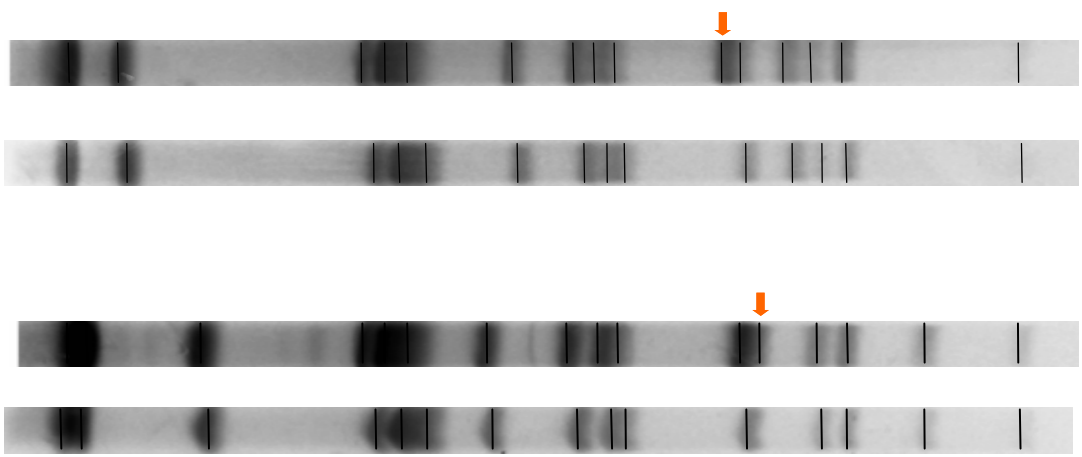


Fig. 4.2 Dendrogram showing genetic relationship of the 60 *S.* Bovismorbificans isolates based on the typing results of the 11-loci MAPLT scheme.

Symbol + indicated positive amplification of the prophage loci; symbol – indicated no PCR products detected for the prophage loci.



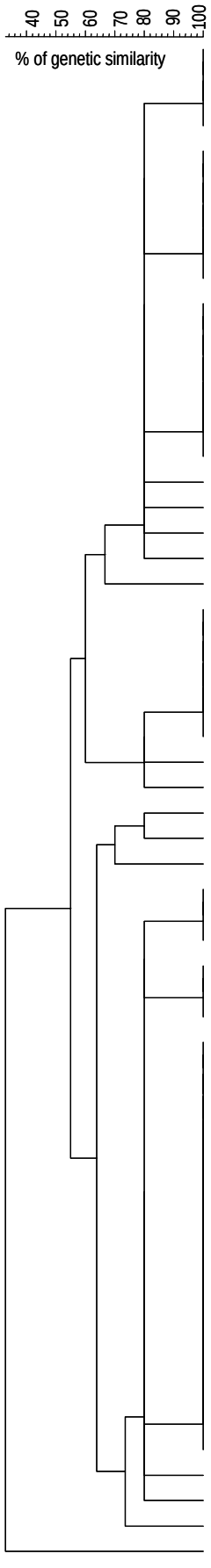
Isolates	MAPLT profiles	PFGE profiles
B06	5	23
B48	5	20
B08	21	13
B30	21	12

Fig. 4.3 Identical MAPLT profiles were observed between the phage type non-typable isolates with isolates of designated phage types. Pair-wise comparisons of the corresponding PFGE patterns also indicated the close genetic relationships where a minor one-fragment difference (red arrow) was observed within each pair of the PFGE patterns.

Table 4.3 MLVA loci tested in this study in the 60 *S.* Bovismorbificans isolates

MLVA loci	No. of alleles	[a] No. of tandem repeats ([b] no. of isolates)	References
STTR-2	2	9 (1), 10 (59)	Lindstedt *et al.,* (2003)
STTR-3	2	9 (1), 11 (59)	Lindstedt *et al.,* (2003)
STTR-5	8	8 (4), 9 (41), 10 (1), 13 (6) 14 (2), 17 (1), 18 (3), 19 (1), 20 (1)	Lindstedt *et al.,* (2003)
STTR-6	7	not amplified (39), 12 (1), 13 (8), 14 (4), 15 (6), 16 (1), 18 (1)	Lindstedt *et al.,* (2003)
STTR-7	1	7 (60)	Lindstedt *et al.,* (2003)
STTR-9	5	3 (2), 4 (26), 5 (29), 6 (2), 11 (1)	Lindstedt *et al.,* (2004)
STTR-10	Not applicable	Not amplified (60)	Lindstedt *et al.,* (2003)
Sal02	1	2 (60)	Ramisse *et al.,* (2004)
Sal04	1	0 (60)	Ramisse *et al.,* (2004)
Sal06	Not applicable	Not amplified (60)	Ramisse *et al.,* (2004)
Sal10	1	2 (60)	Ramisse *et al.,* (2004)
Sal15	1	2 (60)	Ramisse *et al.,* (2004)
Sal20	1	9 (60)	Ramisse *et al.,* (2004)
Sal23	1	3 (60)	Ramisse *et al.,* (2004)

All MLVA loci were amplified using primers listed in Table 2.2
[a] The number of tandem repeats contained within the amplified MLVA loci; [b] Number of isolates amplified with the MLVA alleles



Isolates	Time	Sources	States	PT	PFGE	MAPLT	MLVA	STTR2	STTR3	STTR5	STTR6	STTR9
B25	2006	Human	SA	24	19	6	1					
B28	2006	Canine	QLD	24	19	6	1					
B40	2008	Human	SA	24	19	6	1	734 (10)	492 (11)	338(9)	342 (14)	262 (5)
B42	2008	Egg pulp	SA	24	19	6	1					
B20	2005	Human	NSW	16	6	16	2					
B58	2006	Feed sheep	SA	24	19	6	2					
B59	2006	Human	VIC	24	7	16	2	734 (10)	492 (11)	338 (9)	348 (15)	262 (5)
B41	2008	Human	NSW	24	20	4	2					
B47	2007	Sheep	SA	24	19	6	2					
B48	2007	Sheep	SA	24	20	5	2					
B05	2007	Bovine	SA	UN	19	6	3					
B07	2006	Bovine	WA	UN	19	4	3					
B12	2006	Human	WA	24	22	4	3					
B26	2006	Bovine	WA	24	22	4	3	734 (10)	492 (11)	338 (9)	336 (13)	262 (5)
B27	2006	Human	NSW	24	19	6	3					
B46	2008	Bovine	WA	24	19	4	3					
B49	2007	Human	WA	24	6	7	3					
B06	2006	Pork meat	WA	UN	20	5	4	734 (10)	492 (11)	338 (9)	366 (18)	262 (5)
B29	2007	Bovine	SA	24	19	6	5	734 (10)	492 (11)	338 (9)	330 (12)	262 (5)
B52	2007	Human	VIC	24	3	2	6	734 (10)	492 (11)	338 (9)	0	262 (5)
B60	2006	Human	WA	24	21	4	7	734 (10)	492 (11)	338 (9)	354 (16)	262 (5)
B24	2005	Bovine	VIC	39	19	17	8	734 (10)	492 (11)	338 (9)	336 (13)	271 (6)
B08	2006	Chicken meat	NSW	UN	13	21	9					
B10	2006	Canine	SA	14	14	22	9					
B11	2006	Chicken meat	VIC	14	12	21	9					
B14	2006	Chicken meat	NSW	35	12	21	9	734 (10)	492 (11)	362 (13)	0	262 (5)
B30	2006	Human	NSW	14	12	21	9					
B53	2008	Human	TAS	14	17	18	9					
B04	2007	Broiler litter	QLD	32	1	2	10	734 (10)	492 (11)	332 (8)	0	262 (5)
B55	2007	Human	QLD	14	18	19	11	734 (10)	492 (11)	398 (19)	0	262 (5)
B16	2005	Human	NSW	35	17	13	12	734 (10)	492 (11)	368 (14)	0	244 (3)
B21	2005	Bovine	SA	24	1	2	13	734 (10)	492 (11)	338 (9)	0	244 (3)
B31	2006	Layer litter	NSW	14	8	18	14	734 (10)	492 (11)	368 (14)	0	271 (6)
B32	2006	Chicken meat	QLD	14	16	20	15					
B33	2007	Human	QLD	14	16	20	15	734 (10)	492 (11)	392 (18)	0	253 (4)
B54	2008	Chicken meat	NSW	14	16	20	15					
B17	2005	Bovine	VIC	RDNC	1	15	16					
B19	2005	Bovine	QLD	35	10	14	16	734 (10)	492 (11)	332 (8)	0	253 (4)
B23	2005	Bovine	SA	38	1	15	16					
B09	2006	Lamb meat	VIC	UN	5	3	17					
B13	2006	Bovine	TAS	32	1	2	17					
B15	2005	Human	SA	RDNC	5	12	17					
B18	2005	Broiler litter	VIC	UN	1	14	17					
B22	2005	Human	NT	13	2	10	17					
B34	2005	Egg pulp	SA	13	1	14	17					
B35	2006	Bovine	NSW	13	1	14	17					
B36	2007	Human	SA	13	1	14	17	734 (10)	492 (11)	338 (9)	0	253 (4)
B37	2007	Chicken meat	NSW	13	1	14	17					
B38	2007	Chicken meat	NSW	13	1	14	17					
B39	2008	Human Blood	VIC	24	5	3	17					
B51	2007	Beef meat	VIC	24	24	1	17					
B56	2008	Canine	VIC	13	9	11	17					
B57	2008	Human	NT	13	9	10	17					
B43	2008	Bovine	NSW	24	23	1	17					
B45	2007	Human	WA	24	4	10	17					
B50	2007	Human	WA	24	4	10	17					
B01	2007	Human	QLD	RDNC	15	19	18	734 (10)	492 (11)	404 (20)	0	253 (4)
B44	2007	Human	SA	24	1	14	19	734 (10)	492 (11)	344 (10)	0	253 (4)
B02	2007	Human	NSW	40	11	8	20	734 (10)	426 (9)	338 (9)	0	253 (4)
B03	2007	Human	NSW	11	26	9	21	674 (9)	492 (11)	398 (19)	0	316 (11)

Fig. 4.4 Dendrogram depicting genetic relationship between the 60 *S.* Bovismorbificans isolates based on the typing results from the five previously described MLVA loci.

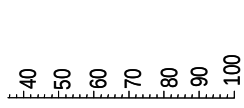
The MLVA results were presented as amplified fragment length in bp. The number of repeat units for each locus was indicated in the brackets.

Table 4.4 Primers for the *S.* Bovismorbificans composite MAPLT / MLVA scheme

	Gene loci	Primer sequences (5'→ 3')
MAPLT	P22-*ninB*	[a]ninBF1: AACCTTTGAAATTCGATCTCCAGC
		[a]ninBR1: CTTCGTCTGACCACTTAACGC
	ST64B-*SB04*	SB04F: TGTCATACGACACCTATACCG
		SB04R: TGTTCTGCACCATGTGCAATG
	186-*cII*	P186cIIF: GACATAGCGGGATTAGTCTGC
		P186cIIR: GTCACAACATGGCGAAGCTG
	186-*gene O*	P18BPF: GTGCGATTCGTAACCTCATCC
		P18BPR: GTTTGTTGGTGCATACCACCT
	186-*gene G*	P186GF: GACGTTCTCTCGATGACGATG
		P186GR: CAATGGCACTACCGGTAACAG
	Prophage in SL254-A2929	SL254tailF: AGGCGGATTACCTGAAACGTC
		SL254tailR: ATATCCACCGCCTTCTTGCTC

	Gene loci	Primer sequences (5'→ 3')
MLVA	STTR-5	[b]STTR5F: GCTGCAGTTAATTTCTGCG
		[b]STTR5R: TCAGTAAAACGGTGATCGC
	STTR-6	[b]STTR6F: CAGATTTTTCACCATCTGCGC
		[b]STTR6R: AGTTGCTTCAGGATATCTGGC
	STTR-9	[b]STTR9F: ATGATCGACCACGATCTTGCC
		[b]STTR9R: CAAACGACCGCTATTCGTCG

[a] Primer sequences described by Ross and Heuzenroeder (2005)
[b] Primer sequences described by Ross and Heuzenroeder (2008)



Isolates	Time	Sources	States	PT	MAPLT/ MLVA	ninB	SB04	P186BP	P186cII	P186gpG	SL254-tail	STTR5	STTR6	STTR9
B17	2005	Bovine	VIC	RDNC	1	+	-	-	-	-	-	332	0	253
B23	2005	Bovine	SA	38	1									
B19	2005	Bovine	QLD	35	2	-	-	-	-	-	-	332	0	253
B44	2007	Human	SA	24	3	-	-	-	-	-	-	344	0	253
B51	2007	Beef meat	VIC	24	4	+	-	-	-	-	+	338	0	253
B43	2008	Bovine	NSW	24	4									
B13	2006	Bovine	TAS	32	5	+	-	-	-	-	-	338	0	253
B09	2006	Lamb meat	VIC	UN	6	+	+	-	-	-	-	338	0	253
B39	2008	Human Blood	VIC	24	6									
B18	2005	Broiler litter	VIC	UN	7	-	-	-	-	-	-	338	0	253
B34	2005	Egg pulp	SA	13	7									
B35	2006	Bovine	NSW	13	7									
B36	2007	Human	SA	13	7									
B37	2007	Chicken meat	NSW	13	7									
B38	2007	Chicken meat	NSW	13	7									
B15	2005	Human	SA	RDNC	8	-	+	-	-	-	-	338	0	253
B22	2005	Human	NT	13	9	-	-	+	-	+	-	338	0	253
B57	2008	Human	NT	13	9									
B45	2007	Human	WA	24	9									
B50	2007	Human	WA	24	9									
B56	2008	Canine	VIC	13	10	-	-	+	+	+	-	338	0	253
B02	2007	Human	NSW	40	11	-	-	+	+	-	-	338	0	253
B32	2006	Chicken meat	QLD	14	12	-	-	-	-	-	+	392	0	253
B33	2007	Human	QLD	14	12									
B54	2008	Chicken meat	NSW	14	12									
B04	2007	Broiler litter	QLD	32	13	+	-	-	-	-	-	332	0	262
B52	2007	Human	VIC	24	14	+	-	-	-	-	-	338	0	262
B21	2005	Bovine	SA	24	15	+	-	-	-	-	-	338	0	244
B03	2007	Human	NSW	11	16	-	-	+	-	-	-	398	0	316
B08	2006	Chicken meat	NSW	UN	17	-	-	-	-	-	+	362	0	262
B11	2006	Chicken meat	VIC	14	17									
B14	2006	Chicken meat	NSW	35	17									
B30	2006	Human	NSW	14	17									
B10	2006	Canine	SA	14	18	-	-	-	-	-	-	362	0	262
B53	2008	Human	TAS	14	19	-	-	+	-	+	+	362	0	262
B16	2005	Human	NSW	35	20	-	+	-	-	-	-	368	0	244
B01	2007	Human	QLD	RDNC	21	+	-	+	-	+	+	404	0	253
B55	2007	Human	QLD	14	22	+	-	+	-	+	+	398	0	262
B31	2006	Layer litter	NSW	14	23	-	-	+	-	+	+	368	0	271
B25	2006	Human	SA	24	24	+	-	-	+	-	+	338	423	262
B28	2006	Canine	QLD	24	24									
B40	2008	Human	SA	24	24									
B42	2008	Egg pulp	SA	24	24									
B05	2007	Bovine	SA	UN	25	+	-	-	+	-	+	338	417	262
B27	2006	Human	NSW	24	25									
B29	2007	Bovine	SA	24	26	+	-	-	+	-	+	338	411	262
B58	2006	Feed sheep	SA	24	27	+	-	-	+	-	+	338	429	262
B47	2007	Sheep	SA	24	27									
B48	2007	Sheep	SA	24	28	+	-	+	+	-	+	338	429	262
B41	2008	Human	NSW	24	29	+	-	+	+	+	+	338	429	262
B06	2006	Pork meat	WA	UN	30	+	-	+	+	-	+	338	447	262
B07	2006	Bovine	WA	UN	31	+	-	+	+	+	+	338	417	262
B12	2006	Human	WA	24	31									
B26	2006	Bovine	WA	24	31									
B46	2008	Bovine	WA	24	31									
B49	2007	Human	WA	24	32	+	-	+	+	-	-	338	417	262
B60	2006	Human	WA	24	33	+	-	+	+	+	+	338	435	262
B20	2005	Human	NSW	16	34	-	-	+	+	-	-	338	429	262
B59	2006	Human	VIC	24	34									
B24	2005	Bovine	VIC	39	35	-	-	-	+	-	+	338	417	271

Fig. 4.5 Dendrogram depicting genetic relationship between the 60 *S.* Bovismorbificans according to the amplification results of 6 MAPLT and 3 MLVA loci.

Positive amplification of the prophage loci was indicated as '+'; negative PCR amplification of the prophage loci was indicated as '-'. The MLVA results were presented as amplified fragment length (in bp).

Table 4.5 Comparison of the differentiating abilities between the 4 methodologies based on the calculated Simpson's index of diversity

	No. of primers used	No. of profiles	Simpson's index of diversity (95% confidence interval)
PFGE	na[1]	26	0.92 (0.88 to 0.96)
MAPLT	11	22	0.94 (0.92 to 0.96)
MLVA	13	21	0.89 (0.83 to 0.94)
MAPLT/MLVA	9	35	0.97 (0.96 to 0.99)

[1]na = not applicable

Table 4.6 Differentiating ability of PFGE, MAPLT, MLVA and MAPLT / MLVA for PTs 24, 14 and 13 isolates

	Simpson's index of diversity (no. of profiles)		
	PT 24 (n = 24)	PT 14 (n = 9)	PT 13 (n = 8)
PFGE	0.83 (12)	0.86 (6)	0.61 (3)
MAPLT	0.85 (10)	0.86 (5)	0.61 (3)
MLVA	0.85 (9)	0.75 (4)	0 (1)
MAPLT / MLVA	0.98 (16)	0.89 (6)	0.61 (3)

All values illustrated in this table were derived from Fig. 4.1 (PFGE); Fig. 4.2 (MAPLT); Fig. 4.4 (MLVA), and Fig. 4.5 (MAPLT / MLVA)

4.3.6 Use of the combined MAPLT / MLVA for outbreak epidemiological typing

As stated earlier, a food-borne outbreak of *S.* Bovismorbificans PT 11 isolates occurred in 2006 (OzFoodNet, 2006). The isolates from this outbreak investigation was employed to assess the ability of the composite assay of MAPLT / MLVA for outbreak epidemiological typing. Prior to subjecting isolates to the typing method, PCR amplification of *sucA* and STTR-5 as previously described was performed to ensure that the *Salmonella* DNA was present and suitable for PCR reactions. Thirteen of the sixteen isolates showed positive amplification of both loci and were characterised using the MAPLT / MLVA scheme. These outbreak isolates were designated as B61 to B73.

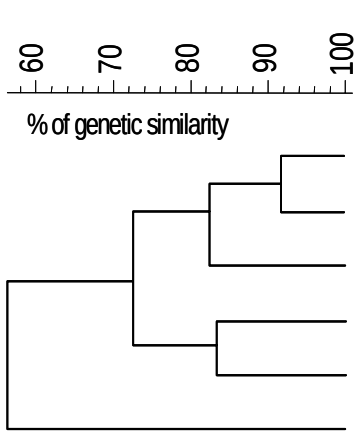
The thirteen isolates all amplified at least one locus of the typing scheme and six profiles were observed (Fig. 4.6). Identical composite typing profile (profile 1) was observed between the outbreak food source salami (B73) and six human isolates indicating epidemiological linkages (Table 4.7). It was noted that the salami isolate B64 was sampled three months earlier than B73 was determined as not being part of the outbreak despite that both isolates generated the same MAPLT / MLVA profile 1. Two isolates B65

and B66 from the assumed human cases had profiles 3 and 2 respectively, which differed from the outbreak profile 1 by one locus each (Fig. 4.6). Previously these patients were confirmed to be outbreak-related on the basis of the identical MLVA data seen with that of the salami sample B73 during the outbreak investigation in 2006 (Table 4.7). There were only a small number of selected isolates typed previously by Microbiological Diagnostic Unit, University of Melbourne using the MLVA scheme for *S.* Typhimurium (Lindstedt *et al.*, 2004). In the case of isolate B65 (profile 3), the amplified locus STTR-9 was one tandem repeat shorter than the previously reported results. In comparison, all three MLVA loci amplified from isolate B66 (profile 2) in this study had the same tandem repeat numbers as the previous results, but this isolate also amplified locus gpG_{186} that was otherwise absent in the outbreak profile 3 (Fig. 4.6).

Table 4.7 The 13 *S.* Bovismorbificans PT 11 isolates included in the 2006 outbreak study

Isolate ID	PT	Source	? outbreak-related	2006 MLVA[(i)]	MAPLT / MLVA[(ii)]
B61	13	Human	assumed no	B	6
B62	11	Human	Uncertain, reported 3 weeks prior	Not done	1
B63	11	Human	possibly but ate a Japanese meal (meat and noodles); salami not mentioned	Not done	4
B64	11	Salami - sampled 1/3/2006	assumed no	Not done	1
B65	11	Human	Epidemiologically matched; confirmed with MLVA	A	3
B66	11	Human	Epidemiologically matched; confirmed with MLVA	A	2
B67	11	Human	assumed yes	Not done	1
B68	11	Human	assumed yes	Not done	1
B69	11	Human	assumed yes	Not done	1
B70	11	Human	assumed yes	Not done	1
B71	11	Human	assumed yes	Not done	5
B72	11	Human	assumed yes; confirmed with MLVA	A	1
B73	11	Salami - sampled 7/6/2006	Outbreak sources	A	1

[(i)] Two different 2006 MLVA profiles were observed and indicated as profile A and profile B in the table
[(ii)] The six MAPLT / MLVA profiles generated in this study were illustrated in Fig. 4.6.



ninB	SB04	P186BP	P186cII	P186gpG	SL254-tail	STTR5	STTR6	STTR9	Profiles	Isolate ID
-	-	+	+	-	-	338	429	182	1	B62, B64 (S), B67, B68, B69, B70, B72, B73 (S)
-	-	+	+	+	-	338	429	182	2	B66
-	-	+	+	-	-	338	429	173	3	B65
-	+	+	-	-	-	338	429	182	4	B63
-	+	+	-	-	-	344	429	182	5	B71
-	-	+	+	+	-	338	0	173	6	B61

Fig. 4.6 Dendrogram showing the relationships of the 6 MAPLT/ MLVA profiles of the isolates associated in the *S.* Bovismorbificans PT 11 outbreak investigation. All were human isolates except B64 and B73 that were isolated from food salami and were labelled with (S).
Positive amplification of the prophage loci was indicated as '+'; negative PCR amplification of the prophage loci was indicated as '-'.

4.4 DISCUSSION

Salmonella serovar Bovismorbificans is one of the common *Salmonella* serovars isolated from humans in Australia. In addition, food-borne outbreaks caused by this serovar have been reported in Australia and elsewhere (Pouhiniemi *et al.*, 1997; Stafford *et al.*, 2002; Gilsdorf *et al.*, 2005; Rimhanen-Finne *et al.*, 2011). However due to its low incidence worldwide in comparison to other serovars such as *S.* Typhimurium and *S.* Enteritidis, little effort has been made to develop newer methods for epidemiological studies of *S.* Bovismobificans infections. At the present time, PFGE remains the 'gold-standard' typing method for *Salmonella*, however alternative typing approaches have been established and increasingly used for *S.* Typhimurium and *S.* Enteritidis. These recently developed methods include MAPLT and MLVA that are both less laborious and simple to use than PFGE. Moreover they show a high level of intra-serovar and intra-phage type differentiation suitable for local epidemiological typing (Lindstedt *et al.*, 2003; Ross and Heuzenroeder, 2008, 2009). In this study, investigations were carried out to evaluate the ability of MAPLT and MLVA in differentiating within *S.* Bovismorbificans.

A set of unrelated *S.* Bovismorbificans isolates derived from various sources, Australian states and time periods were used, and thus the ability of each method in differentiating within this serovar could be measured. The differentiating ability of the methods was measured using the Simpson's index (DI); which indicates the probability of using a method to differentiate between two isolates (Hunter and Gaston, 1988). Referring to Table 4.5, the DIs of PFGE, MAPLT and the combined MAPLT / MLVA were all greater than 0.90 indicating that there is more than 90% chance that these methods will be able to differentiate between two isolates. These results suggested that the aforementioned methods could provide a high level of discrimination within *S.* Bovismorbificans. In addition, the established MAPLT scheme detecting nine prophage loci was shown to have the DI almost the same as that of PFGE although there was a lesser number of MAPLT profiles generated (Table 4.5). In view of that the calculation of DI takes into the account of both the capacity of a system in generating profiles and the relative frequencies of isolates in the profiles (Hunter and Gaston, 1988); the result suggested that PFGE did not classify the isolates as evenly as MAPLT. Therefore, the indices of evenness (Zar, 1984) of PFGE and MAPLT were calculated using an online biodiversity calculator available from http://math.hws.edu/javamath/ryan/DiversityTest.html. The index of evenness of MAPLT (0.91) was slightly higher than that of PFGE (0.86) reflecting a slightly more even distribution of MAPLT profiles. Despite that, the observed uneven classification of isolates by both PFGE and MAPLT may be due to the inclusion of a large proportion of isolates of the same phage type (PT 24, PT 14 and PT 13) in this study. However as these are the phage types dominating in Australia, the results may reflect the actual classification of the Australian *S.* Bovismorbificans isolates by PFGE. Further analysis using a higher

number of isolates of the same phage types and other phage types is required to confirm this observation.

MLVA typing using fourteen previously published loci showed the least overall differentiating capacity among all the molecular methods applied in the study. The separation of isolates was finally based on five loci that showed allelic variation in the number of tandem repeats. The remaining loci displayed no variations or were not detected in any isolates. A previous study of *S.* Infantis also showed similar findings in that of the eight published MLVA loci examined, only three MLVA loci showed useful for subtyping this serovar (Ross and Heuzenroeder, 2008). This study also showed that truncated tandem repeats at MLVA loci occur independently between serovars. For example, truncated repeat units at the 5'end of locus STTR-3 has been reported from a number of serovars including *S.* Typhimurium, *S.* Enteritidis and *S.* Infantis (Lindstedt *et al.*, 2003; Ross and Heuzenroeder, 2008, 2009). However, none of the sequenced STTR-3 loci from this study contained truncated repeats indicating that such repeats may not be present in *S.* Bovismorbificans. The results supported the view that development of high-resolution MLVA typing schemes relies highly on the availability of complete genomes to pinpoint the informative MLVA loci specific for the serovars of interest. While the sequenced genomes might not be in place for MLVA development of certain serovars (e.g. *S.* Bovismorbificans and *S.* Infantis), useful MLVA loci may still be found from the genomic sequences from the already described serovars. However any truncations at tandem repeats and the rate of mutation of the MLVA loci need to be ascertained for each serovar.

While MAPLT was demonstrated to be as discriminative as PFGE in this study, its ability in profile generation could be improved through identification of additional MAPLT primers. An alternative and simpler solution was to incorporate the three discriminative MLVA primer pairs into MAPLT. As illustrated in Table 4.5, the composite assay of MAPLT and MLVA comprising nine primer pairs not only displayed a differentiating ability as high as PFGE, but also generated a higher number of profiles than PFGE. Previously, this approach was also suggested for *S.* Enteritidis, as though MLVA provided the highest level of discrimination for this serovar as a whole, MAPLT performed better within some phage types (Ross and Heuzenroeder, 2009). This is because MAPLT and MLVA detect different aspects of genomic variations in *Salmonella* resulting in different separation of isolates.

However in the case of *S.* Bovismorbificans, the intra-phage type differentiation may not necessarily be improved when using the MAPLT / MLVA scheme as seen for PT 13 (Table 4.6). This result suggested the high genetic similarity between PT 13 isolates where similar levels of separation were observed regardless the typing approaches used that targeted different genomic regions of the organism. Nevertheless, the study indicated the feasibility of replacing PFGE with the composite assay of MAPLT

and MLVA in differentiation within the PT 24 phage group (Table 4.6). This was highlighted since PT 24 is an important *S.* Bovismorbificans phage type that is frequently detected in Australia and has been implicated in food-borne outbreaks in this country and elsewhere (Stafford *et al.*, 2002; Gilsdorf *et al.*, 2005). However the composite assay of MAPLT and MLVA may be tested before application in other countries. This is due to the fact that phage typing relates isolates phenotypically on the basis of the sensitivity to a phage panel. Therefore, it is possible that isolates of the same phage types may not be genetically similar and have different prophage content, especially when isolates occur in different countries with different environmental pressures and exposure to different temperate phages.

Thirteen *S.* Bovismorbificans PT 11 isolates involved in the 2006 salami outbreak were additionally subjected to the composite assay of MAPLT / MLVA to demonstrate its use as an outbreak epidemiological typing tool. PFGE could not be performed for comparison of typing results, as none of these PT 11 isolates could be re-cultivated. Assuming all the sample vials contained only single colonies of the correct *S.* Bovismorbificans strains, this study demonstrated that the composite assay of MAPLT and MLVA was able to show the close genetic relationship between the outbreak isolates. Most of the human isolates assumed to be outbreak-related had the same MAPLT / MLVA profile to the isolate of the outbreak source (B73). As mentioned earlier, isolate B64 was obtained from the same food source three months earlier and was epidemiologically not linked the outbreak. However as it also generated the same profile as B73, this result suggested that the outbreak isolate might have been present in the food chain prior to the onset of the outbreak.

The results also suggested that slight difference by one locus may be observed between outbreak-related isolates as seen from profiles 2 and 3 with the outbreak profile 1. This observation was not surprising as MAPLT or MLVA loci are among the highly variable regions in bacterial genomes and hence have been used widely for resolving differences within homogeneous bacterial groups. Nevertheless, the results strongly supported the proposal of establishing interpretation guidelines to determine the relationship between isolates with highly similar profiles particularly when the applied genetic markers could change relatively quickly (Ross and Heuzenroeder, 2009).

Similarly, the study suggested that the method might also have the ability to identify isolates that were unrelated to an outbreak by showing differences at two or more loci. Previously provided epidemiological data indicated that human isolate B63 may or may not be associated with the outbreak, as the patient did not consume salami but ate a Japanese meal with meat and noodles. Typing data obtained from this study showed a weaker genetic relationship by demonstrating differences at two loci between isolate B63 (profile 4) and the outbreak profile 1 (Fig. 4.6). The MAPLT / MLVA method separated an assumed human case (B71) (profile 5) from the outbreak by showing differences at three

loci including two MAPLT and one MLVA loci (Fig. 4.6). Further testing was performed using the complete MAPLT scheme that showed the presence of phage ST64B loci SB04 and SB38 that were otherwise not present in any of the outbreak-related isolates (data not shown). The result provided further support that isolate B71 might not be involved in the outbreak. Additional epidemiological data would be required to clarify the linkage.

It is clear that further typing of an additional set of known unrelated PT 11 isolates, in particular ones that were detected during the course of the outbreak would be required. This could illustrate the ability of the composite assay of MAPLT / MLVA in discriminating outbreak isolates from the 'background' PT 11 isolates. This would also display the genetic relationship within PT 11 as a whole as measured by the MAPLT / MLVA method, thereby providing guidance in determining the level of genetic similarity between isolates with similar profiles.

Referring to previous studies, phage typing could sometimes separate outbreak-related isolates. In the study of Lindstedt *et al.* (2004), a *S.* Typhimurium U302 isolate was included in a DT 104 outbreak investigation based on the observed epidemiological linkage, which was then supported by the generation of the identical MLVA profile with the outbreak-related isolates. A similar observation was reported from a *S.* Typhimurium DT 29 and DT 44 outbreak investigation where human and food isolates that showed either of the two phage types had identical MLVA profiles, suggesting a single outbreak event. This hypothesis was further supported from the observed DT 29 and DT 44 colonies sub-cultured from one patient sample (Ross *et al.*, 2011). This is in contrast to what was seen in this study. Previous MLVA testing have shown that this PT 13 isolate had a MLVA profile differing to that of the food isolate B71 by two loci including the absence of the STTR-6 locus (data not shown). The MAPLT / MLVA typing additionally showed the PT 13 isolate contained locus gpG_{186} that was not present in the food isolate (Fig. 4.6). Taken together, the genetic differences of isolate B61 was determined from the variations at the residing prophage and MLVA loci thereby independently confirming the assumption of the outbreak investigators that the PT 13 isolate B61 was not part of the outbreak.

4.5 CHAPTER SUMMARY

This chapter demonstrated the potential use of two recently proposed molecular typing techniques MAPLT and MLVA for differentiation within *S.* Bovismorbificans, an endemic serovar in Australia. The differentiating ability of each method was evaluated by comparing with the current 'gold standard' method of PFGE that were performed on the same set of sixty isolates. This study suggested that MAPLT could provide a similar level of differentiation to PFGE for *S.* Bovismorbificans as a whole, and

more importantly within *S.* Bovismorbificans phage types. In contrast, MLVA was less discriminative as most MLVA loci assessed did not show allelic variation or were not harboured in any of the tested isolates. In addition, a composite assay of MAPLT / MLVA was developed which provided the highest discriminatory power using as few as nine primer sets. Furthermore, the composite MAPLT / MLVA scheme was subjected to a retrospective outbreak study and demonstrated the ability to resolve the close genetic relationships between outbreak-related isolates indicating its use for local epidemiological studies.

CHAPTER 5 MOLECULAR TYPING OF *SALMONELLA* HEIDELBERG

5.1 INTRODUCTION

Salmonella enterica serovar Heidelberg (*Salmonella* Heidelberg or *S.* Heidelberg) is among the *Salmonella* serovars that are of significance to many parts of the world. According to a study monitoring the global distribution of *Salmonella* serovars, *S.* Heidelberg was most frequently detected in the United States and Canada (Hendriksen *et al.*, 2011). In the United States, *S.* Heidelberg ranked among the top seven most commonly reported *Salmonella* serovar causing human infections consecutively from 2005 to 2008 (CDC 2005-2008). Between 2006 and 2008 approximately 4% of all serotyped *Salmonella* isolates were *S.* Heidelberg (CDC 2005 - 2008). In Canada, *S.* Heidelberg ranked in the top three most frequently isolated *Salmonella* serovars from humans. Furthermore the proportion of all *Salmonella* isolates identified as *S.* Heidelberg in Canada was much higher (12%) than in the United States (4%) (CDC 2005-2008; Public Health Agency of Canada 2006).

S. Heidelberg has become a significant serovar in Australia since the 1980s. In the years between 1960 and 1980 the number of *S.* Heidelberg isolates from humans was no more than three isolates in any year. However the number of isolates increased and recently reached the average of 68 isolates annually between 2001 and 2009 and ranked among the top ten most frequently isolated *Salmonella* serovars from human source in the past 15 years (ASRC Annual Reports 2001-2009). *S.* Heidelberg has also been the causative agent in several food borne outbreaks involving a number of Australian states. In the 1980s, three food-borne outbreaks occurred in Queensland and New South Wales. In the 1990s a large *S.* Heidelberg outbreak was recorded in Victoria, while more *S.* Heidelberg outbreaks occurred in Queensland and New South Wales at the same time (ASRC Annual Reports 1986-1999).

As stated in Chapter 3 and Chapter 4, bacteriophage (phage) typing is routinely used for subdivision within specific *Salmonella* serovars. In Australia, a phage typing scheme (IMVS phage typing scheme) employing a panel of 11 phages was established in 1990 in response to the increased *S.* Heidelberg isolations (ASRC Annual Report 2004). The IMVS phage typing scheme identifies 27 different phage types. Phage type 1 has been the most persistent and common phage type every year for the last decade in Australia (ASRC Annual Reports 1999-2009). The majority of the PT 1 isolates were derived from humans, comprising from 42 to 100% of all PT 1 isolates received by ASRC in the years 2005 to 2009 (ASRC Annual Reports 2005-2009). Phage type 1 has also been isolated from non-human sources including animal food products (ASRC Annual Reports 1995-2009). This phage typing has assisted outbreak investigations (ASRC Annual Reports 2001, 2006; Harvey *et al.,* 1993). In 2001 phage typing demonstrated that a food-borne outbreak that occurred in a nursing home was due to *S.*

Heidelberg phage type (PT) 1 and was believed to be transmitted from contaminated eggs (ASRC Annual Report 2001). Phage typing also showed that *S.* Heidelberg PT 16 caused an outbreak through consumption of contaminated eggs from airline meals in 1996 (Abelson *et al.*, 2006).

There is a second phage typing scheme independently established and is used for surveillance in Canada (Demczuk *et al.*, 2003). This phage typing scheme also comprises 11 typing phages and is able to distinguish at least 49 phage types within the Canadian *S.* Heidelberg isolates (Demczuk *et al.*, 2003). Similar to what has been observed with the IMVS phage typing scheme, the majority of the Canadian sporadic isolates from humans and non-human sources were classified into a single phage type (PT 19). Approximately 51% of the all the tested isolates were PT19 (Demczuk *et al.*, 2003).

Molecular typing methods are used to further differentiate isolates of the same *Salmonella* phage types during outbreaks. Pulsed-field gel electrophoresis (PFGE) is the current 'gold standard' method for *Salmonella*. However increased numbers of studies indicated the inadequate differentiating ability provided by this method (see section 1.2.4.3.1). In regard to *S.* Heidelberg, Demczuk *et al.*, (2003) described a limited discrimination within *S.* Heidelberg PT19 using PFGE. In that study 52 PFGE profiles were generated but one profile was seen in 55% of all isolates, and from 91% of the predominant PT 19 isolates (Demczuk *et al.*, 2003). One strategy applied to improve the discrimination level of PFGE for *S.* Heidelberg was to analyse the composite PFGE patterns generated individually by two or more restriction enzymes (Zhao *et al.*, 2006; Zheng *et al.*, 2007; Zhao *et al.*, 2008). As demonstrated by Zhao *et al.* (2008), the number of PFGE patterns could be increased by 75% from 61 *Xba*I restriction patterns to 106 combined *Xba*I/*Bln*I patterns from the same group of *S.* Heidelberg isolates. However due to that the lengthy procedure involved in PFGE and the increased complexity of the generated typing results, this approach may seem impractical to apply for outbreak epidemiological studies (refer section 1.2.4.3.3 for detailed discussion).

Recently two PCR-based typing methods have been increasingly applied for sub-serovar or sub-phage type differentiation due to their abilities in providing fine level of strain differentiation, and being rapid, simple and easy to perform compared to PFGE. These methods are multiple-locus (variable-number tandem repeat) analysis (MLVA) and multiple amplification of phage locus typing (MAPLT). MLVA reports the allelic variation displayed by the targeted loci due to the different numbers of tandem repeats contained within (Lindstedt *et al.*, 2003). Individual MLVA typing schemes have been developed for *Salmonella* serovars Typhimurium, Enteritidis, Typhi, and Newport, all of which were shown to be at least as discriminative as PFGE (Lindstedt *et al.*, 2003; Liu *et al.*, 2003; Lindstedt *et al.*, 2004; Witonski *et al.*, 2006; Boxrud *et al.*, 2007; Octavia and Lan, 2009). Similar to MLVA, MAPLT also detects small genetic variations at bacterial genomes, where prophage loci in the *Salmonella* genomes are targeted

(Ross and Heuzenroeder, 2005a). MAPLT schemes have been published for *Salmonella* serovars Typhimurium, Infantis and Enteritidis (Ross and Heuzenroeder, 2005a, 2008, 2009). Detailed discussions regarding MLVA and MAPLT are in section 1.2.4.4.3 and section 1.2.4.4.4 respectively).

This chapter aimed to develop a MAPLT and a MLVA typing scheme for serovar *S.* Heidelberg. In addition, a composite assay of MAPLT and MLVA was set up. The differentiating ability of each of the developed MAPLT, MLVA and the composite assay was evaluated through comparing with PFGE. In particular, the differentiation within *S.* Heidelberg PT 1 group was emphasised due to its high prevalence and hence potential in causing outbreaks in Australia.

5.2 MATERIALS AND METHODS

This chapter involved use of MAPLT, MLVA, PFGE and composite assay of MAPLT / MLVA for differentiation of *S.* Heidelberg isolates. Bacterial DNA extraction and the four typing methodologies were carried out using materials and protocols described in Chapter 2. The prophage loci tested in this chapter were amplified using primers that were previously published, or constructed for use (see Chapter 2 and Chapter 3). The primer sequences can be found in Table 2.1b. The MLVA loci tested and reported in this chapter were either previously published or first identified in this chapter (see below). The published MLVA loci were amplified using primers listed in Table 2.2. The typing data were analysed as described in Chapter 2. The following sections describe the bacterial isolates and the methods that were specifically used in this chapter.

5.2.1 Bacterial isolates

A total of sixty-four epidemiologically-unrelated *S.* Heidelberg isolates were used in this study (Appendix 1.3). All isolates were obtained from the Australian *Salmonella* Reference centre (ASRC), Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia. The serotypes and the phage types of these *Salmonella* isolates were determined previously by the ASRC. The isolates were originally derived from various sources, and geographic locations in Australia between 2006 and 2009.

5.2.2 Identification of novel MLVA loci

Together with previously described MLVA loci, additional MLVA loci were first identified and tested in this chapter. These MLVA loci were identified using the tandem repeat finder software (Benson, 1999) from the genomic sequence of *S.* Heidelberg strain SL 476 (NCBI accession no. CP001120). Default parameters were applied in search for tandem repeats. Nine regions containing direct tandem repeats were further selected to test for their usefulness in strain differentiation (Table 5.1). The tandem repeats selected were 3 to 18 base pairs in length for each repeat unit (Table 5.1). The online-based BlastN

tool (Altschul *et al.*, 1990) (available from http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied to ensure that the DNA sequence of the nine selected MLVA regions were not identical to any previously published MLVA loci. Primers amplifying these nine novel MLVA loci were subsequently designed and can be found in Table 2.2.

Table 5.1 Characteristics of the novel MLVA loci identified from *S.* Heidelberg strain SL 476

Loci	Repeat length (bp)	No. of repeats[a]	Location in genome[b]	Coding region
SHTR-1	7	4	4618625-4619096	Intergenic
SHTR-2	5	10	2562756-2563042	Intergenic
SHTR-3	10	3	2562417-2562779	Intergenic
SHTR-4	9	2	2379490-2379899	hypothetical protein
SHTR-5	9	5	2005751-2006186	hypothetical protein
SHTR-6	9	2	1762657-1763116	Intergenic
SHTR-7	18	2	3440787-3441169	*garD*
SHTR-8	15	2	4118402-4118723	Intergenic
SHTR-9	15	2	2303349-2303697	*lyt*S

[a] Repeat number was adjusted to disregard the additional bases being considered as "partial repeat unit" by the Tandem Repeat Finder (Benson, 1999)

[b] Location of amplified fragments in S. Heidelberg SL 476 with the GenBank accession no CP001120

5.2.3 Evaluation of the usefulness of the novel MLVA loci

Thirty-two of the sixty-four *S.* Heidelberg isolates were used to initially assess the usefulness of each of the nine novel loci (STHR-1 to -9) for strain differentiation. These thirty-two isolates were selected based on their distinct PFGE profiles and phage types indicating genetic diverseness (Table 5.2). Loci showing variation in the number of tandem repeats within the diverse thirty-two isolates were subsequently tested on the remaining isolates.

Table 5.2 A subset of 32 *S.* Heidelberg isolates used to determine the allelic variability of the novel MLVA loci SHTR-1 to -9

Isolates	Time	Sources	[b]States	Phage types	[a]PFGE profiles
H15	2006	Human	SA	1	20
H16	2006	Human	VIC	1	21
H18	2008	Human	NSW	2	4
H19	2007	Human urine	NSW	2	10
H20	2007	Human	VIC	2	14
H21	2007	Porcine intestine	QLD	2	13
H22	2007	Human	QLD	2	18
H23	2008	Human	NSW	3	24
H25	2007	Human blood	O/S travel	3	24
H27	2007	Human	QLD	4	16
H28	2006	Human blood	QLD	4	29
H29	2007	Human	NSW	5	11
H30	2007	Human	SA	7	17
H31	2008	Human	NSW	8	25
H39	2007	Human	NSW	5	11
H41	2006	Human	O/S travel	23	23
H42	2006	Human	WA	23	2
H43	2006	Human	O/S travel	25	19
H44	2007	Human	QLD	26	7
H45	2007	Human	O/S travel	27	1
H46	2006	Human	VIC	6a	26
H47	2008	Human	O/S travel	[1]RDNC	6
H48	2008	Goat meat	QLD	[2]UN	17
H49	2006	Pork meat	QLD	[2]UN	5
H50	2007	Human	VIC	1	3
H52	2007	Human	QLD	4	27
H53	2007	Human	QLD	4	12
H54	2007	Human blood	NSW	1	8
H55	2007	Human	NSW	2	9
H56	2006	Goat meat	VIC	2	17
H57	2006	Human	NSW	4	28
H60	2007	Human	QLD	4	15

The isolates were collected from various sources, time and locations, and were indicated as diversely related by phage typing and PFGE. [a]PFGE profiles were derived from Fig. 5.2. [b] Abbreviations for Australian states: NSW = New South Wales; NT = NewTerritory; QLD = Queensland; SA = South Australia; WA = Western Australia; O/S travel = overseas travel. [1] RCNC = react does not conform, [2] UN = untypable

5.3 RESULTS

5.3.1 MAPLT

In total, twenty-one phage loci were amplified from at least one of the *S.* Heidelberg isolates (Table 5.3). These phage loci were the same as those of the published phages including P22, ST64T, ST64B, Gifsy-1, Gifsy-2, Fels-2, SopEΦ, and a prophage in the sequenced *S.* Newport strain SL254 (NCBI accession no CP001113). All these prophage loci were variably amplified from the isolates, with the exceptions of two Gifsy-2 loci (STM1032 and STM1048) and the *sopE* gene locus that were detected from all test isolates. Gene loci of phage ST64B were routinely detected at a low frequency, for example locus $SB06_{ST64B}$ was amplified in five isolates. In most cases, gene loci derived from the same phages were observed in different isolates (Table 5.3). For example, while only one isolate amplified locus $sieB_{P22}$, seven and nineteen isolates amplified the other two phage P22 loci $ninB_{P22}$ and int_{P22} respectively. The one exception was that the four Fels-2 phage loci examined in this chapter were all amplified from the same twelve isolates (Appendix 2.3).

In total, seventeen MAPLT profiles were seen from the sixty-four *S.* Heidelberg isolates using a minimum set of twelve MAPLT primers (Table 5.4). Incorporating further MAPLT loci that were examined in this study did not enhance the separation of isolates. Of the seventeen MAPLT profiles generated, eight were generated by single isolates. Among the remaining 9 MAPLT profiles, MAPLT profile 2 was generated by the highest number of isolates that were predominately PT 1 isolates (16 of 17 isolates) (Fig. 5.1). Moreover, most PT 1 isolates with MAPLT profile 2 were derived from humans and none were derived from goat meat, which was the predominant source of non-human *S.* Heidelberg isolates by ASRC (ASRC 1986 - 2009). Similarly the PT 2 isolates derived from humans (MAPLT profiles 3 and 6) were distinct from the isolates derived from animal origins (MAPLT profiles 1 and 7). In contrast, the PT 8 isolates generating the MAPLT profile 3 were isolated from both human and non-human sources.

5.3.2 PFGE

Twenty-nine PFGE profiles were generated with twenty-four unique profiles from single isolates (Fig. 5.2). The remaining five PFGE profiles generally clustered isolates of the same phage types. For example, twenty-two of the twenty-five isolates with PFGE profile 17 were PT 1 and included all the PT 1 goat meat isolates. Similarly, all four isolates with PFGE profile 24 were PT 3, and all seven isolates with PFGE profile 25 were PT 8 (Fig. 5.2). While a general typing system concordance between PFGE and phage typing was observed for these phage types, PFGE showed the wide genetic diversity within other phage types. For example, the eight PT 2 isolates generated seven PFGE profiles, and all six PT 4 isolates had distinct PFGE profiles (Fig. 5.2).

Table 5.3 Positive amplifications of prophage gene loci from the 64 *S.* Heidelberg isolates

Prophages	Gene loci	Gene functions	No. of positive isolates
P22/ST64T	*cI*	CI protein	11
	g17	Putative superinfection exclusion protein	16
	gtrC	O-antigen conversion	19
P22	*ninB*	Unknown	7
	int	Integrase	19
	sieB	Superinfection exclusion protein	1
ST64B	SB06	Major capsid protein	2
	SB21	Putative head assembly protein	5
	SB28	Integrase	3
	SB38	CI protein	4

Prophages	Gene loci	Gene functions	No. of positive isolates
Fels-2	STM2697	Tail protein	12
	STM2714	Lysis regulatory protein	12
	STM2719	Terminase small subunit	12
	STM2723	Portal protein	12
Gifsy-1/2	STM2619	Unknown (NinG)	10
	STM2632	Exodeoxyribonuclease	55
Gifsy-1	STM2608	Terminase large subunit	2
Gifsy-2	STM1032	Putative capsid protein	64
	STM1048	Host specificity protein J	64
SopEϕ	*sopE*	Type III secretive protein (SopE)	64
Prophage in SL254	A2929	Tail sheath protein	19

Primer sequences amplifying the prophage gene loci in this table are listed in Table 2.1b

Table 5.4 Primers of the MAPLT scheme for *Salmonella* Heidelberg

Prophages	Gene loci	Encoded proteins	Primer sequences (5'→ 3')
P22/ST64T	*cI*	CI protein	[a]PTc1F: CTTTACCAATCTGAACCGCCG
			[a]PTc1R: CTGAGTTGTTTTGGCATAATTACTCC
	g17	Putative superinfection exclusion	[a]F17: GGCTGTTGTTTCTTCTTTCAGGC
			[a]R17: AGGAAATATGAAATTACGTGTCTGGC
P22	*sieB*	Superinfection exclusion	[a]SIEBF: CGATGAACAACTCATGGTGGC
			[a]SIEBR: AGCGAGGTAAGGTATTTGTCG
Fels-2	STM2714	Lysis regulatory protein	Fels2lysBF: TGACCTTTCCAGACGGCACT
			Fels2lysBR: TGGTTCTGGCGCTGGTACTT
Gifsy-1	STM2608	Terminase large subunit	Gifsy1AF: GATCACGCATCCATTATGTTCAC
			Gifsy1AR: TATTCCCGTACCGCTTACCAC
Gifsy-1/2	STM2632	Exodeoxyribonuclease	Gifsy1RecF: CTAAAGACAATATGACCTGGACG
			Gifsy1RecR: GAATAGCCGATACACGGTTGC
	STM2619	Unknown (NinG)	Gifsy1ninGF: ACACTGAAGATGGATGTTGAAGC
			Gifsy1ninGR: GCCGTAAGTGCGCAAACAAAG

Prophages	Gene loci	Encoded proteins	Primer sequences (5'→ 3')
ST64B	SB06	Major capsid protein	[a]SB06F1: ACGACAAGCGCGTTGAGGC
			[a]SB06R1: GCTTCCACGTTGAAGAAGGC
	SB21	Putative head assembly protein	[a]SB21F1: CTGTATGGTTATATCGATTATCTGG
			[a]SB21R1: GATTTCCTTTGCCCAGATGACG
	SB28	Integrase	[a]SB28F1: TGCAGTCAAGAGGACGTCC
			[a]SB28R1: TGCCGATATGCTGATCTGGC
	SB38	CI protein	[a]BIM1F: ATGGTGGCCTTGTCGACGC
			[a]BIM1R: GCTAACGTGAAGGATTTGTTCCG
Prophage in SL254	A2929	Tail sheath	SL254tailF: AGGCGGATTACCTGAAACGTC
			SL254tailR: ATATCCACCGCCTTCTTGCTC

[a] Primer sequences described by Ross and Heuzenroeder (2005)

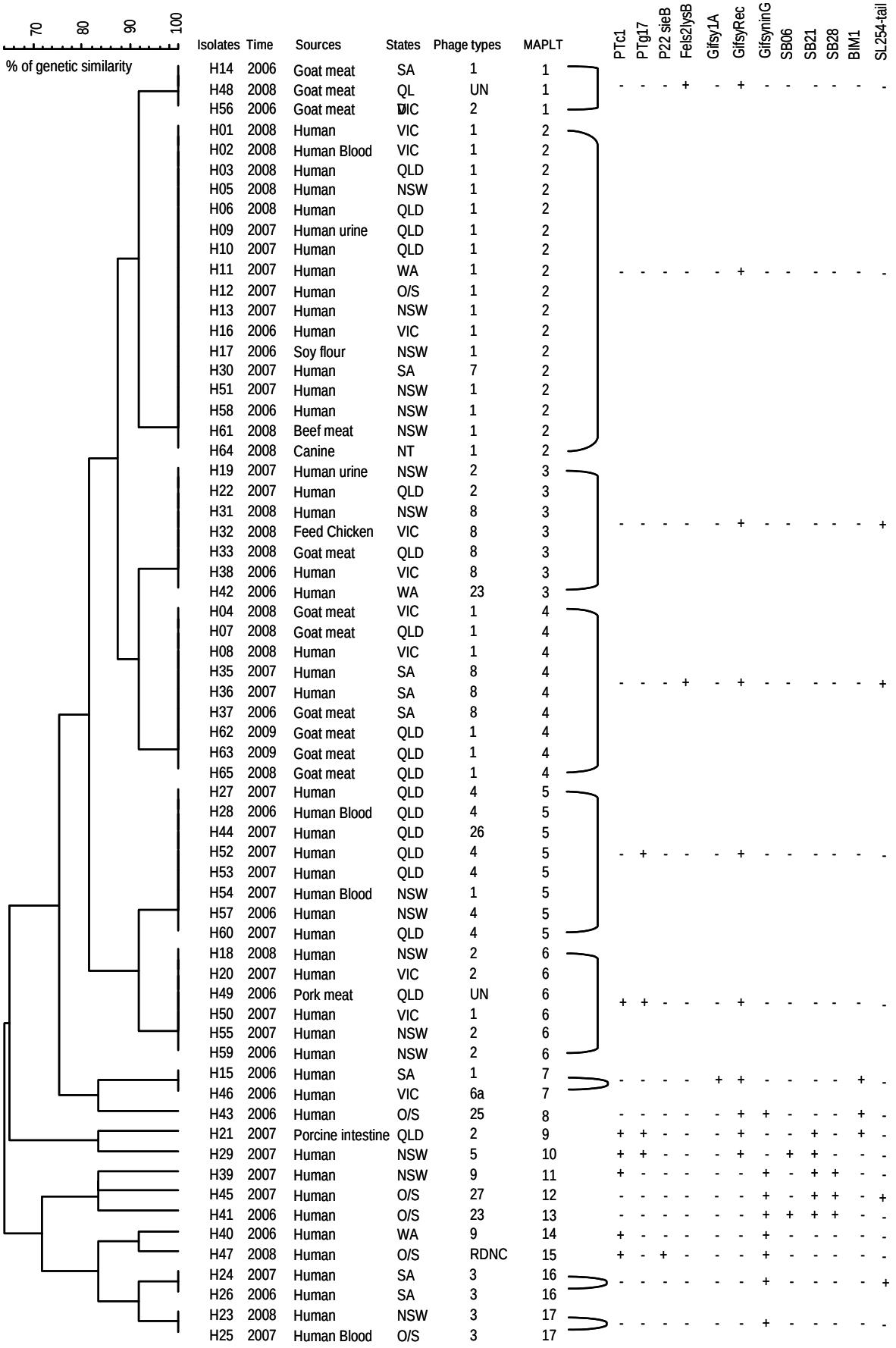
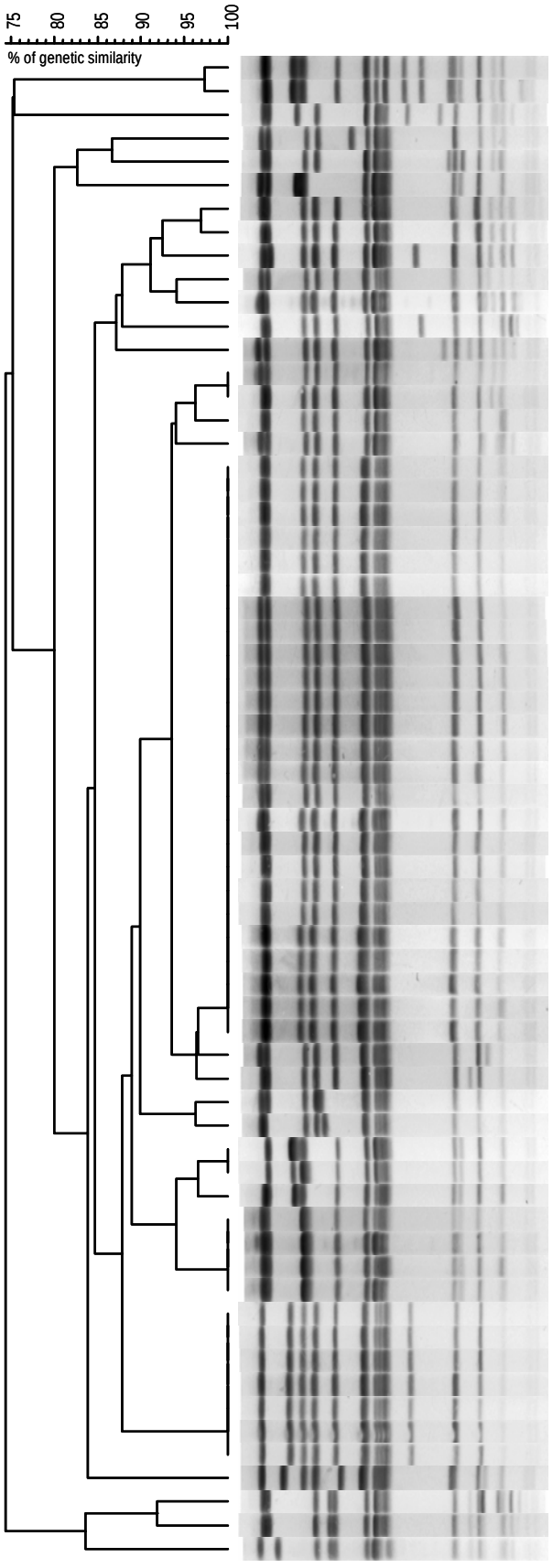


Fig. 5.1 Dendrogram showing the genetic relationship between the 64 S. Heidelberg isolates as generated by MAPLT results. Symbol + indicated positive amplification of the prophage loci; symbol – indicated negative PCR amplification of the prophage loci.

UN = untypable; RDNC = reacts but does not conform.

Abbreviations for Australian states: NSW = New South Wales; NT = Northern Territory; QLD = Queensland; SA = South Australia; WA = Western Australia; O/S = overseas travel



Isolates	Time	Sources	States	Phage types	PFGE	MAPLT
H45	2007	Human	O/S	27	1	12
H42	2006	Human	WA	23	2	3
H50	2007	Human	VIC	1	3	6
H18	2008	Human	NSW	2	4	6
H49	2006	Pork meat	QLD	UN	5	6
H47	2008	Human	O/S	RDNC	6	15
H44	2007	Human	QLD	26	7	5
H54	2007	Human Blood	NSW	1	8	5
H55	2007	Human	NSW	2	9	6
H19	2007	Human urine	NSW	2	10	3
H29	2007	Human	NSW	5	11	10
H53	2007	Human	QLD	4	12	5
H21	2007	Porcine intestine	QLD	2	13	9
H20	2007	Human	VIC	2	14	6
H59	2006	Human	NSW	2	14	6
H60	2007	Human	QLD	4	15	5
H27	2007	Human	QLD	4	16	5
H01	2008	Human	VIC	1	17	2
H02	2008	Human Blood	VIC	1	17	2
H03	2008	Human	QLD	1	17	2
H04	2008	Goat meat	VIC	1	17	4
H05	2008	Human	NSW	1	17	2
H06	2008	Human	QLD	1	17	2
H07	2008	Goat meat	QLD	1	17	4
H08	2008	Human	VIC	1	17	4
H09	2007	Human urine	QLD	1	17	2
H10	2007	Human	QLD	1	17	2
H11	2007	Human	WA	1	17	2
H12	2007	Human	O/S	1	17	2
H13	2007	Human	NSW	1	17	2
H14	2006	Goat meat	SA	1	17	1
H17	2006	Soy flour	NSW	1	17	2
H30	2007	Human	SA	7	17	2
H48	2008	Goat meat	QLD	UN	17	1
H51	2007	Human	NSW	1	17	2
H56	2006	Goat meat	VIC	2	17	1
H58	2006	Human	NSW	1	17	2
H61	2008	Beef meat	NSW	1	17	2
H62	2009	Goat meat	QLD	1	17	4
H63	2009	Goat meat	QLD	1	17	4
H64	2008	Canine	NT	1	17	2
H65	2008	Goat meat	QLD	1	17	4
H22	2007	Human	QLD	2	18	3
H43	2006	Human	O/S	25	19	8
H15	2006	Human	SA	1	20	7
H16	2006	Human	VIC	1	21	2
H39	2007	Human	NSW	9	22	11
H40	2006	Human	WA	9	22	14
H41	2006	Human	O/S	23	23	13
H23	2008	Human	NSW	3	24	17
H24	2007	Human	SA	3	24	16
H25	2007	Human Blood	O/S	3	24	17
H26	2006	Human	SA	3	24	16
H31	2008	Human	NSW	8	25	3
H32	2008	Feed Chicken	VIC	8	25	3
H33	2008	Goat meat	QLD	8	25	3
H35	2007	Human	SA	8	25	4
H36	2007	Human	SA	8	25	4
H37	2006	Goat meat	SA	8	25	4
H38	2006	Human	VIC	8	25	3
H46	2006	Human	VIC	6a	26	7
H52	2007	Human	QLD	4	27	5
H57	2006	Human	NSW	4	28	5
H28	2006	Human Blood	QLD	4	29	5

Fig. 5.2 Dendrogram constructed based on the PFGE patterns of the 64 *S.* Heidelberg isolates. The PFGE patterns were related by the BioNumerics program with the band tolerance level and optimisation set at 1.7% and 0.5% respectively.

5.3.3 MLVA

5.3.3.1 Allelic variability of the published MLVA loci

Of the fourteen published MLVA loci examined in this chapter, only three loci displayed allelic variations (Table 5.5). Locus STTR-5 displayed the highest number of alleles (seven alleles), while loci Sal20 and STTR-3 displayed three alleles and two alleles respectively. Of the remaining eleven MLVA loci, locus Sal04 was amplified with no tandem repeats from any isolate, while loci STTR-6 and STTR-10 were not amplified from any of the isolates.

5.3.3.2 Allelic variability of the novel MLVA loci

A subset of thirty-two genetically and epidemiologically distinct *S.* Heidelberg isolates was selected to determine the allelic variability of these loci within the serovar. Nine novel MLVA loci designated SHTR-01 to -09 were examined for variable amplified fragment lengths (Table 5.1). Of the nine loci only SHTR-05 exhibited allelic polymorphism insofar that twenty-nine isolates amplified a fragment of 427 bp and three isolates amplified a fragment of 436bp. Locus SHTR-03 was amplified in all but five isolates. All the amplified SHTR-3 products were 363bp in length. In regard to the remaining loci, SHTR-01 and -02 were not amplified in any isolates, while SHTR-04, -06, -07, -08 and -09 were amplified by all isolates but the amplified products showed no polymorphism. Consequently only loci SHTR-03 and SHTR-05 were applied to the remaining isolates.

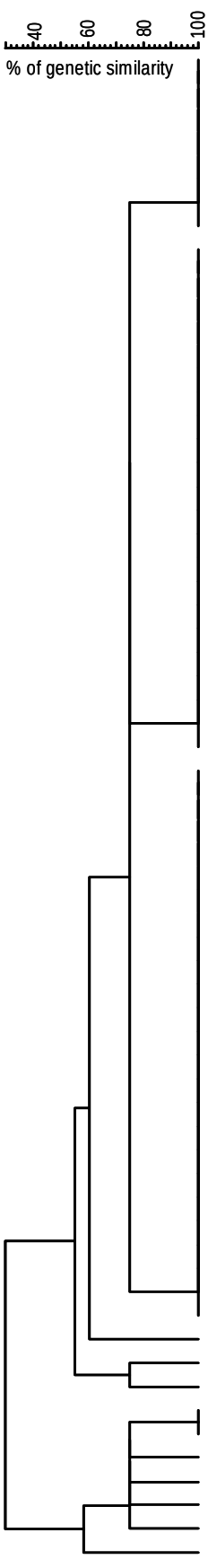
5.3.3.3 MLVA differentiation of the sixty-four *S.* Heidelberg isolates

MLVA typing of all sixty-four isolates was performed using the five loci that displayed allelic polymorphism as described above. However the highest level of differentiation of isolates was obtained by using three loci: STTR-3, STTR-5 and Sal20 (Fig. 5.3). Including the novel MLVA loci SHTR-3, and -5 did not differentiate the isolates any further. Eleven different MLVA profiles were generated and most isolates either exhibited MLVA profiles 1 (n=8), 2 (n=22) and 3 (n=24) (Fig. 5.3). In regard to the intra-phage type differentiation, MLVA was the most effective in separating PT 3 isolates compared to MAPLT and PFGE where all four isolates tested had distinct profiles. By comparion, PFGE failed to differentiate any of the PT 3 isolates and MAPLT only provided partial separation of them into two groups. The PT 1 isolates generated three MLVA profiles: MLVA profile 1 (n=2), profile 2 (n=12) and profile 3 (n=12). The six PT 2 isolates were separated in a very similar way to MAPLT, with three MLVA profiles generated. The exception was that isolates H19, H20 and H21 were grouped together by MLVA, while isolate H21 was separated from the other two isolates by MAPLT. Similarly both MLVA and MAPLT did not separate any of the PT 4 isolates but differentiated the two PT 9 isolates. Alternatively, MLVA showed a similar ability to PFGE in differentiating PT 8 isolates. While all PT 8 isolates exhibited an identical PFGE profile, six out of seven PT 8 isolates displayed MLVA profile 2 and one isolate (H31) had the MLVA profile 4 despite the diverse sources of the isolates.

Table 5.5 Previously described MLVA loci that were used to differentiate the 64 *S.* Heidelberg isolates

MLVA loci	No. of alleles	[a] No. of tandem repeats ([b] no. of isolates)	References
STTR-2	1	10 (64)	Lindstedt *et al.,* (2003)
STTR-3	2	12 (63), 10 (1)	Lindstedt *et al.,* (2003)
STTR-5	7	9 (1), 10 (1), 11 (26), 12 (25), 13 (2) 14 (8), 15 (1)	Lindstedt *et al.,* (2003)
STTR-6	Not applicable	Not amplified (64)	Lindstedt *et al.,* (2003)
STTR-7	1	8 (64)	Lindstedt *et al.,* (2003)
STTR-9	1	2 (64)	Lindstedt *et al.,* (2004)
STTR-10	Not applicable	Not amplified (64)	Lindstedt *et al.,* (2003)
Sal02	1	2 (64)	Ramisse *et al.,* (2004)
Sal04	1	0 (64)	Ramisse *et al.,* (2004)
Sal10	1	2 (64)	Ramisse *et al.,* (2004)
Sal15	1	2 (64)	Ramisse *et al.,* (2004)
Sal20	1	11 (56), 13 (7), 14 (1)	Ramisse *et al.,* (2004)
TR1	1	2 (64)	Liu *et al.,* (2003)
2628542	1	4 (64)	Witonski *et al.,* (2006)

[a] The number of tandem repeats contained within the amplified MLVA loci
[b] Number of isolates amplified with the MLVA alleles
Primer sequences amplifying the MLVA loci in this table are listed in Table 2.2



Isolates	Time	Sources	States	Phage type	MAPLT	PFGE	MLVA	STTR3	STTR5	Sal20
H13	2007	Human	NSW	1	2	17	1			
H18	2008	Human	NSW	2	6	4	1			
H20	2007	Human	VIC	2	6	14	1			
H43	2006	Human	O/S	25	8	19	1			
H49	2006	Pork meat	QLD	UN	6	5	1	519 (12)	368 (14)	367 (11)
H50	2007	Human	VIC	1	6	3	1			
H55	2007	Human	NSW	2	6	9	1			
H59	2006	Human	NSW	2	6	14	1			
H04	2008	Goat meat	VIC	1	4	17	2			
H07	2008	Goat meat	QLD	1	4	17	2			
H08	2008	Human	VIC	1	4	17	2			
H14	2006	Goat meat	SA	1	1	17	2			
H15	2006	Human	SA	1	7	20	2			
H16	2006	Human	VIC	1	2	21	2			
H17	2006	Soy flour	NSW	1	2	17	2			
H32	2008	Feed Chicken	VIC	8	3	25	2			
H33	2008	Goat meat	QLD	8	3	25	2			
H35	2008	Human	SA	8	4	25	2			
H36	2007	Human	SA	8	4	25	2			
H37	2006	Goat meat	SA	8	4	25	2	519 (12)	350 (11)	367 (11)
H38	2006	Human	VIC	8	3	25	2			
H42	2006	Human	WA	23	3	2	2			
H46	2006	Human	VIC	6a	7	26	2			
H48	2008	Goat meat	QLD	UN	1	17	2			
H56	2006	Goat meat	VIC	2	1	17	2			
H61	2008	Beef meat	NSW	1	2	17	2			
H62	2009	Goat meat	QLD	1	4	17	2			
H63	2009	Goat meat	QLD	1	4	17	2			
H64	2008	Canine	NT	1	2	17	2			
H65	2008	Goat meat	QLD	1	4	17	2			
H01	2008	Human	VIC	1	2	17	3			
H02	2008	Human Blood	VIC	1	2	17	3			
H03	2008	Human	QLD	1	2	17	3			
H05	2008	Human	NSW	1	2	17	3			
H06	2008	Human	QLD	1	2	17	3			
H09	2007	Human urine	QLD	1	2	17	3			
H10	2007	Human	QLD	1	2	17	3			
H11	2007	Human	WA	1	2	17	3			
H12	2007	Human	O/S	1	2	17	3			
H19	2007	Human urine	NSW	2	3	10	3			
H21	2007	Porcine intestine	QLD	2	9	13	3			
H22	2007	Human	QLD	2	3	18	3			
H27	2007	Human	QLD	4	5	16	3	519 (12)	356 (12)	367 (11)
H28	2006	Human Blood	QLD	4	5	29	3			
H29	2007	Human	NSW	5	10	11	3			
H30	2007	Human	SA	7	2	17	3			
H44	2007	Human	QLD	26	5	7	3			
H51	2007	Human	NSW	1	2	17	3			
H52	2007	Human	QLD	4	5	27	3			
H53	2007	Human	QLD	4	5	12	3			
H54	2007	Human Blood	NSW	1	5	8	3			
H57	2006	Human	NSW	4	5	28	3			
H58	2006	Human	NSW	1	2	17	3			
H60	2007	Human	QLD	4	5	15	3			
H31	2008	Human	NSW	8	3	25	4	453 (10)	350 (11)	367 (11)
H26	2006	Human	SA	3	16	24	5	519 (12)	362 (13)	367 (11)
H41	2006	Human	O/S	23	13	23	6	519 (12)	356 (12)	367 (11)
H39	2007	Human	NSW	9	11	22	7			
H47	2008	Human	O/S	RDNC	15	6	7	519 (12)	350 (11)	373 (13)
H23	2008	Human	NSW	3	17	24	8	519 (12)	374 (15)	373 (13)
H24	2007	Human	SA	3	16	24	9	519 (12)	362 (13)	373 (13)
H25	2007	Human Blood	O/S	3	17	24	10	519 (12)	344 (10)	373 (13)
H45	2007	Human	O/S	27	12	1	11	519 (12)	338 (9)	373 (13)
H40	2006	Human	WA	9	14	22	12	519 (12)	350 (11)	376 (14)

Fig. 5.3 Dendrogram depicting genetic relationship between the 64 *S.* Heidelberg isolates based on the typing results from the discriminative MLVA loci. Novel MLVA loci SHTR-3 and SHTR-5 were omitted from the assay as they did not provide further discrimination of the isolates.

The MLVA results were presented as amplified fragment length in bp. The number of repeat units for each locus was indicated in the brackets.

5.3.4 Composite assay of MAPLT and MLVA

Typing data of the composite typing assay of MAPLT and MLVA was prepared using twelve loci comprising nine MAPLT loci and three MLVA loci (Table 5.6). Twenty-three profiles were observed in total (Fig. 5.4). Nine profiles were generated by more than one isolate and were mainly the isolates of the same phage types. For example, profile 9 was generated by six isolates, of which four were PT 2. Similarly, profile 12 was generated by eight isolates, of which six were PT 4 (Fig 5.4). The highest number of profiles was observed when using the composite assay in differentiating the predominating PT 1 isolates. The twenty-six PT 1 isolates generated eight MAPLT / MLVA profiles in comparison to six MAPLT profiles, five PFGE profiles, and three MLVA profiles. Similarly, the seven PT 8 isolates generated three MAPLT / MLVA profiles in comparison to two profiles each from MAPLT or MLVA. PFGE did not differentiate any of the PT 8 isolates. However there was no further increase in the number of generated profiles for PT 2 and PT 4 isolates when using the composite assay, compared to using MAPLT and MLVA individually. There were four profiles observed from the PT 2 isolates using MAPLT or the composite assay, each was generated by the same isolates. Likewise, none of the PT 4 isolates were differentiated by MAPLT or MLVA, therefore no PT 4 isolates were differentiated by the composite assay as well (Fig. 5.4).

5.3.5 Comparison of the differentiating abilities between the four molecular typing approaches

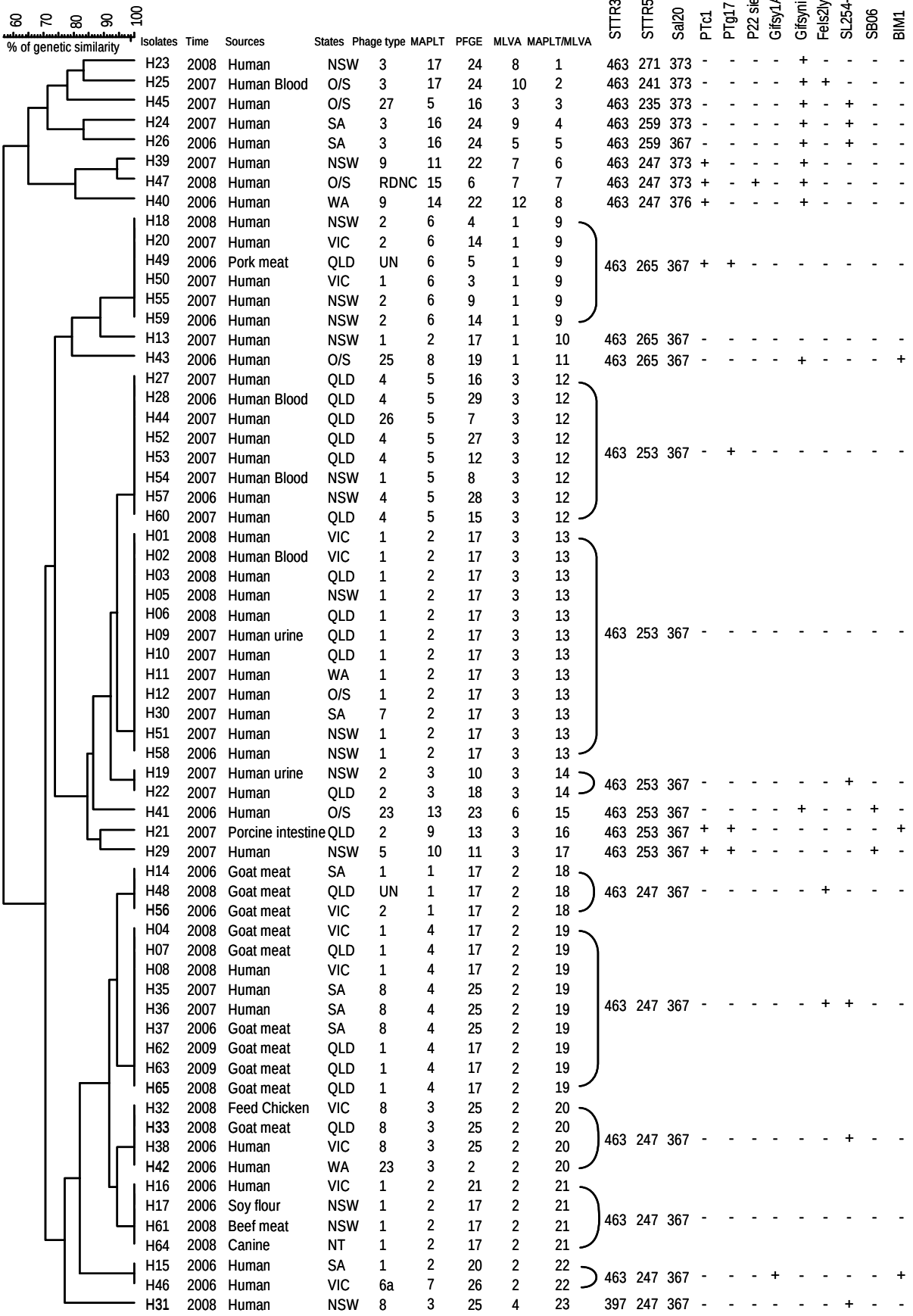
The differentiating ability of each methodology was presented as the Simpson's index of diversity (Table 5.7). MAPLT and PFGE showed to have comparable differentiating ability (MAPLT DI = 0.88; PFGE DI = 0.84). In contrast, MLVA performed the poorest giving the lowest DI value of 0.73 and the lowest number of profiles. The calculated DI value of the composite assay was shown as the highest (DI = 0.92) (Table 5.7). However it was noted that the composite assay generated a few profiles less than PFGE. The DI values of the four molecular methods in differentiating the predominating PT 1 isolates were also determined (Table 5.8). The composite assay performed the best in differentiating the PT 1 isolates giving the highest DI value and the number of profiles.

Table 5.6 Primers of the *S.* Heidelberg composite MAPLT / MLVA scheme

	Gene loci	Primer sequences (5'→ 3')
MAPLT	P22/ST64T-*cI*	[a]PTc1F: CTTTACCAATCTGAACCGCCG
		[a]PTc1R: CTGAGTTGTTTTGGCATAATTACTCC
	P22/ST64T-*g17*	[a]F17: GGCTGTTGTTTCTTCTTTCAGGC
		[a]R17: AGGAAATATGAAATTACGTGTCTGGC
	P22-*sieB*	[a]SIEBF: CGATGAACAACTCATGGTGGC
		[a]SIEBR: AGCGAGGTAAGGTATTTGTCG
	Gifsy1/2-*ninG*	GifsyninGF: ACACTGAAGATGGATGTTGAAGC
		GifsyninGR: GCCGTAAGTGCGCAAACAAAG
	Fels2- STM2714	Fels2lysBF: TGACCTTTCCAGACGGCACT
		Fels2lysBR: TGGTTCTGGCGCTGGTACTT
	Prophage in SL254-A2929	SL254tailF: AGGCGGATTACCTGAAACGTC
		SL254tailR: ATATCCACCGCCTTCTTGCTC

	Gene loci	Primer sequences (5'→ 3')
MAPLT	ST64B-SB06	[a]SB06F1: ACGACAAGCGCGTTGAGGC
		[a]SB06R1: GCTTCCACGTTGAAGAAGGC
	ST64B-SB38	[a]BIM1F: ATGGTGGCCTTGTCGACGC
		[a]BIM1R: GCTAACGTGAAGGATTTGTTCCG
MLVA	STTR-3	[b]STTR3F: CGTTGAAAATAACGGTGGC
		[b]STTR3R: CCTTTATCGATGGTGACGC
	STTR-5	[b]STTR5F: GCTGCAGTTAATTTCTGCG
		[b]STTR5R: TCAGTAAAACGGTGATCGC
	Sal20	Sal20F2: AGCAGCCGACACAACTTAACG
		Sal20R2: ACCATCCAGCGACGTTCATC

[a] Primer sequences described by Ross and Heuzenroeder (2005)
[b] Primer sequences described by Ross and Heuzenroeder (2008)



Isolates	Time	Sources	States	Phage type	MAPLT	PFGE	MLVA	MAPLT/MLVA	STTR3	STTR5	Sal20	PTc1	PTg17	P22 sieB	Gifsy1A	GifsyninG	Fels2lysB	SL254-tail	SB06	BIM1
H23	2008	Human	NSW	3	17	24	8	1	463	271	373	-	-	-	-	+	-	-	-	-
H25	2007	Human Blood	O/S	3	17	24	10	2	463	241	373	-	-	-	-	+	+	-	-	-
H45	2007	Human	O/S	27	5	16	3	3	463	235	373	-	-	-	-	+	-	+	-	-
H24	2007	Human	SA	3	16	24	9	4	463	259	373	-	-	-	-	+	-	+	-	-
H26	2006	Human	SA	3	16	24	5	5	463	259	367	-	-	-	-	+	-	+	-	-
H39	2007	Human	NSW	9	11	22	7	6	463	247	373	+	-	-	-	+	-	-	-	-
H47	2008	Human	O/S	RDNC	15	6	7	7	463	247	373	+	-	+	-	+	-	-	-	-
H40	2006	Human	WA	9	14	22	12	8	463	247	376	+	-	-	-	+	-	-	-	-
H18	2008	Human	NSW	2	6	4	1	9												
H20	2007	Human	VIC	2	6	14	1	9												
H49	2006	Pork meat	QLD	UN	6	5	1	9	463	265	367	+	+	-	-	-	-	-	-	-
H50	2007	Human	VIC	1	6	3	1	9												
H55	2007	Human	NSW	2	6	9	1	9												
H59	2006	Human	NSW	2	6	14	1	9												
H13	2007	Human	NSW	1	2	17	1	10	463	265	367	-	-	-	-	-	-	-	-	-
H43	2006	Human	O/S	25	8	19	1	11	463	265	367	-	-	-	-	+	-	-	-	+
H27	2007	Human	QLD	4	5	16	3	12												
H28	2006	Human Blood	QLD	4	5	29	3	12												
H44	2007	Human	QLD	26	5	7	3	12												
H52	2007	Human	QLD	4	5	27	3	12												
H53	2007	Human	QLD	4	5	12	3	12	463	253	367	-	+	-	-	-	-	-	-	-
H54	2007	Human Blood	NSW	1	5	8	3	12												
H57	2006	Human	NSW	4	5	28	3	12												
H60	2007	Human	QLD	4	5	15	3	12												
H01	2008	Human	VIC	1	2	17	3	13												
H02	2008	Human Blood	VIC	1	2	17	3	13												
H03	2008	Human	QLD	1	2	17	3	13												
H05	2008	Human	NSW	1	2	17	3	13												
H06	2008	Human	QLD	1	2	17	3	13												
H09	2007	Human urine	QLD	1	2	17	3	13	463	253	367	-	-	-	-	-	-	-	-	-
H10	2007	Human	QLD	1	2	17	3	13												
H11	2007	Human	WA	1	2	17	3	13												
H12	2007	Human	O/S	1	2	17	3	13												
H30	2007	Human	SA	7	2	17	3	13												
H51	2007	Human	NSW	1	2	17	3	13												
H58	2006	Human	NSW	1	2	17	3	13												
H19	2007	Human urine	NSW	2	3	10	3	14												
H22	2007	Human	QLD	2	3	18	3	14	463	253	367	-	-	-	-	-	-	+	-	-
H41	2006	Human	O/S	23	13	23	6	15	463	253	367	-	-	-	-	+	-	-	+	-
H21	2007	Porcine intestine	QLD	2	9	13	3	16	463	253	367	+	+	-	-	-	-	-	-	+
H29	2007	Human	NSW	5	10	11	3	17	463	253	367	+	+	-	-	-	-	-	+	-
H14	2006	Goat meat	SA	1	1	17	2	18												
H48	2008	Goat meat	QLD	UN	1	17	2	18	463	247	367	-	-	-	-	-	+	-	-	-
H56	2006	Goat meat	VIC	2	1	17	2	18												
H04	2008	Goat meat	VIC	1	4	17	2	19												
H07	2008	Goat meat	QLD	1	4	17	2	19												
H08	2008	Human	VIC	1	4	17	2	19												
H35	2007	Human	SA	8	4	25	2	19												
H36	2007	Human	SA	8	4	25	2	19	463	247	367	-	-	-	-	-	+	+	-	-
H37	2006	Goat meat	SA	8	4	25	2	19												
H62	2009	Goat meat	QLD	1	4	17	2	19												
H63	2009	Goat meat	QLD	1	4	17	2	19												
H65	2008	Goat meat	QLD	1	4	17	2	19												
H32	2008	Feed Chicken	VIC	8	3	25	2	20												
H33	2008	Goat meat	QLD	8	3	25	2	20												
H38	2006	Human	VIC	8	3	25	2	20	463	247	367	-	-	-	-	-	-	+	-	-
H42	2006	Human	WA	23	3	2	2	20												
H16	2006	Human	VIC	1	2	21	2	21												
H17	2006	Soy flour	NSW	1	2	17	2	21												
H61	2008	Beef meat	NSW	1	2	17	2	21	463	247	367	-	-	-	-	-	-	-	-	-
H64	2008	Canine	NT	1	2	17	2	21												
H15	2006	Human	SA	1	2	20	2	22												
H46	2006	Human	VIC	6a	7	26	2	22	463	247	367	-	-	-	+	-	-	-	-	+
H31	2008	Human	NSW	8	3	25	4	23	397	247	367	-	-	-	-	-	-	+	-	-

Fig. 5.4 Dendrogram depicting genetic relationship between the 64 *S.* Heidelberg according to the amplification results of 9 MAPLT and 3 MLVA loci.

Positive amplification of the prophage loci was indicated as '+'; negative PCR amplification of the prophage loci was indicated as '-'. The MLVA results were presented as amplified fragment length (in bp).

Table 5.7 Comparison of the differentiating abilities between the 4 methodologies based on the Simpson's index of diversity for the 64 *S.* Heidelberg isolates

	No. of primers used	No. of profiles	Simpson' index of diversity (95% confidence interval)
PFGE	na[1]	29	0.84 (0.74 to 0.92)
MAPLT	12	17	0.88 (0.83 to 0.92)
MLVA	6	12	0.73 (0.67 to 0.80)
MAPLT/MLVA	12	23	0.92 (0.89 to 0.95)

[1]na = not applicable

Table 5.8 Differentiating ability of PFGE, MAPLT, MLVA and MAPLT / MLVA for PT 1 isolates

	Simpson's index of diversity (no. of profiles)
MAPLT	0.58 (6)
PFGE	0.29 (5)
MLVA	0.59 (3)
MAPLT / MLVA	0.77 (8)

All values illustrated in this table were derived from Fig 5.1 (MAPLT); Fig 5.2 (PFGE); Fig 5.3 (MLVA), and Fig 5.4 (MAPLT /MLVA)

5.4 DISCUSSION

Salmonella Heidelberg is one of the commonly isolated *Salmonella* serovars in Australia and in other countries. In Australia phage typing is routinely carried out to monitor subtypes of *S.* Heidelberg and hence to facilitate early outbreak detection. A further differentiation of strains within phage types employs the current 'gold-standard' typing method PFGE during outbreaks. However the differentiating ability of PFGE has been challenged by the apparent genetic similarity exhibited by strains in predominating phage type groups such as PT 1 isolates in Australia. Furthermore, the PFGE protocol is time-consuming, laborious and data is open to subjective interpretation. In this study, three PCR-based typing methods that are rapid and simple to perform and that generate objective data were evaluated for their ability in discriminating *S.* Heidelberg strains.

The 12-loci MAPLT scheme appeared as capable as PFGE in differentiating within serovar *S.* Heidelberg despite the lower number of profiles observed. More significantly, the MAPLT scheme was shown to have a higher ability in subdividing the predominant PT 1 isolates in comparison to PFGE. Further improvement in discriminating the PT 1 isolates was achieved by combining MAPLT with MLVA, where the highest number of profiles and a higher DI value were observed. The 16 PT 1 isolates with MAPLT profile 2 were further divided into 3 groups of 11 isolates, 4 isolates and 1 isolate respectively (Fig. 5.4). Significantly, all the non-human PT 1 isolates were separated from the human PT 1 isolates that was not achieved by applying MAPLT, MLVA or PFGE alone. It was observed that while MAPLT identified the different prophage content between isolates derived from goat meat and human isolates, MLVA also identified the allelic differences at locus STTR-5 between the three isolates obtained from the beef, the soy flour and the canine sources. This observation suggests the usefulness of the combined molecular assay in determining transmission of *S.* Heidelberg PT 1 isolates from a wider range of animal sources to humans in comparison to MAPLT alone. However further investigation needs to be carried out to confirm the observations as this study only included a small number of PT 1 isolates from diverse animal or food origins.

DOP-PCR procedure as described in Chapter 3 has been carried out on isolates having MAPLT profile 2 to identify additional prophage loci for further discrimination. Phages showing sequence similarity to phages Gifsy-1 and Gifsy-2 were obtained (data not shown). This is consistent with the MAPLT results insofar that all PT 1 isolates amplified with at least one Gifsy-1 or Gifsy-2 locus that encodes a putative exodeoxyribonuclease gene (Fig. 5.1). Three additional gene loci including two from phage Gifsy-2 (genes encoding putative capsid protein and host specificity protein J) and one shared between phages Gifsy-1 and GIfsy-2 (gene encoding putative phage tail protein) were screened. These three Gifsy phage loci were detected in most isolates including all PT 1 isolates (Appendix 2.3). A locus within the

phage virulence gene *sopE* was also tested as this gene has been described in a number of Gifsy-related phages (Mirold *et al.*, 2001). The *sopE* locus was amplified in all isolates (Appendix 2.3). The results demonstrated the abundance of mosaically-related phages residing in *S.* Heidelberg that are genetically related to Gifsy phages. Furthermore the results suggested that the Gifsy phages would likely be the vehicle for gene *sopE* contributing to the virulence of *S.* Heidelberg isolates in Australia.

While a number of previously described MLVA loci were identified in *S.* Heidelberg, only a few of these loci demonstrated allelic polymorphism. This limited this method for typing *S.* Heidelberg based on available sequence data. Most of the published loci displayed allelic polymorphism between or within other *Salmonella* serovars but only three these loci showed allelic variations in *S.* Heidelberg (Lindstedt *et al.*, 2003; Liu *et al.*, 2003; Lindstedt *et al.*, 2004; Ramisse *et al.*, 2004; Witonski *et al.*, 2006). Locus STTR-5 was shown to be the most variable. This result was expected as locus STTR-5 has exhibited significant allelic polymorphism in all the analysed serovars including Typhi, Typhimurium, Enteritidis, Newport and Infantis (Lindstedt *et al.*, 2003; Ramisse *et al.*, 2004; Witonski *et al.*, 2006; Boxrud *et al.*, 2007; Ross and Heuzenroeder, 2008, 2009). Locus Sal20 displayed three alleles with two frequently detected and one detected from one PT 9 isolate only. Referring to Ramisse *et al.*, (2004), *S.* Paratyphi A showed a similar degree of variation at the Sal20 locus with *S.* Heidelberg in terms of the number of alleles (2 alleles) detected. Alternatively this MLVA locus was conserved in *S.* Typhimurium but nine alleles were detected in *S.* Typhi (Ramisse *et al.*, 2004). A similar observation was made for STTR-3 where high allelic diversity was shown in *S.* Typhimurium and *S.* Enteritidis (Lindstedt *et al.*, 2003; Boxrud *et al.*, 2007; Ross and Heuzenroeder, 2009). However in this study and another study describing MLVA typing of *S.* Infantis (Ross and Heuzenroeder, 2008), only one isolate was found to have a different STTR-3 allele. Taken together these results indicate that the allelic frequency displayed from MLVA loci by one serovar does not necessarily mean a similar result for other serovars. As a consequence each MLVA locus would need to be examined individually to determine its usefulness in typing other serovars of interest.

Using the default parameters of the Tandem Repeat Finder (Benson, 1999), 77 tandem repeat containing loci were recognised from the sequenced genome of *S.* Heidelberg strain SL 476 (NCBI accession no. CP001120). As observed from previous studies, truncations are commonly associated with long tandem repeat units and more allelic variations in terms of tandem repeat numbers were usually seen in loci with short tandem repeat units (Liu *et al.*, 2003; Lindstedt *et al.*, 2004; Ramisse *et al.*, 2004; Boxrud *et al.*, 2007). Therefore this study concentrated on the investigation of the degree of variation in loci containing direct and short tandem repeat length. Nine loci were further selected however only one locus (SHTR-5) showed allelic variations with two alleles observed. Two loci, SHTR-1 and SHTR-2, were not amplified in any isolates in the subset, while locus SHTR-3 was amplified from

twenty-seven of the thirty-two tested isolates. Locus SHTR-1 is located upstream from a P4-like integrase gene, while SHTR-2 and -3 are located downstream from a phage capsid gene. Since these 3 loci were found located adjacent to the phage-related elements (i.e. mobile genetic elements), they are likely to be of phage origin as well. This may explain why these loci were present in the sequenced *S.* Heidelberg genome but were not detected or variably detected in the tested isolates. Similarly, STTR-6 is a Gifsy-1 phage-related locus that was not detected in any isolates although other Gifsy-1 loci were differentially present in the collected isolates. This observation clearly demonstrated the mosaic nature of phage genomes.

The inability of the selected panel of MLVA loci (Table 5.1 and Table 5.5) to separate *S*. Heidelberg indicated the need to further identify discriminative MLVA loci before this method alone can be a useful option for typing purposes. In this study, nine of the seventy-seven novel MLVA loci with short direct repeat units (3-18bp) were examined but none provided further resolution. Therefore the remaining novel MLVA loci with longer repeat units may need to be examined even though random truncations at the repeat units may be present complicating determination of the number of repeat units contained within. For example, locus STTR-3 from the current MLVA typing scheme for *S.* Typhimurium was found to contain a combination of tandem repeats of 27bp and 33bp in lengths (Lindstedt *et al.*, 2003; Ross and Heuzenroeder, 2008, 2009). To utilise this locus for strain differentiation whilst maintaining the ease of result interpretation, the actual length of the amplified STTR-3 is generally reported without the calculation of the number of repeat units (Gilbert, 2008; Larsson *et al.*, 2009).

Recently, another complete genomic sequence of *S.* Heidelberg strain B182 has been deposited in GenBank (NCBI accession no. CP003461). Subsequently, a sequence comparison between *S.* Heidelberg strains SL 476 and B182 can be carried out enabling identification of MLVA loci showing variations between the two *S.* Heidelberg strains. The availability of multiple complete genomes of a serovar can also ensure the detection of most if not all the MLVA loci present in a serovar considering that some MLVA loci may not be present in every strain of a serovar as discussed in Chapter 3. An online-based bioinformatics tool at http://minisatellite.u-psud.fr under the “Strain Comparison Page” is freely accessible to fulfill the purpose (Denæud and Vergnaud, 2004).

5.5 CHAPTER SUMMARY

This study demonstrated the use of MAPLT and MLVA in discriminating *S.* Heidelberg isolates, in particular between the predominating *S.* Heidelberg PT 1 isolates. In comparison to PFGE, MAPLT demonstrated a higher ability in differentiating the PT 1 isolates. Furthermore the results suggested that MAPLT may be a valuable epidemiological typing tool for determining transmission of *S.* Heidelberg PT 1 isolates from suspect food or environmental source (such as goat meat) to humans. As MAPLT and MLVA separated isolates of the same phage type differently it was concluded that the composite assay of MAPLT and MLVA is possibly the most useful and practical approach when a high level differentiation is required. Further assay development should be carried out using known epidemiologically-related and unrelated isolates to validate MAPLT or the composite assay for outbreak epidemiological studies.

CHAPTER 6 GENETIC CHARACTERISATION OF BACTERIOPHAGES IN *SALMONELLA*

6.1 INTRODUCTION

Bacteriophages or phages are believed to be the most abundant biological entities on earth with an estimate of 10^{31} phage particles in the environment (Ashelford *et al.*, 2003; Suttle, 2005). Bacteriophages can be classified in a number of ways, most commonly by their morphology and the nucleic acid types contained in the viral particles. Current data indicates that tailed phages containing double-stranded DNA (dsDNA) form the majority of phages, comprising 96% of the total number of phages that have been investigated (Ackermann and Kropinski, 2007; Hatfull, 2008; Ackermann, 2009). An extensive range of over 150 bacterial genera are known to be infected with dsDNA tailed-phages (Ackermann, 2009). *Salmonellae* is one such genus frequently infected with dsDNA tailed-phages that are temperate in nature. As a consequence of lysogenic infection, the phage DNA integrates into and replicates along with the bacterial genome during cell division (Miller and Day, 2008).

As the genomic sequences of more phages become available, they are further classified into phage families according to the similarity displayed in their genomic architectures. In other words, this approach classifies phages into the same families when they have genes of similar functions arranged in the same order, regardless of whether the genes unrelated at the nucleotide level or not (Campbell, 1994). The lambdoid family is one phage family to which the characterised *Salmonella* temperate phages belong, including the well-studied *Salmonella* phage P22 (Susskind and Botstein, 1978). Temperate bacteriophage λ is the type virus of this family, to which all family members show relationships in terms of their overall genomic architecture.

This genetic grouping of phages was proposed based on the modular theory of bacteriophage evolution (Susskind and Botstein, 1978) in that the diversity of phages is principally driven by horizontal exchanges of genes that share similar functions occupying corresponding locations in the genomes (Brüssow *et al.*, 2004). This theory rationalised the mosaic features between lambdoid phages that was observed in DNA heteroduplex mapping and sequence data analysis where there were DNA regions showing nearly identical DNA sequences that were interrupted with regions showing little to no sequence identity between each pair of the examined lamdboid phages (Simon *et al.*, 1971; Juhala *et al.*, 2000; Mmolawa *et al.*, 2003; Price-Carter *et al.*, 2011). These same observations also supported the view that horizontal gene exchanges among phages of a family (e.g. lambdoid phages) infecting closely related groups of hosts (e.g. enteric bacteria) would be a frequent occurrence (Hendrix *et al.*, 1999).

Nevertheless temperate phages infecting one bacterial host may be composed of individual genes from phages infecting a wide range of phylogenetically distant bacterial hosts (Hendrix *et al.*, 1999). One example is the *S.* Typhimurium phage ST64B, where most genes showed similarity at the translated protein level to phages infecting hosts outside the family *Enterobacteriaceae* including Gram-positive bacteria (Mmolawa *et al.*, 2003). A similar observation was seen for the cryptic prophage ϕflu in *Haemophilus influenzae* that had genes showing sequence similarity to phages infecting *Haemophilus*, *Bacillus*, *Salmonella*, *Mycobacterium*, *Escherichia*, *Streptococcus*, and *Lactococcus* (Hendrix *et al.*, 1999). These observations suggested that there could have been many steps of modular exchanges occurring over a long period of time that brought these genes together, therefore the similarity between the translated proteins between phages of distant hosts is often weak (Hendrix *et al.*, 1999). Therefore, while gene exchanges between phages occur continuously and that the sources of the genes could be derived from phages infecting distantly related hosts, prophage integration often contributes to inter-strain genetic differences within defined bacterial groups such as *Salmonella* serovars (Deng *et al.*, 2003; Hermans *et al.*, 2005; Cooke *et al.*, 2007).

Upon lysogenic infection survival fitness of the bacterial hosts may be enhanced through lysogenic conversion, due to putative virulence determinants carried by the phages. Several putative and experimentally confirmed virulence determinants have been detected on *Salmonella* phages. Section 1.3.6.1 provides a detailed discussion regarding the *Salmonella* phage-encoded virulence factors. Some of these genes have been shown to significantly contribute to the overall *Salmonella* pathogenicity, such as the *sodCI* gene (De Groote *et al.*, 1997; Farrant *et al.*, 1997) and the *gtgE* gene (Ho *et al.*, 2002). Both these virulence genes were mapped on phage Gifsy-2 of *S.* Typhimurium strain LT2 (Figueroa-Bossi *et al.*, 2001), and when mutated separately caused a 5-fold and a 7-fold attenuation *in vivo* respectively (Ho *et al.*, 2002). Combined mutations of these two genes showed a greater than 100-fold attenuation that was similar to when the test strain was cured of Gifsy-2 (Ho *et al.*, 2002). Phage Gifsy-1 residing in *S.* Typhimurium LT2 also carries virulence genes, such as the *gipA* gene that encodes a protein needed for growth in the intestinal phase of infection (Stanley *et al.*, 2000).

Referring to the typing data from the previous chapters, it was observed that Gifsy phage loci were frequently detected in the studied serovars: phage Gifsy-1 loci were commonly detected in the *S.* Virchow isolates, and phage Gifsy-2 loci were common in *S.* Heidelberg isolates. The aim of this chapter is to examine the significance of Gifsy-like phages in terms of their contribution to genetic variation and virulence to *Salmonella* serovars.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial isolates

Table 6.1 Bacterial isolates used in this chapter

Isolates	*Salmonella* serovars & [a]PT	Purpose	Source
V10	*S.* Virchow PT 8	phage induction	Chapter 3
H03	*S.* Heidelberg PT 1	phage induction	Chapter 5
1727	*S.* Enteritidis PT 1	indicator strain*	Tan 2010

[a]PT = phage type; n/a = not applicable

*used for propagation of the induced phages from isolates V10 and H03

During the course of this study, phage lysates and subsequent phage propagation were performed on a number of *S.* Virchow, *S.* Heidelberg and *S.* Bovismorbificans isolates (see below for procedure). Phages induced from isolate *S.* Virchow V10, namely PV10 and isolate *S.* Heidelberg H03, designated PH03 were obtained in high titres and therefore were selected for whole genome sequencing. Prior to DNA sequencing, the DOP-PCR procedure was carried out to confirm that the induced phages PV10 and PH03 were Gifsy-1 related and Gifsy-2 related respectively (data not shown). In contrast, DOP-PCR sequence data of the induced *S.* Bovismorbificans phage lysates failed to show DNA identity to phages Gifsy-1 or Gifsy-2 and these therefore were omitted from the study.

6.2.2 Purification of bacteriophages

Bacteriophages to be characterised were induced from their bacterial hosts according to the procedure as described in section 3.2.1.1 (small scale induction). A culture of indicator strain *S.* Enteritidis 1727 was prepared by growing a 1 in 100 diluted overnight culture in LB broth in the presence of 5mM calcium chloride until the OD_{600} reached 0.3. To propagate phages, 100µl of the phage lysate was added to 200µl of broth culture of indicator strain and the mixture was incubated at 37C for 15 min for phage absorption. The phage-bacteria mixture was then added to 3ml of 0.5% molten LB agar, mixed gently and poured into a 90mm (diameter) LB plate. After overnight incubation at 37C, single plaques were harvested and resuspended individually into vials containing 500µl of SM buffer. The vials were incubated at room temperature for at least 2 hours to allow phage elution prior to storage at 4C until used. Plaque morphologies produced by each lysate are shown in Fig. 6.1a -b.

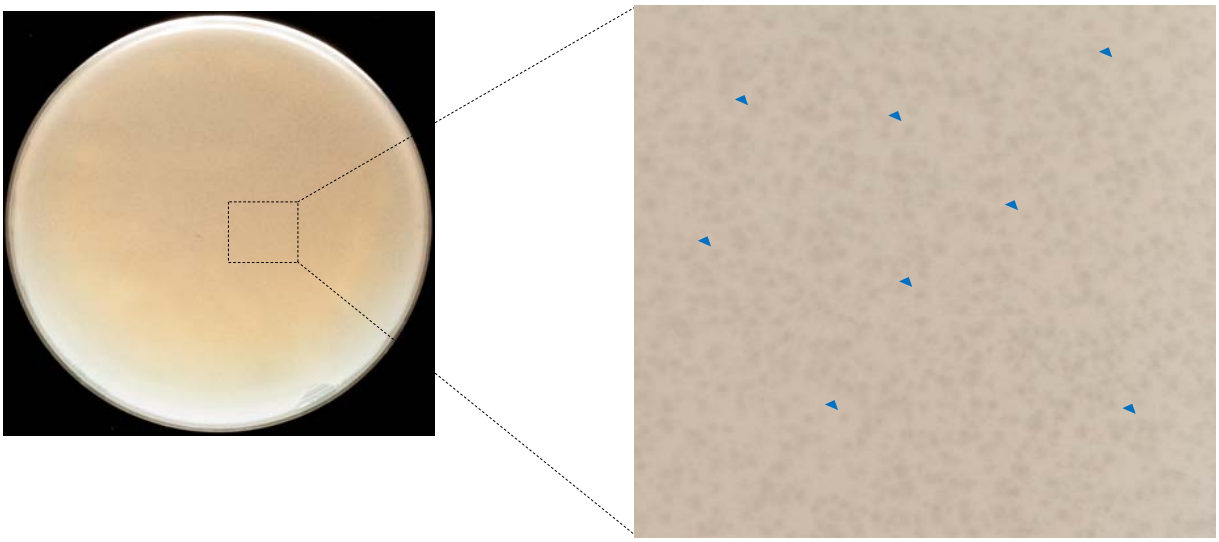


Fig. 6.1a Plate lysis of bacteriophage PV10

Propagation of phage PV10 was achieved through lysis of a plate lawn of indicator bacteria. Growth of phages was evident as turbid plaques observed throughout the plate (examples were marked with blue triangles). Plaques produced by PV10 were approximately 0.5mm diameters in size.

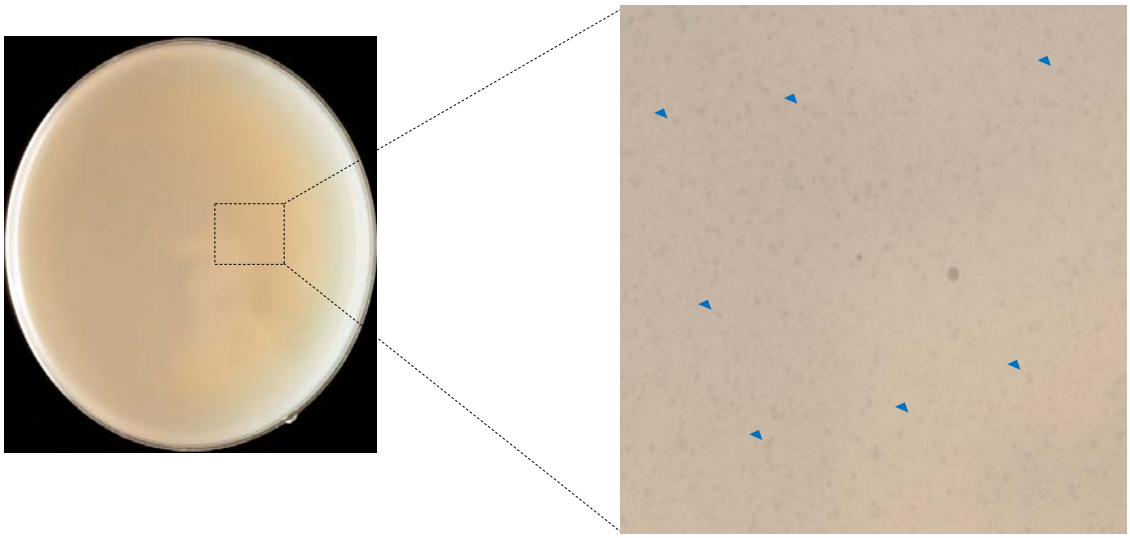


Fig. 6.1b Plate lysis of bacteriophage PH03

Propagation of phage PH03 was achieved through lysis of a plate lawn of indicator bacteria. Growth of phages was evident as turbid plaques observed throughout the plate (examples were marked with blue triangles). Plaques produced by PH03 were approximately 0.2mm diameters in size.

6.2.3 Propagation of extracted bacteriophages

Bacteriophages extracted from each plaque were propagated twice due to the small starting volume of the phage suspension. As tiny plaques (0.2 to 0.5mm diameters in size) were observed from crude lysates PV10 and PH03 (Fig. 6.1a-b), 5% w/v glycerol was added to both top and bottom LB layer in order to improve burst sizes (Santos *et al.*, 2009). Increasing the multiplicity of infection (MOI) through use of diluted bacterial cultures, and slowing down bacterial growth by incubating phage plates at room temperature were also applied to improve phage titres.

For the first round of propagation, the plate lysates were prepared on four of 90mm (diameter) LB plates. The procedure used was based on section 6.2.2 but omitted the steps involving plaque isolation. Instead, 1ml of SM buffer was added to each plate lysate after overnight incubation and the plates were left at room temperature for 2 hours to allow phage elution. The top agar layers were transferred to sterile centrifuge tubes that were centrifuged at 3220 *x g* for 20 min. The supernatant from each tube was recovered and filtered through 0.45µm filters. At the second round of propagation, the phages obtained from the first round of propagation (4ml of phage suspension) were used to prepare eight 150mm (diameter) plates of phage lysates. The procedure used followed that in section 3.2.2.3. Titres of phages were then determined as described in section 3.2.2.4.

6.2.4 Bacteriophage DNA extraction, quantity and quality evaluation

Extraction of bacteriophage DNA was performed using the procedure described in section 3.2.3.1, and the amount of DNA was determined as described in section 3.2.3.2.

The integrity of each extracted DNA sample was visualised using electrophoresis. An aliquot of 5µl was used for each sample. Restriction enzyme digestion with enzyme *Hind*III was carried out to estimate the phage genome size. Each reaction mixture contained 10µl of DNA, 1x reaction buffer, 1% bovine serum albumin and 2 U *Hind*III restriction enzyme. The total reaction volume was 20µl. The reactions were incubated in 37C for 2 hours prior to electrophoresis. Ten microlitre of each DNA sample was mixed with an equal amount of 2x loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 20% glycerol, 2mM EDTA). Electrophoresis was conducted on 0.8% (w/v) gels in a Gibco BRL model Horizon® 11.14 horizontal gel electrophoresis apparatus (Life Technologies, Gaithersburg, MD, US). Gels were run in 1xTAE buffer (40mM sodium acetate, 40mM Tris and 2mM EDTA). Visualisation of DNA bands was with ultraviolet light in a Gel Doc system BioRad Laboratories, Hercule, CA, US) after the gels were stained with 1x GelRed™ solution.

DNA molecular weight marker phage SPP-1 digested with *EcoR*I was run in each gel and contained DNA fragments of the following sizes (in kilobases, kb): 8.557, 7.427, 6.106, 4.899, 3.639, 2.799, 1.953, 1.882, 1.515, 1.412, 1.164, 0.992, 0.710, 0.492, 0.359 and 0.081. The SPP-1 DNA marker was purchased from Geneworks, Thebarton, South Australia.

Gel images of the intact and the *Hind*III digested phage DNA for the two phage samples are illustrated in Fig. 6.2a- b.

6.2.5 Bacteriophage genome sequencing

The phage genomes were sequenced using next-generation sequencing technology on an Illumina® Genome Analyzer IIx. The generated sequence reads were assembled into contigs in the SeqMan NGen™ version 3 (DNASTAR Inc., Madison, WI, US). Both sequencing and assembly of sequence reads were performed by Geneworks (Thebarton, South Australia, Australia).

6.2.6 DNA sequence analysis

The assembled sequence reads were viewed using SeqMan Pro from Lasergene® version 10 (DNASTAR Inc., Madison, WI, US) to ascertain any discrepancies at bases between reads at the same position. Contig assembly was performed on BioNumerics version 6.6.4 (Applied Maths, Kortrijk, Belgium). Prediction of open reading frames (ORFs) was performed on BioNumerics and GeneMark.hmm Heuristic Approach (Besemer and Borodovsky, 1999) (available from http://exon.gatech.edu/heuristic_hmm2.cgi). Functions of the ORFs were assigned by scanning the deduced proteins of the ORFs for homologues using BlastP (Altschul *et al.*, 1990) (available from http://blast.ncbi.nlm.nih.gov/Blast.cgi) against GenBank database of non-redundant protein sequences. Program BlastN (Altschul *et al.*, 1990) was used to determine the degree of similarity at nucleotide level between the ORFs and the homologues. An online Artmetis Comparison Tool (ACT) (Carver *et al.*, 2005) (available from http://www.webact.org/WebACT/generate) was used to display pair-wise comparisions between the sequenced phage genomes with phage genomes deposited in the NCBI database. The genomic regions that are ≥ 100bp in length showing ≥ 80% DNA identity will be highlighted in blue in the diagrams. Software Kodon version 3.5 (Applied Maths. Kortijk, Belgium) was used to visualise and draw genetic maps of the analysed phages.

Fig.6.2a - PV10

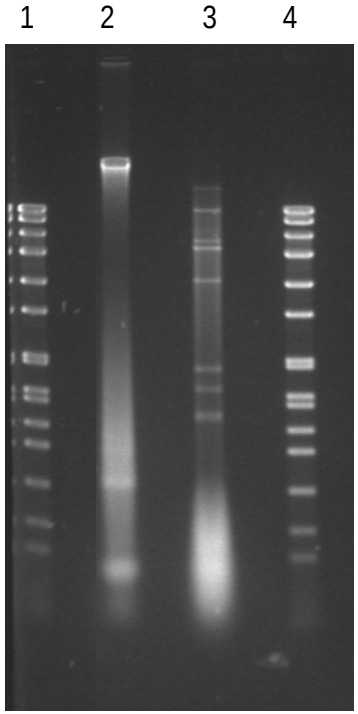


Fig.6.2b - PH03

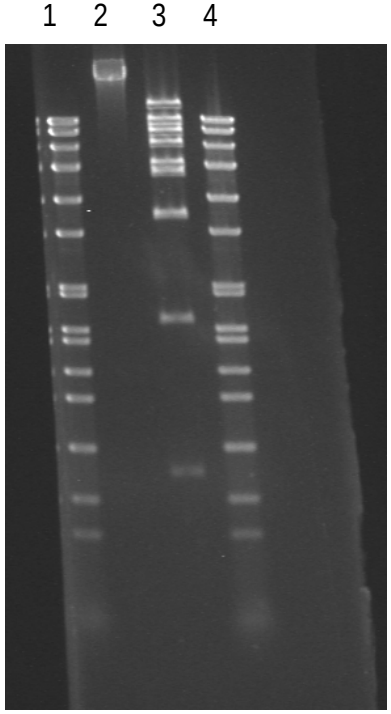


Fig. 6.2a-b Bacteriophages PV10 (a) and PH03 (b) uncut genomic DNA and genomic DNA digests with restriction enzyme *Hind*III
Electrophoresis was run on 0.8% TAE gels. In each gel: Lane 1, 4: molecular weight marker SPP-1 digested with *EcoR*I (Geneworks, Thebarton, SA); Lane 2: phage DNA uncut; Lane 3: phage DNA digested by *Hind*III.
Both DNA samples were shown to be intact and have adequate quality allowing restriction enzyme digestion.

6.3 RESULTS AND DISCUSSION

6.3.1 Genomic sequence analysis of PV10

The sequenced genome of PV10 was 47,419bp in size with 56 putative open reading frames (ORFs). Through BlastP searches, assignment of putative PV10 genes wasnfirmed and their functions were inferred (Table 6.2). Overall, the amino acid sequences inferred from most ORFs showed high levels of similarity (>85% identity) to those of phage Gifsy-1; while the predicted proteins of the remaining ORFs showed similarity to those of phage Gifsy-2, as well as unamed prophages harboured in various *Salmonella* serovars and other enteric bactera.

Similar to the published phage genomes, the putative genes of phage PV10 are organised into modules with each module responsible for a particular function. It was observed that the functional modules were organised in a similar order to phage lambda, suggesting that phage PV10 is a member of the lambdoid family (Fig. 6.3). At the nucleotide level, the PV10 genome showed a mosaic relationship to the previously studied phages Gifsy-1 and Gifsy-2 (Table 6.2; Fig. 6.4a and b). There are thirteen ≥100-bp genomic segments of PV10 showing ≥ 90% sequence similarity to phage Gifsy-1. This is equivalent to 69.3% of the genomic sequence of PV10. When compared with phage Gifsy-2, eighteen ≥100-bp genomic segments of PV10 showed ≥ 81% sequence similarity to Gifsy-2, which is equivalent to 38.4% of the genomic sequence of PV10.

With regard to the putative ORFs encoding proteins for the head and tail parts of PV10, all but three genes showed over 90% identity throughout the complete length of the ORFs to phage Gifsy-1. The exceptions were ORFs 16, 23 and 25, which encode a tail tape measure protein, a side tail protein and a side tail fibre assembly factor respectively. In contrast, the putative ORFs encoding proteins essential for viral growth shared a lesser number of genes with phage Gifsy-1 (Table 6.2). For example, the immunity region of PV10 is composed of ORFs 41, 42 and 43 encoding putative CI, CRO and CII proteins respectively. Only the putative gene *cII* shared DNA sequence similarity with that of Gifsy-1. Similarly, of the three putative ORFs (ORFs 53, 54 and 55) responsible for cell lysis, only ORF55 that is a homolog of λ gene *Rz*, showed sequence similarity to the corresponding gene of Gifsy-1. For the twenty ORFs encoding hypothetical proteins and proteins that are not essential for viral growth, only ten showed significant DNA similarity to those of phage Gifsy-1.

Table 6.2 PV10 ORFs and putative functions

ORF	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related sequences	% (nucleotide identities)	% (aa[2] identities)	BlastP e value
1	*1*	*546*	+	*546*	*162*	*Terminase small subunit;*	*AAL21504; Gifsy-1*	*99% (544/546)*	*100% (162/162)*	*2e-133*
2	*518*	*2449*	+	*1932*	*643*	*Terminase large subunit*	*AAL21503; Gifsy-1*	*99% (1928/1932)*	*99% (641/643)*	*0.0*
3	*2433*	*2636*	+	*204*	*67*	*Head-to-tail joining protein*	*AAL21502; Gifsy-1*	*99% (203/204)*	*100% (67/67)*	*5e-40*
4	*2633*	*4213*	+	*1581*	*526*	*Portal protein*	*AAL21501; Gifsy-1*	*99% (1573/1581)*	*99% (524/526)*	*0.0*
5	*4203*	*5699*	+	*1497*	*498*	*Prohead protease*	*AAL21500; Gifsy-1*	*99% (1482/1497)*	*98% (489/498)*	*0.0*
6	*5712*	*6059*	+	*348*	*115*	*Head decoration protein*	*AAL21499; Gifsy-1*	*99% (347/348)*	*100% (115/115)*	*3e-76*
7	*6114*	*7142*	+	*1029*	*342*	*Major capsid protein*	*AAL21498; Gifsy-1*	*100% (1029/1029)*	*100% (342/342)*	*0.0*
8	*7200*	*7559*	+	*360*	*119*	*Accessory DNA packaging protein*	*AAL21497; Gifsy-1*	*100% (360/360)*	*100% (119/119)*	*7e-78*
9	*7570*	*7953*	+	*384*	*127*	*Minor capsid protein*	*AAL21496; Gifsy-1*	*100% (384/384)*	*100% (127/127)*	*7e-85*
10	*7981*	*8559*	+	*579*	*192*	*Head-tail assembly protein*	*AAL21495; Gifsy-1*	*100% (579/579)*	*100% (192/192)*	*1e-133*
11	*8608*	*9738*	-	*1131*	*376*	*GipA protein*	*AAF98319; Gifsy-1*	*100% (1131/1131)*	*100% (376/376)*	*0.0*
12	*9847*	*10248*	+	*402*	*133*	*Tail shaft stabilisation protein*	*AAL21493; Gifsy-1*	*99% (401/402)*	*99% (132/133)*	*6e-91*
13	*10259*	*11002*	+	*747*	*248*	*Major tail subunit*	*AAL21492; Gifsy-1*	*100% (747/747)*	*100% (248/248)*	*4e-173*
14	*11053*	*11448*	+	*396*	*131*	*Tail assembly chaperone*	*AAL21491; Gifsy-1*	*100% (396/396)*	*100% (131/131)*	*3e-91*

ORF	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related sequences	% (nucleotide identities)	% (aa[2] identities)	Blast P e value
15	*11445*	*11783*	+	*339*	*112*	*Part of tail assembly chaperone gene G*	*AAL21490; Gifsy-1*	*99% (338/339)*	*100% (112/112)*	*2e-75*
16	11755	14796	+	3042	1013	Tail tape measure protein	AAL21489; Gifsy-1	98% (2475/2530)	93% (961/1013)	0.0
							AAL19975; Gifsy-2	91% (1978/2172)	85% (803/948)	0.0
17	*14799*	*15128*	+	*330*	*109*	*Tail tip assembly protein*	*AAL21488; Gifsy-1*	*100% (330/330)*	*100% (109/109)*	*7e-75*
							AAL19976; Gifsy-2		*79% (85/109)*	*6e-44*
18	*15138*	*15836*	+	*699*	*232*	*Tail tip assembly protein*	*AAL21487; Gifsy-1*	*99% (698/699)*	*99% (231/232)*	*3e-171*
19	*15981*	*16580*	+	*600*	*199*	*Tail tip assembly protein*	*AAL21486; Gifsy-1*	*99% (593/600)*	*98% (195/199)*	*3e-143*
20	*16478*	*17125*	+	*648*	*215*	*Tail tip assembly protein*	*AAL21485; Gifsy-1*	*98% (638/648)*	*99% (212/215)*	*2e-152*
21	*17188*	*20550*	+	*3363*	*1120*	*Host specificity protein*	*AAL21484; Gifsy-1*	*99% (3308/3363)*	*98%(1053/1078)*	*0.0*
22	*20589*	*20831*	+	*243*	*80*	*Hypothetical protein*	*YP_001700592; Gifsy-1*	*99% (240/243)*	*98% (78/80)*	*2e-50*
							YP_001700595; Gifsy-2	*99% (240/243)*	*99% (79/80)*	*3e-51*
23	20885	23323	+	2439	812	Side tail fibre protein	AAL21483; Gifsy-1	94% (1391/1484)	92% (743/812)	0.0
							AAL19983; Gifsy-2	97% (2354/2435)	98% (793/812)	0.0
24	*23320*	*24144*	+	*825*	*274*	*Tail assembly-like protein*	*AAL21482; Gifsy-1*	*91% (753/825)*	*90% (247/274)*	*0.0*

ORF	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related sequences	% (nucleotide identities)	% (aa[2] identities)	Blast P e value
25	24134	24715	+	582	193	Side tail fibre assembly factor	AAL21481; Gifsy-1	85% (487/570)	87% (166/191)	2e-116
							AAL19984; Gifsy-2	88% (503/570)	90% (167/191)	5e-122
26	24912	25634	-	723	240	Type III secretive protein	YP_002045056; prophage of S. Heidelberg SL476	100% (723/723)	100% (240/240)	1e-176
27	25847	26065	+	219	72	DNA invertase	AFH45051; prophage of *S.*Heidelberg str. B182	100% (219/219)	100% (72/72)	2e-43
28	26285	27505	+	1221	406	Transposase	AFH45052; prophage of *S.*Heidelberg str. B182	100% (1221/1221)	100% (406/406)	0.0
29	27502	27927	-	426	141	Hypothetical protein	AFH45053; prophage of *S.*Heidelberg str. B182	99% (425/426)	99% (140/141)	7e-94
							AAL21479; Gifsy-1	78% (180/232)	55% (74/134)	1e-34
30	28363	29703	-	1341	446	Integrase	AAL19939; Gifsy-2	99% (1339/1341)	99% (429/430)	0.0
31	29700	29948	-	249	82	Excisionase	AAL19940; Gifsy-2	100% (249/249)	100% (82/82)	2e-53
32	*29989*	*30228*	-	*240*	*79*	*Hypothetical protein*	*AAL21528; Gifsy-1*	*99% (238/240)*	*100% (79/79)*	*1e-50*
							AAL19941; Gifsy-2	*99% (238/240)*	*100% (79/79)*	*1e-50*

ORF	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related sequences	% (nucleotide identities)	% (aa[2] identities)	Blast P e value
33	*30271*	*31428*	-	*1158*	*385*	*Recombination protein*	*AAL21527; Gifsy-1*	*99% (1153/1158)*	*99% (368/369)*	*0.0*
							AAL19942; Gifsy-2	*99% (1153/1158)*	*99% (368/369)*	*0.0*
34	31391	34591	-	3201	1066	Recombination protein	EDZ01339; prophage from *S.* Virchow SL491	100% (3201/3201)	100% (1066/1066)	0.0
							AAL21526; Gifsy-1	99% (1339/1346)	91% (559/616)	0.0
							AAL19943; Gifsy-2	99% (1339/1346)	91% (559/616)	0.0
35	*34718*	*35068*	-	*351*	*116*	*Hypothetical protein*	*AAL21525; Gifsy-1*	*99% (347/351)*	*97% (113/116)*	*4e-79*
36	35090	35260	-	171	56	Hypothetical protein	ABX67578; *S.* Paratyphi B str SPB7	99% (170/171)	98% (55/56)	2e-32
37	*35253*	*35369*	-	*117*	*38*	*Hypothetical protein*	*AAL21523; Gifsy-1*	*99% (116/117)*	*100% (38/38)*	*2e-18*
							AAL19945; Gifsy-2	*97% (114/117)*	*97% (37/38)*	*6e-18*
38	35657	35863	+	207	68	Hypothetical protein	AFH45004; prophage of *S.*Heidelberg str. B182	98% (203/207)	99% (67/68)	5e-36
39	35903	36709	-	807	268	Hypothetical protein	CCC29984; prophage of *S. bongori* NCTC 12419	99% (806/807)	100% (268/268)	0.0
40	36706	37554	-	849	282	Hypothetical protein	CCC29985; prophage of *S. bongori* NCTC 12419	99% (848/849)	100% (282/282)	0.0

ORF	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related sequences	% (nucleotide identities)	% (aa[2] identities)	Blast P e value
41	37726	38121	-	396	131	Repressor protein	AFH45005; prophage of *S.*Heidelberg str. B182	97% (386/396)	99% (130/131)	4e-89
42	38226	38462	+	237	78	Regulatory protein CRO	AFH45006; prophage of *S.*Heidelberg str. B182	99% (234/237)	99% (77/78)	5e-50
43	*38428*	*38802*	+	*375*	*124*	*Regulatory protein CII*	*AAL21521; Gifsy-1*	*97% (365/375)*	*98% (121/124)*	*9e-84*
							AAL19947; Gifsy-2	*97% (365/375)*	*98% (121/124)*	*9e-84*
44	38995	39912	+	918	305	Replication protein	ABV13029; prophage of *C. koseri* ATCC BAA-895	84% (788/942)	85% (267/313)	0.0
45	39909	40604	+	696	231	Replication protein	ACF62348; prophage of *S.* Newport str SL254	84% (591/701)	84% (194/231)	7e-141
46	40618	41118	+	501	166	Putative morphogenetic function	ABX66407; prophage of *S.* Paratyphi B SPB7	90% (209/233)	62% (88/143)	7e-47
47	*41965*	*42198*	+	*234*	*77*	*Damage-inducible protein*	*AAL21515; Gifsy-1*	*99% (232/234)*	*99% (76/77)*	*2e-48*
							AAL19953; Gifsy-2	*99% (232/234)*	*99% (76/77)*	*2e-48*
48	42447	42563	+	117	38	Hypothetical protein	AFH45016; prophage of *S.*Heidelberg str. B182	100% (117/117)	100% (38/38)	1e-19

ORF	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related sequences	% (nucleotide identities)	% (aa[2] identities)	Blast P e value
49	*42598*	*43200*	+	*603*	*200*	*Hypothetical protein*	*AAL21514; Gifsy-1*	*97% (583/603)*	*99% (198/200)*	*1e-143*
							AAL19954; Gfisy-2	*97% (583/603)*	*99% (198/200)*	*4e-144*
50	*43200*	*43406*	+	*207*	*68*	*Hypothetical protein*	*YP_001700624; Gifsy-1*	*99% (203/207)*	*98% (65/66)*	*2e-41*
							YP_001700663; Gifsy-2	*99% (204/207)*	*98% (65/66)*	*2e-42*
51	*43409*	*44020*	+	*612*	*203*	*Unknown (NinG)*	*AAL21513; Gifsy-1*	*99% (606/612)*	*99% (201/203)*	*7e-144*
							AAL19955; Gifsy-2	*99% (606/612)*	*99% (201/203)*	*5e-143*
52	*44154*	*44843*	+	*690*	*229*	*Antitermination protein Q*	*AAL21511; Gifsy-1*	*91% (436/480)*	*77% (170/220)*	*1e-125*
53	45038	45385	+	348	115	Holin	ABX66420; prophage of *S.* Paratyphi B SPB7	98% (342/348)	100% (115/115)	2e-76
							AAL21139; Gifsy-1	80% (145/182)	76% (56/74)	3e-33
54	45388	46014	+	627	208	Endolysin	ABX66421; prophage of *S.* Paratyphi B SPB7	99% (623/627)	99% (206/208)	3e-151
55	*46011*	*46496*	+	*468*	*161*	*Endopeptidase*	*AAL21506; Gifsy-1*	*99% (389/399)*	*90% (138/153)*	*3e-81*
56	*46732*	*47133*	-	*402*	*133*	*Hypothetical protein*	*AAL21505; Gifsy-1*	*99% (399/402)*	*99% (132/133)*	*2e-90*

Putative functions of the ORFs were first inferred based on their homology shown to proteins of phages Gifsy-1 and Gifsy-2 then to other proteins in NCBI databases.

% of nucleotide identities were based on BlastN search, while % of amino acid identities were based on BlastP search.

ORFs showing ≥ 90% DNA similarity to Gifsy-1 genes are italicised

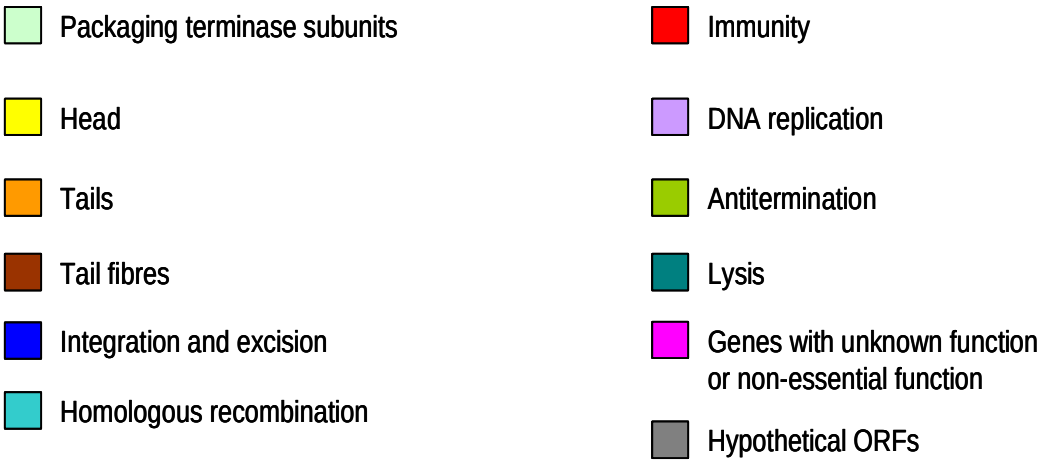
[1]nt = nucleotide sequences; [2]aa = amino acid sequences

Fig. 6.3 Diagrammatic presentations of temperate phages PV10 with Gifsy-1 and λ genomes. The gene map of PV10 shows the putative ORFs labelling from 1 to 56 and also the directions of transcription presented in Table 6.2.

The gene map of phage λ is constructed based on the genomic sequence deposited in GenBank (NCBI no. NC_001416). The genes are labelled also in reference to Hendrix and Casjens (2006).

The gene map of phage Gifsy-1 is constructed according to the genomic sequence in Genbank (NCBI no. AE006468 from nt position 2728552 - 2777042) with modifications to show the genome organisation when the phage is in the infective form. However this remains to be proven. Genes are annotated in reference to NCBI genome file NC_101392 which contains the refined annotation of Gifsy-1, and Boyd and Brüssow (2002) using phage λ nomenclature when possible.

Each map is drawn to scale individually. Symbol -\\- in Gifsy-1 map indicates where the modification was made and the region was not scaled.



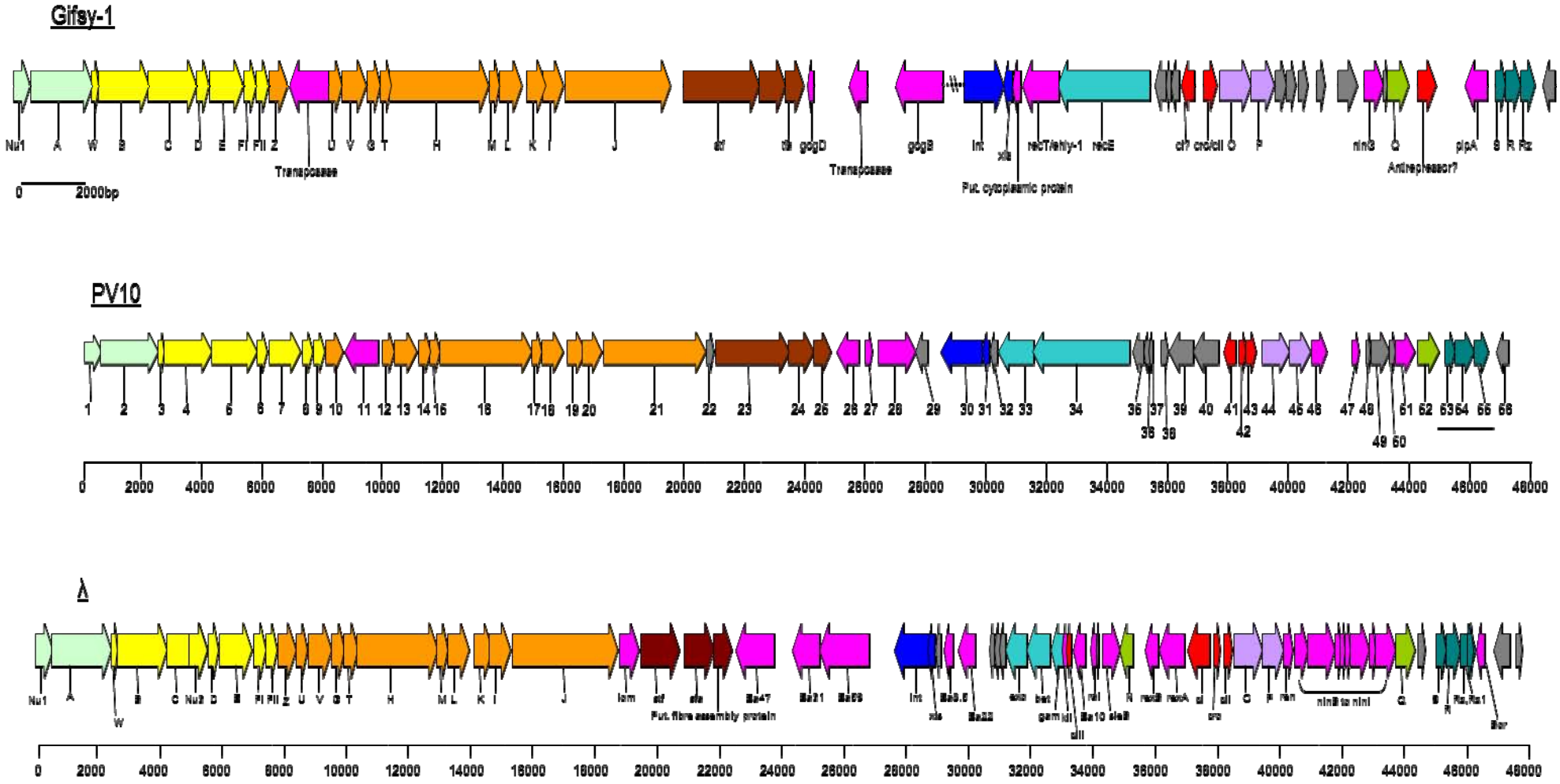

Gifsy-1
Nu1 A W B C D E FI FII Z U V G T H M L K I J stf tfa gogD gogB Int xis recT/ehly-1 recE cI? cro/cII O P nin3 Q pipA S R Rz
Transposase
Transposase
Put. cytoplasmic protein
Antirepressor?
0 2000bp
PV10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
0 2000 4000 6000 8000 10000 12000 14000 16000 18000 20000 22000 24000 26000 28000 30000 32000 34000 36000 38000 40000 42000 44000 46000 48000
λ
Nu1 A W B C Nu3 D E FI FII Z U V G T H M L K I J Ea59 Ea21 Ea59 Int Ea8.5 Ea22 exo bet gam cII cIII Ea10 sieB R recB recA ol ori O P nin ninB to nin Q S R Rz,Rz1 Bor
0 2000 4000 6000 8000 10000 12000 14000 16000 18000 20000 22000 24000 26000 28000 30000 32000 34000 36000 38000 40000 42000 44000 46000 48000

Fig. 6.4a

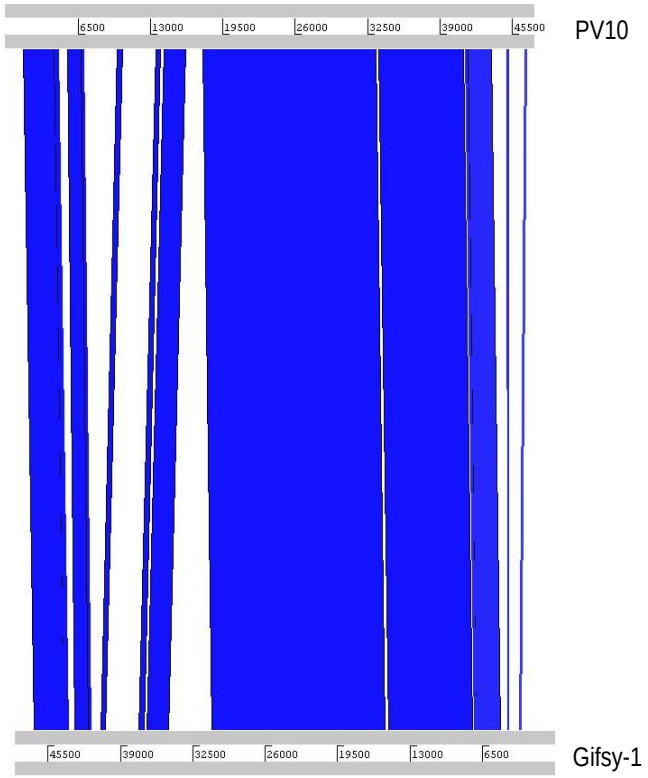


Fig. 6.4b

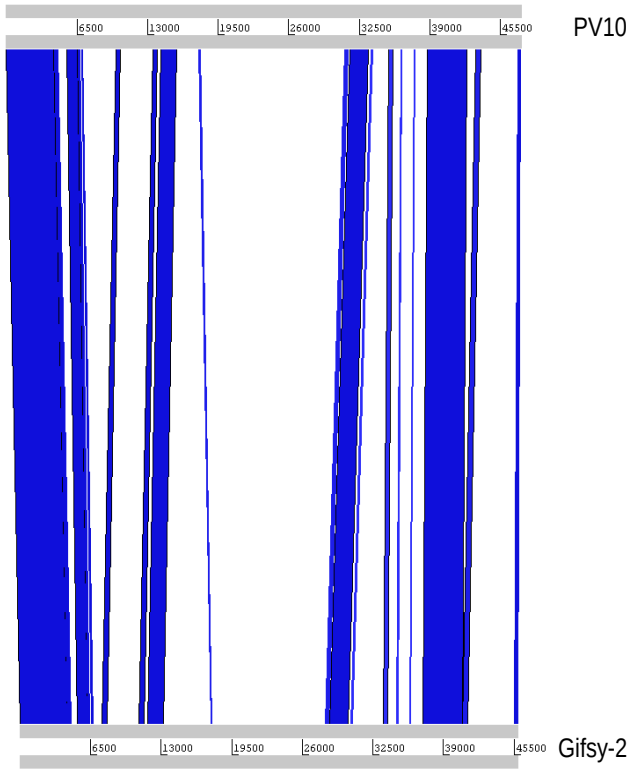


Fig. 6.4 a- b Pair-wise genomic comparisons of PV10 with Gifsy-1 and Gifsy-2. The coloured areas indicate sections of genomes showing ≥ 90% DNA similarity. The two diagrams show that phage PV10 has mosaic genetic relationship with phages Gifsy-1 and Gifsy-2, and that PV10 is closely related to phage Gifsy-1.

6.3.1.2 Genetic relationships between PV10 and *Salmonella* (cryptic) phages in NCBI database

Through the examination of putative genes of PV10 by a blastN search, it was observed that most putative gene sequences of PV10 are widespread in the genus *Salmonella*. For example, $ORF2_{PV10}$ encoding a putative large terminase subunit is not only highly similar to the corresponding gene of prophage Gifsy-1 in *S.* Typhimurium LT2 (NCBI accession no. AE006468) but also showed high sequence similarity (99%) to the corresponding putative loci in the sequenced *S.* Newport str. SL254 (NCBI accession no. CP001113), *S.* Chloleraesuis str. SC-B67 (NCBI accession no. AE017220) and *S.* Welteverden str. 2007-60-3289-1, contig 25 (NCBI accession no. FR775212). Similarly, $ORF43_{PV10}$ encoding a putative CII protein was found to have 97-98% sequence similarity to gene loci from an extensive range of *Salmonella* serovars: *S.* Choleraesuis, *S.* Dublin, *S.* Heidelberg, *S.* Typhi, *S.* Paratyphi B, *S.* Paratyphi C, *S.* Weltevreden, *S.* Newport, *S.* Schwarzengrund, *S.* Typhimurium, as well as *S. bongori*. A lower frequency of occurrence was observed for $ORF39_{PV10}$ which has no known function and only showed sequence identity to gene locus SBG0889 of *S. bongori* NCTC12419 (NCBI accession no. FR877557).

Recently a draft genome of *S.* Virchow str. SL491 has been deposited in the NCBI database. A pair-wise comparison between the genomes of PV10 and a cryptic prophage in contig 2 of the sequenced *S.* Virchow strain (NCBI accession no. ABFH02000002) was performed and showed that these two phages were almost identical (Fig. 6.5). Taken together, the results suggested the significance of Gifsy-1 related phages (e.g. phage PV10) to *Salmonella* possibly in providing virulence determinants to the host cells (will be discussed in section 6.3.4).

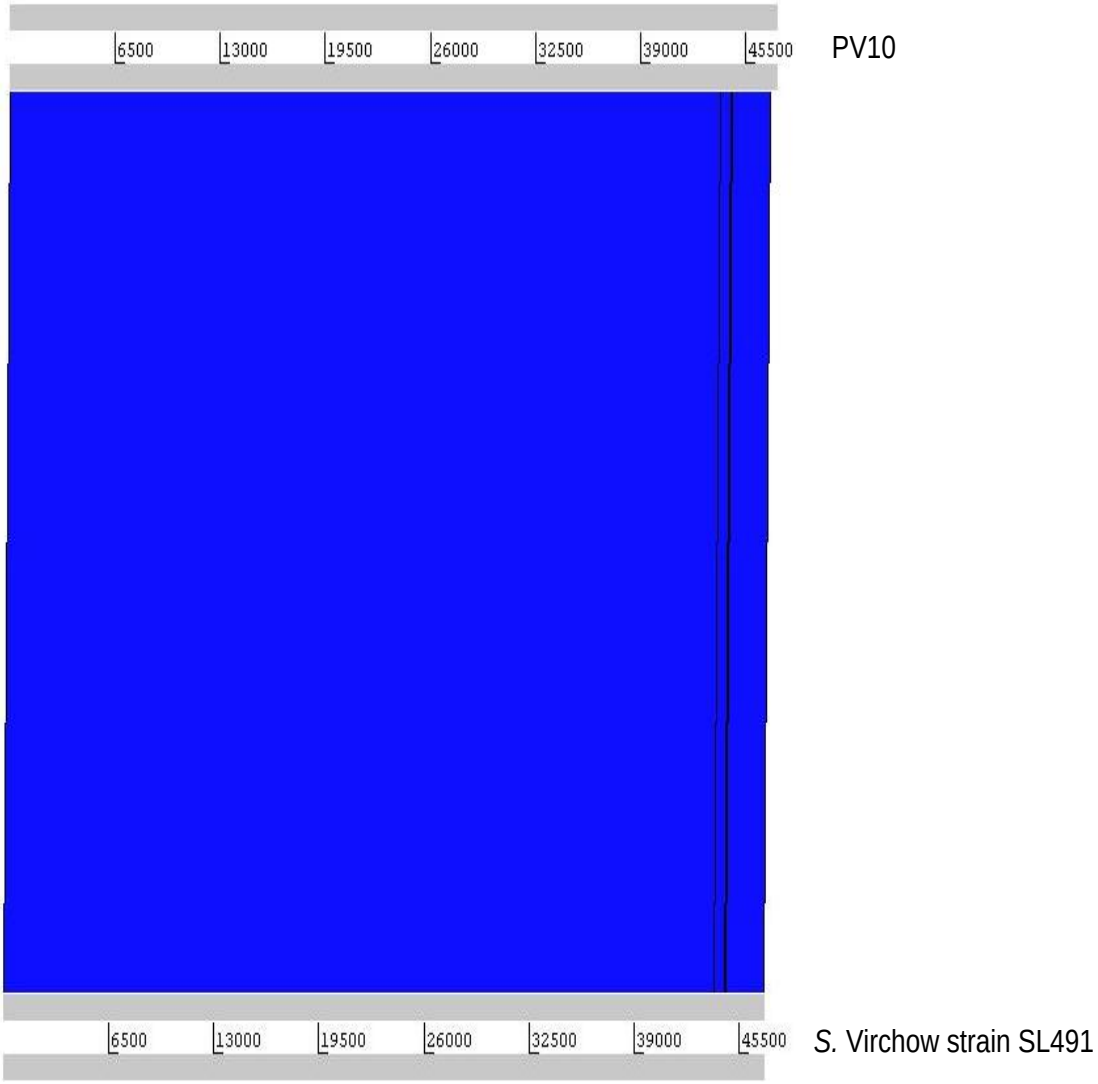


Fig. 6.5 Pair-wise genomic comparison of PV10 and a cryptic prophage locating between 1003742-1050713 of S. Virchow strain SL491 genomic sequence contig 2 (NCBI accession no. ABFH02000002). The coloured area indicates genomic regions that showed ≥ 99% DNA similarity.

6.3.2 Genomic sequence analysis of PH03

The genome of *S.* Heidelberg phage PH03 was sequenced and found to be 46,675bp in size with 61 predicted ORFs. The deduced products of these ORFs were used in searches for similarity with sequences in NCBI database using BlastP algorithm. The majority of the translated proteins of these ORFs showed strong similarity (>90% identity) to those of phage Gifsy-2, and some to the cryptic prophages residing in the sequenced genomes of *Salmonella* and other enteric bacteria. Hypothetical proteins of two ORFs ($ORF45_{PH03}$ and $ORF47_{PH03}$) had no known homologues in the database. Functions of the ORFs were assigned based on the demonstrated sequence similarity to the proteins in the NCBI database (Table 6.3).

A graphical map of phage PH03 genome is presented in Fig. 6.6. As indicated in Fig. 6.6, PH03 is probably a lambdoid phage having putative genes organised similarly as phage lambda. At the nucleotide level, the genome of PH03 showed a strong mosaic relationship to that of Gifsy-2. Overall, sixteen >100-bp regions of the PH03 genome showed ≥ 89% to the Gifsy-2 genome (Fig. 6.7). This is equivalent to 70.6% of the total genomic sequence of PH03. Thirty-four ORFs of PH03 showed ≥ 89% DNA sequence similarity to the corresponding genes of Gifsy-2 (Table 6.3). These include all the putative ORFs for head and tail with the exception of $ORF24_{PH03}$, the putative ORFs for homologous recombination, integration and excision, antitermination and lysis. In contrast, within the three ORFs in the immunity region ($ORF38_{PH03}$, $ORF39_{PH03}$ and $ORF40_{PH03}$), only $ORF40_{PH03}$ that encodes the putative CII protein shares >90% DNA similarity to that of Gifsy-2. In the case of the ORFs involved with DNA replication (ORF41 and ORF42), neither shares significant DNA similarity to the corresponding genes of Gifsy-2. In total, there are thirty ORFs in the PH03 genome conferring functions not required for phage growth or having no known functions. Twelve of these ORFs showed high DNA sequence similarity to the corresponding genes of Gifsy-2.

6.3.2.1 Genetic relationships between PH03 and *Salmonella* (cryptic) phages in NCBI database

Similar to phage PV10, mosaic genomic relationships between phage PH03 and cryptic prophages of a range of *Salmonella* serovars were frequently observed. For example, while $ORF41_{PH03}$ showed no DNA similarity to the corresponding Gifsy-2 gene, it showed 98 - 99% DNA similarity to gene loci in the sequenced *S.* Heidelberg strains B182 (NCBI no. CP003416), and SL476 (NCBI no. CP001120); *S.* Weltereden strain 2007-60-3298-1, contig 26 (NCBI no. FR775213), *S.* Typhimurium strains ST4/74 (NCBI no. CP002487), SL1344 (NCBI no. FQ312003), and 14028S (NCBI no. CP001363), and *S. bongori* NCTC12419 (NCBI no. FR877557). It was noted that $ORF42_{PH03}$ that locates adjacent to $ORF41_{PH03}$ also showed high DNA similarity at its entire ORF length to the prophage loci in the aforementioned *S.* Heidelberg and the *S.* Weltereden strains. However, only 329bp of the 5' end of $ORF42_{PH03}$ is shared with the corresponding prophage genes in the remaining four *Salmonella* strains.

Table 6.3 PH03 ORFs and putative functions

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identities)	BlastP e value
1	*122*	*718*	+	*597*	*198*	*Terminase small subunit*	*AAL19964; Gifsy-2*	*100% (534/534)*	*100% (177/177)*	*4e-125*
2	*675*	*2813*	+	*2139*	*712*	*Terminase large subunit*	*AAL19965; Gifsy-2*	*99% (2132/2139)*	*99% (708/712)*	*0.0*
3	*2810*	*3016*	+	*207*	*68*	*?head-to-tail joining protein*	*YP_001700676; Gifsy-2*	*99% (206/207)*	*100% (68/68)*	*1e-39*
4	*3013*	*4560*	+	*1548*	*515*	*Portal protein*	*AAL19966; Gifsy-2*	*99% (1533/1548)*	*99% (431/437)*	*0.0*
5	*4475*	*6562*	+	*2088*	*695*	*ATP-dependent Clp protease*	*AAL19967; Gifsy-2*	*99% (2075/2091)*	*99% (684/693)*	*0.0*
6	*6653*	*6976*	+	*345*	*114*	*RecA/RadA recombinase*	*AAL19968; Gifsy-2*	*100% (324/324)*	*100% (107/107)*	*6e-67*
7	*6969*	*7268*	+	*300*	*99*	*ATP-binding transporter-like protein*	*AAL19969; Gifsy-2*	*100% (300/300)*	*100% (99/99)*	*4e-63*
8	*6710*	*7063*	-	*354*	*117*	*Antivirulence protein*	*AAG03023; Gifsy-2*	*100% (354/354)*	*100% (117/117)*	*5e-82*
9	*7249*	*7815*	+	*576*	*188*	*Head-tail assembly protein*	*AAL19970; Gifsy-2*	*99% (566/567)*	*99% (187/188)*	*3e-132*
10	*7812*	*8213*	+	*402*	*133*	*Tail shaft stabilisation protein*	*AAL19971; Gifsy-2*	*99% (401/402)*	*99% (132/133)*	*9e-91*
11	*8225*	*8974*	+	*750*	*249*	*Major tail protein*	*AAL19972; Gifsy-2*	*99% (749/750)*	*99% (248/249)*	*9e-177*
							AAL21492; Gifsy-1	*72% (474/661)*	*69% (160/233)*	*4e-97*

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identities)	BlastP e value
12	*9020*	*9418*	*+*	*399*	*132*	*Tail assembly chaperone*	*AAL19973; Gifsy-2*	*99% (398/399)*	*99% (131/132)*	*7e-89*
							AAL214912; Gifsy-1	*67% (177/264)*	*52% (70/134)*	*2e-37*
13	*9481*	*9744*	*+*	*264*	*87*	*Tail assembly chaperone; part of G+G' frameshift hybrid protein*	*AAL19974; Gifsy-2*	*100% (264/264)*	*100% (87/87)*	*9e-54*
14	*9725*	*12811*	*+*	*3087*	*1028*	*Tail tape measure protein*	*AAL19975; Gifsy-2*	*98% (3027/3087)*	*98% (979/995)*	*0.0*
							AAL21489; Gifsy-1	*91% (2524/2760)*	*87% (878/1023)*	*0.0*
15	*12808*	*13140*	*+*	*333*	*110*	*Tail tip assembly protein*	*AAL19976; Gifsy-2*	*99% (332/333)*	*99% (109/110)*	*8e-76*
16	*13239*	*13736*	*+*	*498*	*166*	*Attachment and invasion protein*	*AAL19977; Gifsy-2*	*99% (494/496)*	*99% (163/165)*	*8e-110*
17	*13853*	*14386*	*-*	*534*	*177*	*Cu-Zn superoxide dismutase*	*AAL19978; Gifsy-2*	*100% (534/534)*	*100% (177/177)*	*6e-123*
18	*14476*	*15171*	*+*	*696*	*231*	*Tail tip assembly protein*	*AAL19979; Gifsy-2*	*99% (693/696)*	*99% (230/231)*	*1e-170*
19	*15181*	*15918*	*+*	*738*	*245*	*Tail tip assembly protein*	*AAL19980; Gifsy-2*	*99% (737/738)*	*99% (244/245)*	*0.0*
20	*15816*	*16520*	*+*	*705*	*234*	*Tail tip assembly protein*	*AAL19981; Gifsy-2*	*99% (702/705)*	*99% (232/234)*	*1e-166*
							AAL21485; Gifsy-1	*70% (452/645)*	*61% (130/214)*	*4e-86*
21	*16592*	*19942*	*+*	*3351*	*1116*	*Host specificity protein*	*AAL19982; Gifsy-2*	*96% (3232/3351)*	*96% (783/814)*	*0.0*
							AAL21484; Gifsy-1	*75% (2557/3394)*	*75% (793/1080)*	*0.0*

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identities)	BlastP e value
22	*19981*	*20230*	+	*250*	*80*	*Hypothetical protein*	*YP_001700695; Gifsy-2*	*100% (250/250)*	*100% (80/80)*	*8e-52*
							YP_001700592; Gifsy-1	*96% (240/250)*	*96% (77/80)*	*7e-50*
23	20277	22652	+	2376	791	side tail fibre protein	AAL19983; Gifsy-2	[#]see below	92% (744/813)	0.0
							AAL21483; Gifsy-1	96% (2285/2377)	97% (766/791)	0.0
24	22649	23473	+	825	274	Tail-assembly like protein	AAL21482: Gifsy-1	91% (723/825)	90% (247/274)	0.0
25	*23463*	*24044*	+	*582*	*193*	*Side tail fibre assembly protein*	*AAL19984; Gifsy-2*	*89% (506/571)*	*87% (167/191)*	*5e-122*
							AAL21481; Gifsy-1	*85% (487/570)*	*87% (166/191)*	*2e-116*
26	24241	24963	-	723	240	Type III secretive protein; SopE	YP_002045056; prophage of *S.* Heidelberg SL476	100% (723/723)	100% (240/240)	1e-176
27	25176	25394	+	219	72	DNA invertase	AFH45051; prophage of *S.* Heidelberg B182	100% (219/219)	100% (72/72)	2e-43
28	25614	26834	+	1221	406	Transposase	AFH45052; prophage of *S.* Heidelberg B182	100% (1221/1221)	100% (406/406)	0.0

[#]The nucleotide identity between ORF23$_{PH03}$ and the corresponding gene of Gifsy-2 was displayed in two sections: nt position 20277-21755 of PH03 showed 95% (1412/1483); while nt position of PH03 21777-22648 showed 91% (792/872) to the corresponding parts of the Gifsy-2 gene.

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identities)	BlastP e value
29	26831	27328	-	498	165	Hypothetical protein (transposase like protein)	AFH45053; prophage of *S.* Heidelberg B182	99% (497/498)	99% (140/141)	1e-95
30	*27689*	*29029*	*-*	*1341*	*446*	*Integrase*	*AAL19939; Gifsy-2*	*99% (1339/1341)*	*99% (429/430)*	*0.0*
31	*29026*	*29274*	*-*	*249*	*82*	*Excisionase*	*AAL19940; Gifsy-2*	*100% (249/249)*	*100% (82/82)*	*2e-53*
32	*29315*	*29554*	*-*	*240*	*79*	*Hypothetical protein*	*AAL19941; Gifsy-2*	*100% (240/240)*	*100% (79/79)*	*1e-50*
							AAL21528; Gifsy-1	*99% (239/240)*	*100% (79/79)*	*1e-50*
33	*29597*	*30754*	*-*	*1158*	*385*	*Recombination protein*	*AAL19942; Gifsy-2*	*99% (1152/1158)*	*99% (383/385)*	*0.0*
							AAL21527; Gifsy-1	*99% (1152/1158)*	*99% (383/385)*	*0.0*
34	*30717*	*33644*	*-*	*2928*	*975*	*Recombination protein*	*AAL19943; Gifsy-2*	*97% (2674/2760)*	*94% (911/973)*	*0.0*
							AAL21526; Gifsy-1	*97% (2674/2760)*	*94% (911/973)*	*0.0*
35	33785	33943	-	159	52	Hypothetical protein	ABX67578; *S.* Paratyphi B SPB7	98% (123/126)	85% (44/52)	3e-22
							YP_001700650; Gifsy-2	77% (124/162)	71% (37/52)	4e-18
							AAL21524; Gifsy-1	78% (124/160)	71% (37/52)	6e-19
36	*33936*	*34052*	*-*	*117*	*38*	*Hypothetical protein*	*AAL14995; Gifsy-2*	*98% (115/117)*	*97% (37/38)*	*6e-18*
							AAL21523; Gifsy-1	*100% (117/117)*	*100% (38/38)*	*2e-18*
37	34360	34503	-	144	47	Hypothetical protein	ABX67576; *S.* Paratyphi B SPB7	100% (144/144)	100% (47/47)	2e-24

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identities)	BlastP e value
38	34789	35172	-	384	127	Repressor protein?	ABX67575; *S.* Paratyphi B SPB7	99% (380/384)	99% (126/127)	8e-86
39	35276	35512	+	237	78	Regulatory protein CRO?	ABX67574; *S.* Paratyphi B SPB7	99% (235/237)	99% (77/78)	2e-48
40	*35478*	*35852*	+	*375*	*124*	*Regulatory protein CII*	*AAL19947; Gifsy2*	*99% (372/375)*	*99% (123/124)*	*2e-85*
							AAL21521; Gifsy-1	*99% (372/375)*	*99% (123/124)*	*2e-85*
41	35944	36849	+	906	301	Replication protein	AFH45008; prophage of *S.* Heidelberg B182	99% (900/906)	99% (299/301)	0.0
42	36846	37547	+	702	233	Replication protein	AFH45009; prophage of *S.* Heidelberg B182	99% (699/702)	99% (231/233)	1e-170
43	37544	37993	+	450	149	ParB -like nuclease	AFH45010; prophage of *S.* Heidelberg B182	99% (445/450)	98% (146/149)	1e-104
44	37969	38481	+	513	170	Hypothetical protein ?Ea22-like protein	CBJ02151; Prophage of *E.coli* H10407	no match	49% (49/99)	2e-17
45	38521	38835	+	315	104	Hypothetical protein	no similarity found	no match	no match	no match

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identities)	BlastP e value
46	38832	39035	+	204	67	DNA polymerase III subunit theta	ADF60873; *E.* cloacae ATCC13047	78% (114/146)	59% (39/66)	1e-18
47	39073	39363	+	291	96	Hypothetical protein	no similarity found	no match	no match	-
48	39414	39689	+	276	91	Hypothetical protein	EHS95781; *Klebsiella oxytoca* 10-5243	no match	59% (23/29)	4e-06
49	39732	40763	-	1032	343	Hypothetical protein	YP_005819205; Erwinia sp. Ejp617	71% (621/871)	73% (248/338)	0.0
50	41036	41308	+	273	90	DNA damage-inducible protein	AAL19953; Gifsy-2	67% (185/277)	68% (52/77)	2e-31
							AAL21515; Gifsy-1	67% (185/277)	68% (52/77)	2e-31
51	41557	41673	+	117	38	Hypothetical protein	AFH45016; prophage of *S.* Heidelberg B182	100% (117/117)	100% (38/38)	1e-19
52	*41708*	*42310*	+	*603*	*200*	*Hypothetical protein*	*AAL19954; Gifsy-2*	*96% (578/603)*	*98% (196/200)*	*1e-142*
							AAL21514; Gifsy-1	*98% (588/603)*	*98% (196/200)*	*4e-142*
53	*42310*	*42516*	+	*207*	*68*	*Hypothetical protein*	*YP_001700663; Gifsy2*	*99% (204/207)*	*98% (65/66)*	*7e-42*
							TP_001700624; Gifsy-1	*98% (203/207)*	*95% (63/66)*	*1e-40*

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identites)
54	42519	42953	+	435	144	NinG; Unknown	AAL19955; Gifsy2 AAL21513; Gifsy-1	[§]See below	66% (133/203) 66% (133/203)
55	*42950*	*43096*	+	*147*	*48*	*Hypothetical protein*	*YP_001700655; Gifsy-2* *YP_001700622; Gifsy-1*	*99% (146/147)* *90% (53/59)*	*100% (48/48)* *82% (31/38)*
56	*43086*	*43883*	+	*798*	*265*	*Antitermination protein*	*AAL19956; Gifsy-2*	*99% (788/789)*	*99% (263/265)*
57	*44005*	*44268*	+	*264*	*87*	*Hypothetical protein*	*AAL19957; Gifsy-2*	*98% (259/264)*	*99% (73/74)*
58	44549	44737	+	189	62	Hypothetical protein	YP_006087295; prophage of *S.* Heidelberg B182	100% (189/189)	100% (62/62)
59	44934	45242	+	309	102	Holin	AFH45022; prophage of *S.* Heidelberg B182	100% (309/309)	100% (102/102)
60	45220	45759	+	540	179	Endolysin	AFH45023; prophage of *S.* Heidelberg B182	100% (540/540)	100% (179/179)
61	45928	46065	+	138	45	Hypothetical protein	AFH45024; prophage of S. Heidelberg B182	100% (138/138)	100% (44/45)

[§] The nucleotide identity between ORF54$_{PH03}$ and the corresponding gene of Gifsy-2 was displayed in two sections: nt position 42710-42949 of PH03 showed 98% (240/244); while nt position of PH03 42519-42720 showed 99% (200/202) to the corresponding parts of the Gifsy-2 gene. The same nt positions showed 99% (243/244) and 98% (198/202) to the corresponding parts of the Gifsy-1 gene respectively.

ORFs	Start	End	Strand	Length (nt[1])	Length (aa[2])	Putative functions	Related protein sequences	% (nt[1] identities)	% (aa[2] identities)	BlastP e value
62	*46067*	*46561*	*+*	*495*	*164*	*Endopeptidase*	*AFH45025; prophage of S. Heidelberg B182*	*100% (495/495)*	*100% (164/164)*	*1e-115*
							AAL19963; Gifsy-2	*92% (384/416)*	*80%(129/161)*	*8e-87*

Putative functions of the ORFs were first inferred based on their homology shown to proteins of phages Gifsy-1 and Gifsy-2 then to other proteins in NCBI databases

% of nucleotide identities were based on BlastN search, while % of amino acid identities were based on BlastP search

ORFs showing ≥ 90% DNA similarity to Gifsy-2 genes are italicised

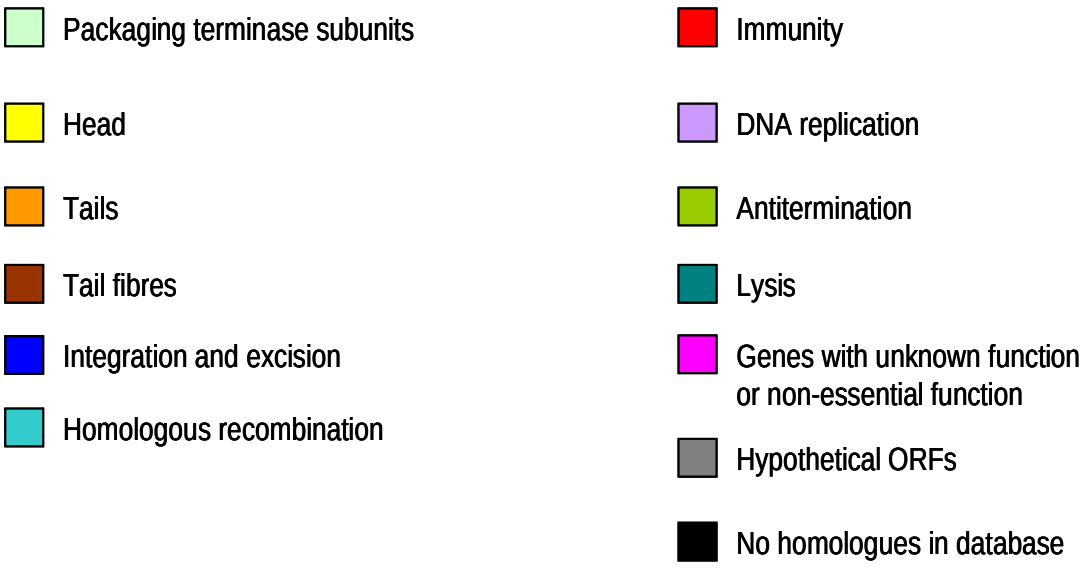
[1]nt = nucleotide sequences; [2]aa = amino acid sequences

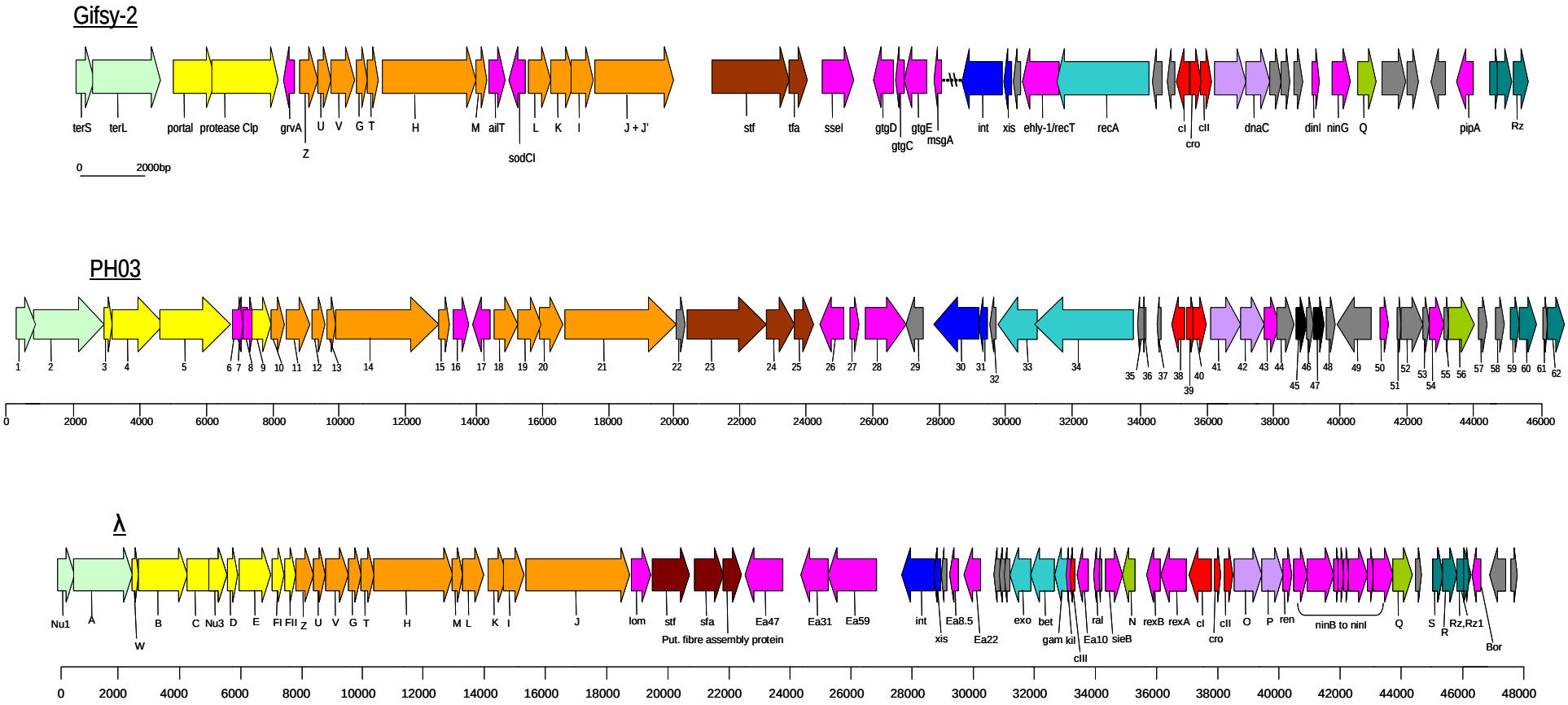
Fig. 6.6 Diagrammatic presentations of temperate phages PH03, Gifsy-2 and λ genomes. The gene map of PH03 shows the putative ORFs labelling from 1 to 62 and also the directions of transcription as described in Table 6.3.

The gene map of phage λ is constructed based on the genomic sequence deposited in GenBank (NCBI no. NC_001416). The genes are also labelled in reference to Hendrix and Casjens (2006).

The gene map of phage Gifsy-2 is constructed according to the genomic sequence in Genbank (NCBI no. AE006468 from nt position 1098228 - 1143714) with modifications to show the genome organisation when the phage is in the infective form. However this remains to be proven. Genes are annotated as per NCBI genome file NC_101393 which contains the refined annotation of Gifsy-2, and Boyd and Brüssow (2002) using phage λ nomenclature when possible.

Each map is drawn to scale individually. Symbol -\\-- in Gifsy-2 map indicates where the modification was made and the region was not scaled.




Gifsy-2
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terL
portal
protease
Clp
grvA
Z
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G
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sodCI
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K
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J + J'
stf
tfa
ssel
gtgD
gtgC
gtgE
msgA
int
xis
ehly-1/recT
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Put. fibre assembly protein
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Ea31
Ea59
int
xis
Ea8.5
Ea22
exo
bet
gam
kil
cIII
ral
Ea10
sieB
N
rexB
rexA
cI
cro
cII
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P
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ninB to ninI
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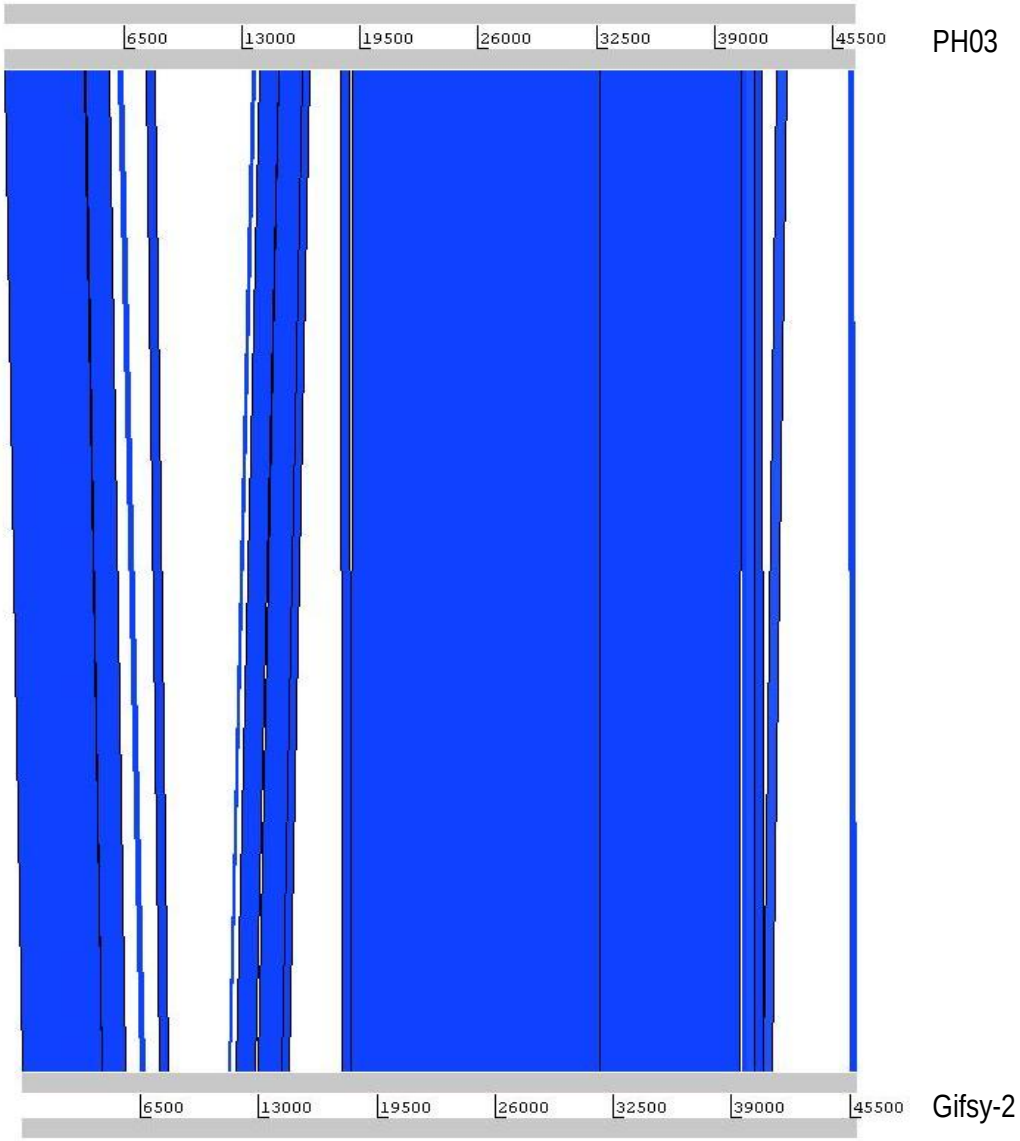


Fig. 6.7 Pair-wise genomic comparison of PH03 with Gifsy-2. The coloured areas indicate sections of genomes showing ≥ 89% DNA similarity. As demonstrated, phage PH03 has a mosaic genetic relationship with Gifsy-2 where portions of the genome with high DNA similarity with Gifsy-2 are separated by portions that have no similarity.

Additionally, there are ORFs of PH03 showing DNA sequence similarity to those of the sequenced *Salmonella* serovars other than the examples already cited above. For example, both $ORF38_{PH03}$ and $ORF39_{PH03}$ in the immunity module of PH03 share 99% of DNA sequence similarity to the corresponding prophage loci in strain *S.* Paratyphi B SPB7 (NCBI no. CP000886).

6.3.2.2 Pair-wise comparison between Gifsy-2 related phages of *S.* Heidelberg

The genetic relationship between *S.* Heidelberg phage PH03 with two cryptic Gifsy-2 like phages in the sequenced *S.* Heidelberg strains SL476 and B182 was further examined. As demonstrated in Fig. 6.8, a large portion of the PH03 genome showed DNA sequence similarity to the prophages in the two sequenced strains. In total, nine >100-bp regions of PH03, which is equivalent to 79.3% of the complete genome, share ≥ 98% sequence similarity with the prophage in strain B182. Similarly, there are also nine >100-bp regions of PH03 that share ≥ 98% sequence similarity with the prophage in strain SL476. This is equivalent to 78.4% of the complete genomic sequence of PH03. In addition, as suggested by the mirror image of the pair-wise comparison diagrams, these are the same genomic regions that share the sequence similarity between the three phages. In contrast, the genomic regions that are different between the phages consist of seventeen ORFs of phage PH03 from $ORF30_{PH03}$ to $ORF39_{PH03}$; and from $ORF44_{PH03}$ to $ORF50_{PH03}$. These are the putative ORFs responsible for site-specific and homologous recombinations, immunity and several ORFs of unknown functions locating between the replication and lysis modules (Fig. 6.8).

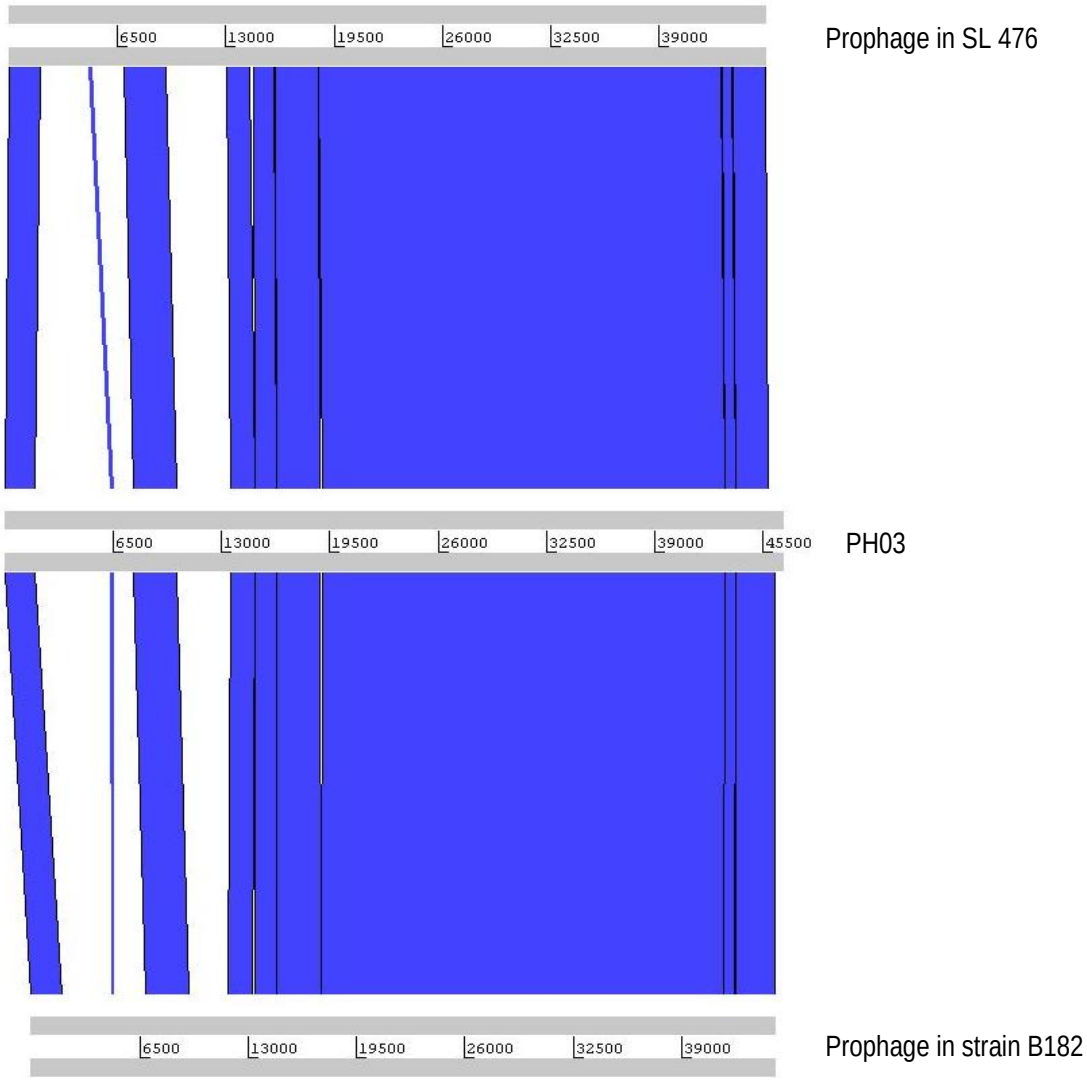


Fig. 6.8 Pairwise comparison of between PH03 and the crytpic Gifsy-2 like prophages in *S.* Heidelberg strains SL476 and B182. Result showed that same regions of both prophage genomes with high DNA similarity to PH03.

For *S.* Heidelberg strain SL476 (NCBI no. CP001120): the crytpic prophage locates between nucleotide positions 102597 and 1147937.

For *S.* Heidelberg strain B182 (NCBI no. CP003416): the cryptic prophage locates between nucleotide position 1834892 and 1879458.

6.3.3 Usefulness of Gifsy-like phage elements for differentiation of *Salmonella* strains

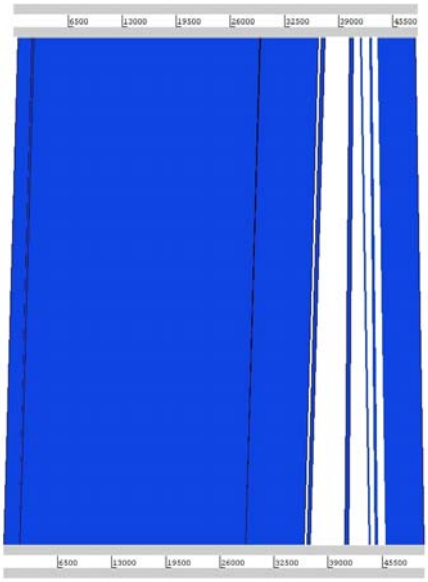
As discussed in section 6.1, prophages contribute to the genetic variations between *Salmonella* strains as their genomes are basically the mosaic compositions of genes that may be acquired from phylogenetically diverse bacterial and phage groups (e.g. phage ST64B) through horizontal gene exchange. Nevertheless, phage evolution driven by such horizontal gene exchange is likely to occur readily leading to phage groups showing apparent DNA sequence similarity in parts of their genomes (e.g. Phages P22 and ST64T). In this chapter, two phages were sequenced where PV10 was found to be genetically related to phage Gifsy-1, and PH03 was related to Gifsy-2. Since PV10 and PH03 only partially share DNA sequences with Gifsy-1 and Gifsy-2 respectively, this suggests the potential use of Gifsy gene loci for strain differentiation at serovars levels.

To verify the usefulness of Gifsy-related phage loci for further development of MAPLT methods, pair-wise comparisons of Gifsy-related phages of the same serovars were performed. It was observed that *S.* Virchow phage PV10 and the cryptic Gifsy-1 related prophage of *S.* Virchow strain SL 491 are almost genetically identical. Similar results were obtained when comparing between three *S.* Heidelberg Gifsy-2 related prophages: PH03 and the two prophages from *S.* Heidelberg strains SL476 and B182. As shown in Fig. 6.8, DNA sequences of the Gifsy-2 related prophages from strains SL476 and B182 were virtually identical but exhibited genetic mosaic relationship with PH03. The genomic region that is different between the published Gifsy-2 related prophages and phage PH03 was observed between the nucleotide positon 29537 to 41263 of the PH03 genomic sequence. This is approximately from $ORF33_{PH03}$ (the recombination module) to $ORF50_{PH03}$ (non-essential genomic region inbetween the replication and lysis modules) in the PH03 phage genome (Fig. 6.6).

Currently complete genomic sequences of seven *S.* Typhimurium strains are available. Pair-wise genomic comparison of Gifsy-1 and Gifsy-2 from strain LT2 and the Gifsy-related phages from the other six strains were performed in order to ascertain gene shuffling locations between the genomes (Fig. 6.9). Genomic mosaicism was observed between phage Gifsy-1 and the six Gifsy-1 related phages between approximately the nucleotide position 37351 and 43678 of the Gifsy-1 genomic sequence. Functionally, this is between locus STM2622 in the recombination module and locus STM2632 in the non-essential region inbetween the replication and lysis modules of the Gifsy-1 genome. It is interesting to note that this is the same genomic region where PH03 showed sequence divergence with the Gifsy-2 related prophages in the published *S.* Heidelberg strains. In contrast, the six *S.* Typhimurium strains that contain Gifsy-2 related prophages are genetically almost identical to Gifsy-2. Between Gifsy-2 and the related prophages in strains ST4/74, SL1344 and D23580, there are only small differences in their genomic sizes with differences ranging from 192 to 820bp in length (Fig. 6.10).

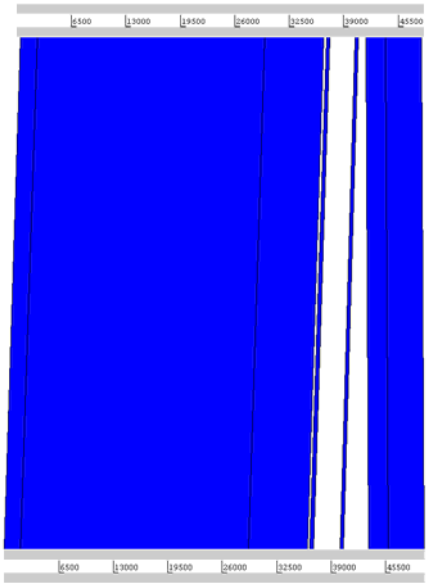
Fig. 6.9 Genomic comparison of the Gifsy-1 related phages from the seven sequenced *S.* Typhimurium strains against Gifsy-1 from strain LT2. The shaded area indicated DNA homology at < 90% level. Most parts of the Gifsy-1 related phages are virtually homologous to Gifsy-1 and the DNA variations appear at the same section of the genome.

Strain ID (NCBI accession no.)	Nucleotide positions in the genome
LT2 (AE006468)	2728976 - 2776816
ST4/74 (CP002487)	2726693 - 2777229
798 (CP003386)	2726084 - 2776182
SL1344 (FQ312003)	2726717 - 2777229
D23580 (FN424405)	2753352 – 2803472
UK-1 (CP002614)	2778902 - 2728740
14028s (CP001363)	2780529 - 2830650



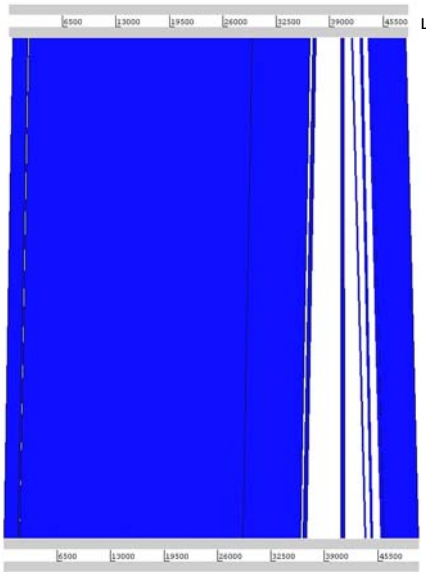
LT2

ST4/74



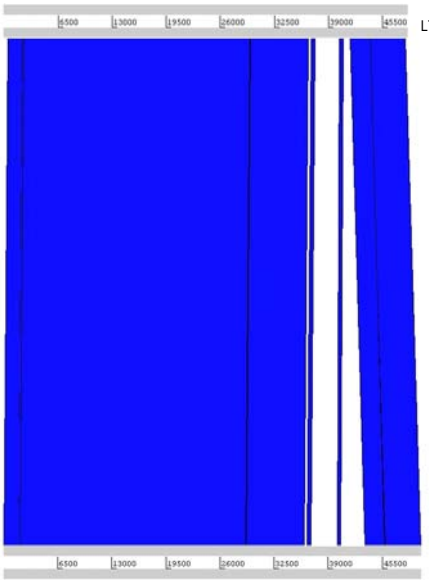
LT2

798



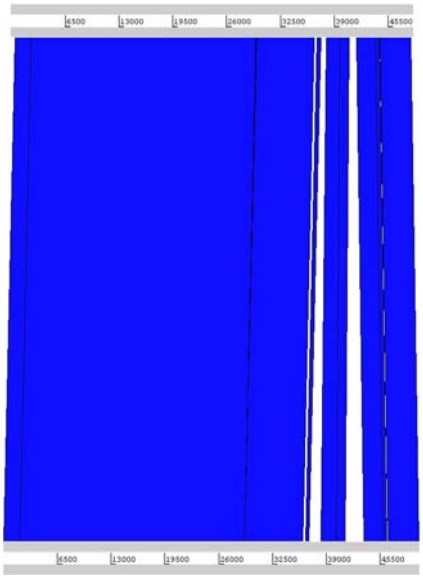
LT2

SL1344



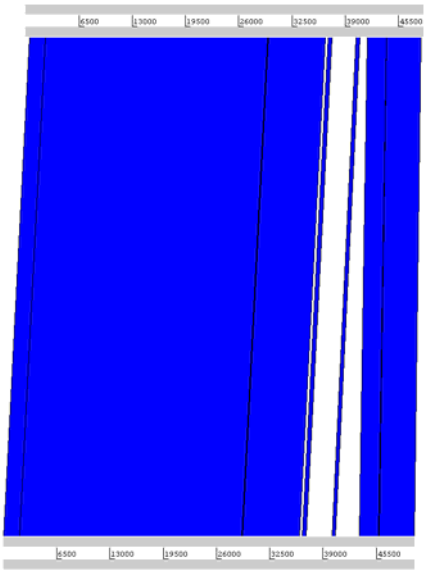
LT2

D23580



LT2

UK-1

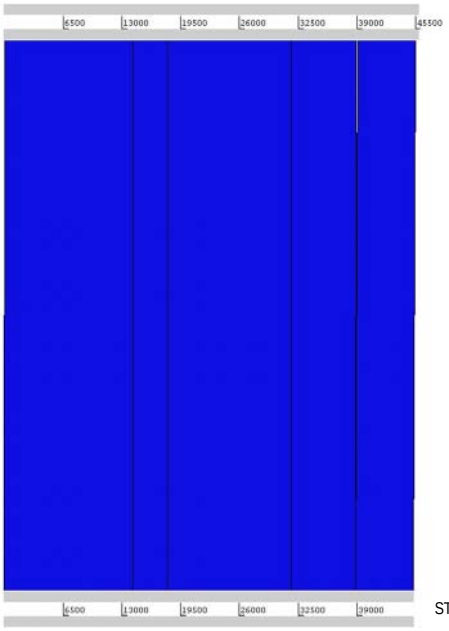


LT2

14028S

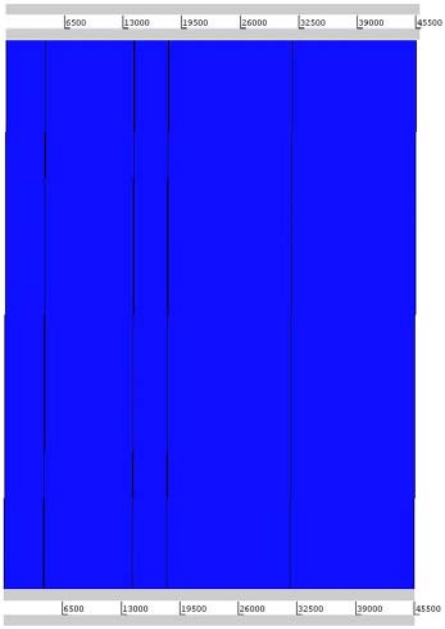
Fig. 6.10 Gifsy-2 phage elements were identified from the seven sequenced *S.* Typhimurium strains. Pair-wise comparisons between the cryptic prophages and Gifsy-2 showed that their genomes are virtually identical as demonstrated by the shaded areas.

Strain ID (NCBI accession no.)	Nucleotide positions in the genome
LT2 (AE006468)	1098228 - 1143714
ST4/74 (CP002487)	1054798 - 1100036
798 (CP003386)	1054777 - 1100292
SL1344 (FQ312003)	1054795 - 1100036
D23580 (FN424405)	1094166 - 1140404
UK-1 (CP002614)	1055877 - 1101349
14028s (CP001363)	1056587 - 1102059



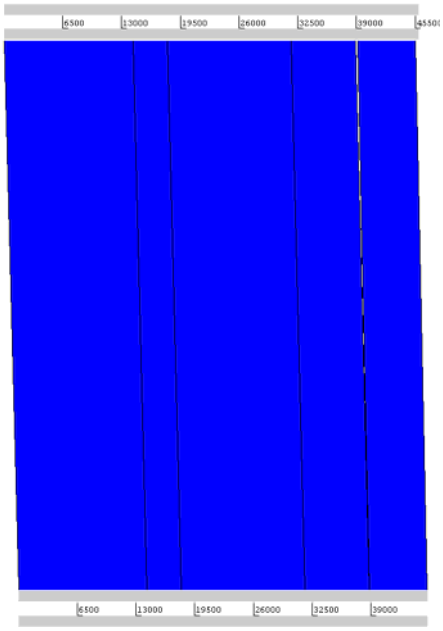
LT2

ST4/74



LT2

798



LT2

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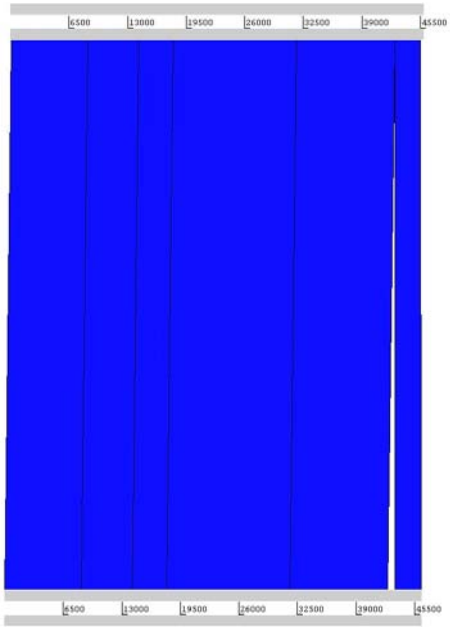

LT2

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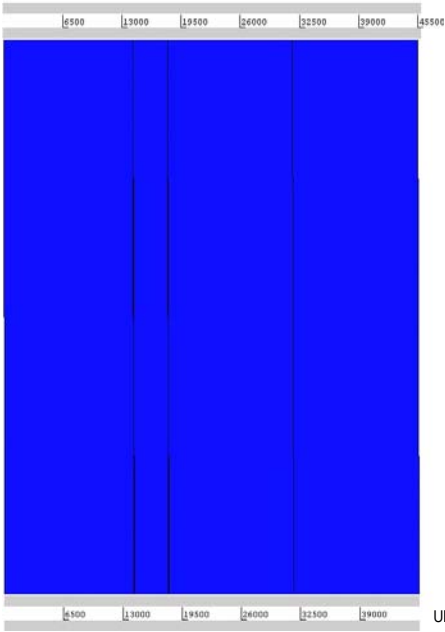
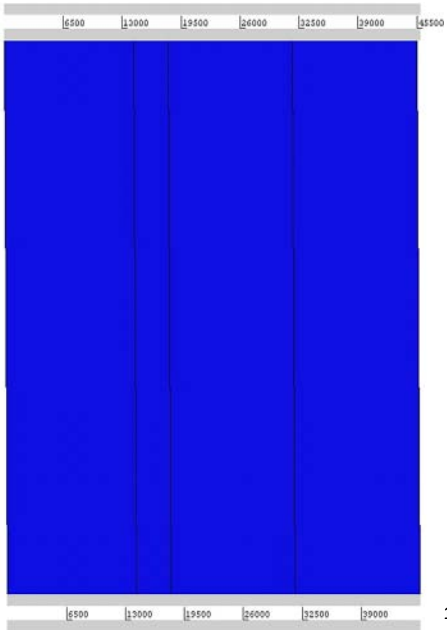


LT2

UK-1



LT2

14028s

Based on the results, Gifsy related phages are common in the genus *Salmonella* and they exhibit a much lower degree of modular variation between the Gifsys related phages from strains of the same serovars. In other words, the results suggest there is little value in detecting additional Gifsys related gene loci for further improvement of the established MAPLT methods. Nevertheless, the genomic comparative analyses suggest that the modular variation is more likely to occur outside the morphogenesis modules (head and tails) of the phage genomes. This finding is in agreement with that of Schicklmaier *et al.,* (1999) where mosaic variation was also demonstrated between the *S.* Typhimurium P22 related phages between or within the immunity, replication and lysis modules but not at the structural (head) module using hybridisation with the P22 DNA probes derived from the aforementioned modules. Taken together, the results suggest that when targeting multiple gene loci of a phage for MAPLT development, gene loci outside the head and tail modules should be preferrentially targeted, thereby increasing the probability of strain differentiation as a result of the different modular composition between the related phages.

6.3.4 Putative virulence genes carried by Gifsy-related phages PV10 and PH03

Virulence genes have been identified in phages Gifsy-1 and Gifsy-2 in *S.* Typhimurium strains. Therefore it is not surprising to observe that the Gifsy-related phages PV10 and PH03 examined in this study also carry putative virulence genes. This study also demonstrated the phage-borne virulence factors that are shared between serovars, and demonstrated some virulence factors that may be specific to *S.* Typhimurium only.

In regard to phage PV10, the study showed that it contains only one of the three known virulence genes that are carried by Gifsy-1. This putative virulence gene *gipA* is encoded by $ORF11_{PV10}$ and is 100% identical in DNA sequence to $gipA_{Gifsy\text{-}1}$. The function of protein GipA remains unknown but has been shown to be required for *Salmonella* proliferation in Peyer's patches in the small intestine (Stanley *et al.*, 2000). The remaining two virulence genes of Gifsy-1 that were not detected in PV10 are located between the tail fibre module and the integration module, which correspond to the b region of phage λ containing genes not necessary for phage growth (Daniels *et al.*, 1983). One of these genes is $gogB_{Gifsy\text{-}1}$ which is a leucine-rich effector protein of the *Salmonella* pathogenicity island SPI-1 and SPI-2 virulence associated type III secretion systems (Coombes *et al.*, 2005). The other gene, $gogD_{Gifsy\text{-}1}$ was found to be similar to *S.* Typhimurium loci *pagJ* and *pagK*, which function to increase survival of *Salmonella* after phagocytosis (Gunn *et al.*, 1995; Figueroa-Bossi *et al.*, 2001).

In the case of PH03, a number of putative virulence genes were found that showed commonality with its close relative *S.* Typhimurium phage Gifsy-2, based on the observed sequence similarity. These include the major virulence determinant *sodCI* gene that encodes a periplasmic Cu/Zn superoxide dismutase protecting the organisms against macrophage oxidative burst (De Groote *et al.*, 1997; Farrant *et al.*, 1997; Figueroa-Bossi *et al.*, 2001). As noted in section 6.1, deletion of gene *sodCI* from Gifsy-2 has been showed to cause a decrease in the extra-intestinal virulence of *S.* Typhimurium by 5-fold (Ho *et al.*, 2002). In PH03, this protein is putatively encoded by ORF17, which exhibited 100% identity in DNA sequence to $sodCI_{Gifsy\text{-}2}$. Immediately upstream to the putative gene *sodCI* of PH03 is ORF16, which is 100% identical to the Gifsy-2 *ailT* gene. Gene *ailT* is a homolog of gene lom_{λ} that encodes a bacterial outer membrane protein conferring serum resistance (Barondess and Beckwith, 1990; Figueroa-Bossi *et al.*, 2001). The Gifsy-2 antivirulence gene sequence *grvA* was also found in PH03. The putative GrvA protein is encoded by $ORF8_{PH03}$ and is at the same location as the *grvA* gene in Gifsy-2, which is between a putative capsid precursor gene ($ORF5_{PH03}$) and a putative head-tail putative gene and ($ORF9_{PH03}$). Contribution of GrvA protein to *Salmonella* pathogenicity is yet to be ascertained, however a study of Ho and Slauch (2001) showed that *grvA* gene expression is dependent on the presence of gene *sodCI*.

In contrast, the five genes locating in the b region of Gifsy-2 were not found in PH03. These include the virulence genes *gtgE*, *gtgB* and *gtgF* that have been studied previously in terms of their contribution to the overall virulence to the Salmonella strains (Miao *et al.*, 1999; Worley *et al.*, 2000; Ho *et al.*, 2002). Gene $gtgE_{Gifsy\text{-}2}$ encodes a SPI-2 secreted type III effector protein that has been shown contributing significantly to the virulence of *Salmonella* (Ho *et al.*, 2002). However, deletions of $gtgB_{Gifsy\text{-}2}$, which also encodes a SPI-2 secreted Type III effector protein, and $gtgF_{Gifsy\text{-}2,}$ which is similar to the macrophage survival gene *msgA* only decreased the virulence of *Salmonella* subtly (Ho *et al.*, 2002).

Common in both PV10 and PH03 genomes, the corresponding b regions were found to contain a putative virulence gene *sopE* ($ORF26_{PV10}$ and $ORF26_{PH03}$) and genes associated with DNA rearrangement ($ORF27_{PV10;}$ $ORF27_{PH03}$) and transposition ($ORF28_{PV10;}$ $ORF28_{PH03}$), suggesting this region may have been a mobile element and indicated the horizontal acquisition of the *sopE*. This result correlated with a previous study where the *sopE* gene cassette from *Salmonella* strains including *S.* Gallinarum, *S.* Enteritidis and *S.* Hadar strains was between the integrase and tail regions of the cryptic lambdoid phages resembling Gifsy-1 and -2 (Mirold *et al.*, 2001)

The encoded putative protein SopE is a SPI-1 secreted type III effector protein that assists entry of *Salmonella* into intestinal epithelial cells through stimulating cell membrane ruffling (Wood *et al.*, 1996; Hardt *et al.*, 1998). Gene *sopE* was first described in *S.* Dublin that is a bovine-adapted serovar (Wood *et al.*, 1996). Later, it was isolated from phage SopEϕ harboured in the epidemic *S.* Typhimurium isolates (Mirold *et al.*, 1999). The same study also demonstrated that deletion of SopEϕ only decreased the invasiveness of the isolates slightly, which in turns indicates the auxillary role of *sopE* to the overall pathogenicity of *S.* Typhimurium (Mirold *et al.*, 1999). Taken together, the findings indicated a role of phages in driving micro-evolution within *Salmonella* serovars that leads to the emergence of temporally epidemic strains (Mirold *et al.*, 1999).

Additionally, phages may have introduced different repertoires of virulence genes into different serovars and therefore potentially make one serovar more virulent and frequently encountered than another. As shown in this and previous studies, phages Gifsy-1 or Gifsy-2 in *S.* Typhimurium (Figueroa-Bossi *et al.*, 1997) and their relatives in other *Salmonella* serovars would have been major contributors to the overall virulence of the serovars, since they are both widespread and versatile vehicles of bacterial virulence genes. However the amount and the invasiveness of the virulence genes carried by these Gifsy phages would likely to be different between serovars. This concept is supported by examining the 'hot-spots' for virulence genes in lambdoid phage genomes particularly the b region of the genomes. In *S.* Virchow phage PV10, the b region contains a putative *sopE* gene in place of two virulence genes *gogB* and *gogD* in *S.* Typhimurium Gifsy-1. Similarly the b region of *S.* Heidelberg PH03 also contains a putative *sopE* gene in place of genes *gtgE*, *gtgB* and *gtgF* in *S.* Typhimurium Gifsy-2. While it has been shown that gene *gtgE* contributes significantly to *Salmonella* virulence compared to *sopE*, replacement of *gtgE* with *sopE* in *S.* Heidelberg would have made this serovar much less virulent than *S.* Typhimurium and partially explains the higher prevalence of *S.* Typhimurium infections in comparison to *S.* Heidelberg.

6.4 CHAPTER SUMMARY

This study involved analysis of two temperate phages: *S.* Virchow phage PV10 and *S.* Heidelberg phage PH03 that displayed mosaic genetic architecture and most closely resemble the *S.* Typhimurium phages Gifsy-1 and Gifsy-2 respectively. Further examinations demonstrated that gene loci of Gifsy-1, Gifsy-2, PV10 and PH03 are also commonly found in the sequenced genomes of strains from other serovars, suggesting the widespread nature of Gifsy-related phages within genus *Salmonella*. However, since the level of modular variation seemed low in particular between the Gifsy phages harboured in isolates of the same serovars; it suggested that exploitation of additional Gifsy related loci is not an effective option to further improve the established MAPLT methods. There were putative virulence genes mapped in both phages PV10 and PH03 despite that the amount and the functionality of these

putative virulence genes were different to those found in Gifsy-1 and Gifsy-2. One noteworthy example is that the Gifsy-2 related *S.* Heidelberg phage PH03 was found to have a comparatively less potent *sopE* gene instead of three virulence genes including *gtgE* in the corresponding region of Gifsy-2 genome. The results suggest the role of Gifsy-related phages in the epidemiology of *Salmonella* making one serovar more prevalent than another.

CHAPTER 7 GENERAL SUMMARY AND DISCUSSION

Bacterial typing methods are in place for surveillance of *Salmonella* infections and for investigating outbreak investigations. Serotyping based on the Kauffmann and White serological scheme is employed initially and has subdivided *Salmonella* into over 2500 serovars (Popoff *et al.*, 2004). Traditionally bacteriophage (phage) typing is also carried out to further subdivide within certain *Salmonella* serovars to differentiate outbreak isolates from sporadic isolates. However discriminating ability can be limited when a *Salmonella* serovar endemic in a geographic region is found to produce a small number of phage types. Therefore molecular methods provide finer levels of discrimination are applied to distinguish outbreak isolates from endemic isolates of same phage types or serovars. In this thesis, three recent molecular typing approaches: multiple amplification of prophage locus typing (MAPLT), multi-locus variable-number tandem repeat (VNTR) analysis (MLVA) and the composite assay of MAPLT and MLVA were developed for typing *Salmonella* serovars Virchow, Bovismorbificans and Heidelberg that are endemic and have caused outbreaks in Australia. The differentiating ability of each method was evaluated through comparison with the 'gold standard' method, pulsed-field gel electrophoresis (PFGE). The aim was to ascertain if PFGE could be replaced as the method of choice for local epidemiological typing.

In agreement with previous studies, combining the use of the most variable MAPLT and MLVA loci provided a higher level of discrimination when compared to PFGE within the three serovars investigated in this study. More importantly, finer differentiation within predominant phage types was frequently achieved. For example, a composite MAPLT / MLVA assay for *S.* Enteritidis was devised using data from the study of Ross and Heuzenroeder (2009) demonstrating a superior discrimination within phage types 4 and 26 in comparison with PFGE. The DI value of the composite assay was 0.99 for both PT 4 and PT 26. In contrast, the DI value was 0.48 for PT 4 and 0.66 for PT 26 respectively when using PFGE. Likewise, poor discrimination within *S.* Virchow PT 8 was also observed using PFGE (DI = 0.56) that was enhanced greatly when using MAPLT / STTR-5 (DI = 0.87).

This study further indicated the usefulness of combining MAPLT / MLVA for outbreak epidemiological typing. Using the 10-loci MAPLT / STTR-5 scheme for *S.* Virchow, the PT 8 isolates included in the retrospective outbreak study were separated in the same manner as PFGE. Both methods were able to cluster all the outbreak-related isolates into the same MAPLT / STTR-5 or PFGE profile. Slightly different results were seen when using the 9-loci MAPLT / MLVA scheme for *S.* Bovismorbificans PT 11 outbreak typing. All outbreak-related isolates generated the same or highly similar MAPLT / MLVA profiles differing by one locus. The results suggested that while MAPLT / MLVA could display the close genetic relationship between outbreak isolates, minor variations in the profiles may be observed. The

significance of these minor variations at the profiles seemed to be dependent on the serovars or the phage-types that are being investigated. For example, there is a close genetic relationship between *S.* Virchow PT 8 isolates. This was suggested independently by PFGE and MAPLT that the majority of PT 8 produced the same PFGE or MAPLT profile. Therefore minor differences in typing data may be critical in differentiating between outbreak-related and endemic isolates.

In contrast, minor variations of single loci at MAPLT / MLVA profiles were observed in the epidemiologically-related *S.* Bovismorbificans PT 11 isolates. However there is no additional typing data in regard to the overall genetic distance between the recent PT 11 isolates as only one unrelated PT 11 isolate was available for this study. PFGE was not performed to measure the genetic similarity between these (non-viable) PT 11 isolates. Nevertheless the observations are consistent with previous findings that rapidly-evolving loci such as MLVA loci may change during the course of outbreaks leading to single-locus variants in the outbreak profiles (Noller *et al.*, 2003; Hopkins *et al.*, 2007).

Combining all the observations discussed above, it could be concluded that MAPLT / MLVA typing approach can be applied for outbreak epidemiological typing in conjunction with result interpretation guidelines that may be different between phage types or serovars. The stringency of guidelines in advising if an isolate is outbreak-related could be more correctly established by measuring the *in vivo* stability of the applied MAPLT or MLVA loci within the phage types or serovars. This could be performed through passing bacterial isolates through animal models, and subsequently comparing the typing profiles before and after the passage (Ross and Heuzenroeder, 2009). Alternatively, *in vivo* stability of typing profiles could be indicated through typing multiple isolates collected from the same patients (Kahl *et al.*, 2005; Hopkins *et al.*, 2007). Subsequently, the MAPLT / MLVA schemes and the proposed guidelines would need to be subjected in outbreak investigations to confirm their usefulness and accuracy.

It has become apparent recently that combined use of two typing methods may be the most suitable option for high-resolution typing of *Salmonella*. A study carried out by Gan *et al.* (2009) showed the improved differentiation of *S.* Sofia into 16 types when combining PFGE patterns of two restriction enzymes *Xba*I and *Spe*I and PCR band patterns of a repetitive element PCR (rep-PCR) using primer REP1. When these methods were carried out alone there were only eight *Xba*I, six *Spe*I and five REP1 profiles observed (Gan *et al.*, 2010). Alternatively, Chiu and co-workers (2010) combined PFGE with randomly amplified polymorphic DNA (RAPD), a different fragment - based method, and successfully differentiated the 95 epidemiologically unrelated *S.* Choleraesuis isolates with a DI of 0.9999. Similarly in another study there were three enzymes used (*Xba*I, *Avr*II and *Spe*I) in PFGE in conjunction with RAPD (Chiu *et al.*, 2011). As a contrast, Broschat *et al.* (2010) demonstrated that the combined use of

PFGE with MLVA could provide a high degree of improved differentiation between epidemiologically related isolates collected from the same host species and location. Therefore the authors suggested extending the combined use of PFGE and MLVA for outbreak epidemiological studies.

Reviewing the published combination of methods has illustrated the significance of PFGE in typing *Salmonella* as it was repeatedly employed in one of the combined methods. For the first two studies, two or more restriction enzymes for PFGE were used in addition to the application of one other fragment - based method (Gan *et al.*, 2010; Chiu *et al.*, 2011). While demonstrating excellent discriminating abilities, the time and labour involved for the procedure would be significantly increased. Moreover, the subjectivity of the typing data would be further increased with the number of band patterns as personal judgement is involved when interpreting fragment patterns. In comparison, use of PFGE in conjunction to MLVA as suggested by the third study was shown to be more practical (Broschat *et al.*, 2010). It is clear that the successful use of this combined approach for strain differentiation would rely equally upon the differentiating abilities of PFGE and the MLVA typing schemes that may not however be optimised for certain *Salmonella* serovars, such as *S.* Bovismorbificans, as complete genome sequence are not yet available for tandem repeat search. Similarly, combining use of MLVA with PFGE would not be useful in differentiating isolates of predominating phage types that have already been shown to have identical PFGE profiles despite that the isolates are epidemiologically diverse, such as *S.* Heidelberg PT1 (Chapter 5).

In comparison to those combined typing methods, combining MAPLT with MLVA appears the most simple and practical approach to develop and provide sufficient differentiation to use in outbreak settings, despite the fact that the composite MAPLT / MLVA scheme must be tailor-made for each serovar and appears less versatile than PFGE. This is because MAPLT and MLVA are both PCR-based and hence when used simultaneously are simple to perform and are able to provide results rapidly. As a result, sources of outbreaks could be identified rapidly to prevent further cases of infections.

Nevertheless, development or fine adjustment to the discriminating ability of a MAPLT / MLVA scheme is less challenging in comparison to setting up a stand-alone MLVA scheme particularly for serovars where the complete genomes are not available. This study indicated that many of the MLVA loci useful for one serovar showed no or less variations in the other serovars (chapters 3 to 5). Furthermore, not all MLVA loci are universally present in *Salmonella* that is possibly due to the different genomic organisations between serovars (Ross and Heuzenroeder 2009), and between strains of same serovars. For example, STTR-6 has only been seen in some strains of *S.* Typhimurium and *S.* Bovismorbificans. It is likely due to that STTR-6 locus is of phage origin, which is carried by Gifsy-1 (Lindstedt *et al.,* 2003).

Therefore the presence of STTR-6 depends on if the strains are integrated with Gifsy-1 or the related phages. In contrast, novel prophage loci with discriminative properties could be identified using DOP-PCR. As shown in the previous and the current studies, novel prophage loci IPS - 01 (Ross and Heuzenroeder, 2008) and DOP13.7 (Chapter 3) were identified from the locally - collected isolates that are useful in differentiating *S.* Infantis and *S.* Virchow isolates respectively.

In addition to identifying prophage loci that contribute to the inter-strain genetic diversity within the serovars, this study also demonstrated the widespread nature of phages related to Gifsy-1 and Gifsy-2 within the genus *Salmonella* (Chapter 6). Further examination of the Gifsy-related phages from different *Salmonella* serovars demonstrated that they carry different repertoires of virulence-associated genes that promote the pathogenicity of the carried *Salmonella* serovars to different degrees. For example, the highly virulent gene *gtgE* that are commonly observed in *S.* Typhimurium are absent in the cryptic prophages of the sequenced *S.* Heidelberg strains and in the sequenced *S.* Heidelberg phage PH03. Therefore it is proposed that Gifsy-related phages have a role in shaping the epidemiology of *Salmonella* serovars in humans. Further examinations should be carried out to determine whether there is significant variation in the Gifsy-carried virulence determinants in the *Salmonella* serovars of different prevalence in human infections. Nevertheless the pathogenicity of a *Salmonella* serovar and the frequency of which it is implicated in human infections are likely to be the composite effect of many genes important for virulence that are located in the bacterial chromosomes and the horizontally transferred elements such as bacteriophages (Litrup *et al.*, 2010). Therefore additional investigations should also be conducted to study curing the resident Gifsy-1 or Gifsy-2 related phages in various *Salmonella* serovars and determining any changes in the virulence level in an animal model. This experiment would demonstrate the importance of Gifsy phages in *Salmonella* virulence.

7.1 CONCLUDING REMARKS

The current study involved development of typing methods that target horizontally acquired genetic elements (prophage loci) and the fast changing genomic regions (MLVA loci). While these two types of genetic loci can be utilised to infer epidemiological relationships between closely related strains, such as to separate outbreak isolates from sporadic isolates in outbreak studies, the typing data generated do not necessarily infer the evolutionary relationships between tested strains. To establish the vertical evolutionary relationships within bacterial groups, typing methods targeting neutral sequence diversity have been used (Kotetishvili *et al.*, 2002; Sangal *et al.*, 2010), an example of these would be MLST. However due to the lack of discrimination, SNP (single-nucleotide polymorphism) typing has gradually replaced MLST for studying global epidemiology among highly clonal bacterial groups such as *S.* Typhi (Octavia and Lan, 2007) and *S.* Typhimurium (Hu *et al.*, 2006; Pang *et al.*, 2012).

Recently the fast and high-throughput new sequencing technologies, collectively named next-generation sequencing (NGS), have become available and are being applied to provide complete genomic sequences of *Salmonella* strains (Andrew-Polymenis *et al.*, 2009). Clearly, as NGS continues to advance, the cost and time required for whole genome sequencing (WGS) will be further reduced. It is expected that in the foreseeable future NGS methods and the subsequent whole genome comparions will be widely applied in epidemiology of bacteria. This is because the typing data are not only highly discriminative allowing unambiguous differentiation of closely related strains (e.g. outbreak and sporadic strains); but also providing real-time evolutionary data of the strains (Gardy *et al.*, 2011; Lienau *et al.*, 2011; Mellmann *et al.*, 2011). Currently, NGS has a significant role in the local epidemiology of bacteria by revealing genetic variations within highly clonal pathogens in a specific locality. This will facilitate the development of high-resolution molecular assays (Bakker *et al.*, 2011; Leekitcharoenphon *et al.*, 2012) or further improvements on the existing assays including the composite MAPLT / MLVA assays.

REFERENCES

Aarts, H. J. M., van Lith, L. A. J. T. & Keijer, J. 1998. High-resolution genotyping of *Salmonella* strains by AFLP-fingerprinting. *Lett Appl Microbiol,* 26, 131-135.

Abe, M. 1974. The replication of prophage P1 DNA. *Mol Gen Genet,* 132, 63-72.

Abedon, S. T. 2008. Phages, ecology, evolution. *In:* Abedon, S. T. (ed.) *Bacteriophage ecology: Population growth, evolution, and impact of bacterial viruses.* United Kingdon: Cambridge University Press.

Abelson, P., Potter Forbes, M. & Hall, G. 2006. The annual cost of foodborne illness in Australia. Canberra: Australian Government Department of Health and Ageing.

Ackermann, H. W. 2003. Bacteriophage observations and evolution. *Res Microbiol,* 154, 245 - 251.

Ackermann, H. W. 2006. Classification of bacteriophages *In:* Calendar, R. (ed.) *The Bacteriophages.* The United Kingdom: Oxford University Press.

Ackermann, H. W. & Kropinski, A. M. 2007. Curated list of prokaryote viruses with fully sequenced genomes. *Res Microbiol,* 158, 555-566.

Ackermann, H. W. 2009. Phage classification and characterization. *Methods Mol Biol,* 501, 127-140.

Ackers, M. L., Puhr, N. D., Tauxe, R. & Mintz, E. D. 2000. Laboratory-based surveillance of *Salmonella* serotype Typhi infections in United States: Antimicrobial resistance on the rise. *JAMA,* 283, 2668-2673.

Adak, B. & Threlfall, E. J. 2005. Outbreak of drug-resistant *Salmonella* Virchow phage type 8 infection. *CDR Wkly (Online),* 15, 2-3.

Ahmed, R., Soule, G., Demczuk, W. H., Clark, C., Khakhria, R., Ratnam, S., Marshall, S., Ng, L., -K., Woodward, D. L., Johnson, W. M. & Rodgers, F. G. 2000. Epidemiologic typing of *Salmonella enterica* serotype Enteritidis in a Canada-wide outbreak of gastroenteritis due to contaminated cheese *J Clin Microbiol,* 38, 2403 - 2406.

Allison, G. E., Angeles, D., Tran-Dinh, N. & Verma, N. K. 2002. Complete genomic sequence of SfV, a serotype-converting temperate bacteriophage of *Shigella flexneri*. *J Bacteriol,* 184, 1974-1987.

Altschul, S., Gish, W., Miller, W., Myers, E. & Lipman, D. 1990. Basic local alignment search tool. *J Mol Biol,* 215, 403-410.

Andrew-Polymenis, H. L., Santiviago, C. A. & McClelland, M. 2009. Novel genetic tools for studying food-borne *Salmonella*. *Curr Opin Biotechnol,* 2009, 149-157.

Arbeit, R. 2003. Laboratory procedures for the epidemiologic analysis of microorganisms. *In:* Baron, E. J., Murray, P. R., Pfaller, M. A., Tenover, F. & Yolken, R. H. (eds.) *Manual of Clinical Microbiology.* Washington, D. C.: ASM Press.

Ashelford, K. E., Day, M. J. & Fry, J. C. 2003. Elevated abundance of bacteriophage infecting bacteria in soil. *Appl Environ Microbiol,* 69, 285-289.

Australian *Salmonella* Reference Centre. Annual Reports 1986-2009. Adelaide, Australia: Institute of Medical and Veterinary Science.

Baggesen, D. L., Wegener, H. C. & Madsen, M. 1997. Correlation of conversion of *Salmonella enterica* serovar Enteritidis phage type 1, 4, or 6 to phage type 7 with loss of lipopolysaccharide. *J Clin Microbiol,* 35, 330 - 333.

Baggesen, D. L., Sandvang, D. & Aarestrup, F. M. 2000. Characterization of *Salmonella enterica* serovar Typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. *J Clin Microbiol,* 38, 1581-1586.

Bakker, H. C., Switt, A. I., Cummings, C. A., Hoelzer, K., Degoricija, L., Rodriguez-Rivera, L. D., Wright, E. M., Fang, R., Davis, M., Root, T., Schoonmaker-Bopp, D., Musser, K. A., Villamil, E., Waechter, H., Kornstein, L., Furtado, M. R. & Wiedmann, M. 2011. A whole-genome single nucleotide polymorphism-based approach to trace and identify outbreaks linked to a common *Salmonella enterica* subsp. *enterica* serovar Montevideo pulsed-field gel electrophoresis type. *Appl Environ Microbiol,* 77, 8648-8655.

Barondess, J. J. & Beckwith, J. 1990. A bacterial virulence determinant encoded by lysogenic coliphage lambda. *Nature,* 346, 871-874.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A. & Horvath, P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science,* 315, 1709-1712.

Barrett, T. J., Gerner-Smidt, P. & Swaminathan, B. 2006. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. *Foodborne Pathog Dis,* 3, 20-31.

Bell, J., Turnidge, J., Ballow, C. & Jones, R. 2003. Multicentre evaluation of the *in vitro* activity of Linezolid in the Western Pacific. *J Antimicrob Chemother,* 51, 339-345.

Bennett, C. M., Dalton, C., Beers-Deeble, M., Milazzo, A., Kraa, E., Davos, D., Puech, M., Tan, A. & Heuzenroeder, M. W. 2003. Fresh garlic: A possible vehicle for *Salmonella* Virchow. *Epidemiol Infect,* 131, 1041-1048.

Benson, G. 1999. Tandem repeats finder: A program to analyze DNA sequences. *Nucleic Acids Res,* 27, 573-580.

Beranek, A., Mikula, C., Rabold, P., Arnhold, D., Berghold, C., Lederer, I., Allerberger, F. & Kornschober, C. 2009. Multiple-locus variable-number tandem repeat analysis for subtyping of *Salmonella enterica* subsp. *enterica* serovar Enteritidis. *Int J Med Microbiol,* 299, 43-51.

Besemer, J. & Borodovsky, M. 1999. Heuristic approach to deriving models for gene finding. *NAR,* 19, 3911-3920.

Bhan, M., Bahl, R. & Bhatnager, S. 2005. Typhoid and paratyphoid fever. Lancet, 366, 749-762.

Bichara, M., Wagner, J. & Lambert, I. B. 2006. Mechanisms of tandem repeat instability in bacteria. *Mutat Res,* 598, 144-163.

Bopp, C. A., Brenner, F. W., Fields, P. I., Wells, J. G. & Strockbine, N. A. 2003. *Esherichia*, *Shigella*, and *Salmonella*. *In:* Baron, E. J., Murray, P. R., Pfaller, M. A., Tenover, F. C. & Yolken, R. H. (eds.) *Manual of Clinical Microbiology.* 7th ed. Washington, D.C.: ASM Press.

Boxrud, D., Pederson-Gulrud, K., Wotton, J., Medus, C., Lyszkowicz, E., Besser, J. & Bartkus, J. M. 2007. Comparison of multiple-locus variable-number tandem repeat analysis, pulsed-field gel electrophoresis, and phage typing for subtype analysis of *Salmonella enterica* serotype Enteritidis. *J Clin Microbiol,* 45, 536-543.

Boyd, E. F., Wang, F.-S., Whittam, T. S. & Selander, R. K. 1996. Molecular genetic relationships of the *Salmonellae*. *Appl Environ Microbiol,* 62, 804-808.

Boyd, E. F. & Brussow, H. 2002. Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *Trends Microbiol,* 10, 521 - 529.

Bradley, D. E. 1967. Ultrastructure of bacteriophages and bacteriocins. *Bacteriol Rev,* 31, 230-314.

Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R. & Swaminathan, B. 2000. *Salmonella* nomenclature. *J Clin Microbiol,* 38, 2465 - 2467.

Broschat, S. L., Call, D. R., Davis, M. A., Meng, D., Lockwood, S., Ahmed, R. & Besser, T. E. 2010. Improved identification of epidemiologically related strains of *Salmonella enterica* by use of a fusion algorithm based on pulsed-field gel electrophoresis and multiple-locus variable-number tandem-repeat analysis. *J Clin Microbiol,* 48, 4072-4082.

Brown, J. C. S., Ward, L. J. H. & Davey, G. P. 1994. Rapid isolation and puriification of lactococcal bacteriophage DNA without the use of caesium chloride gradients. *Lett Appl Microbiol,* 18, 292-293.

Brüssow, H. & Desiere, F. 2001. Comparative phage genomics and the evolution of *Siphoviridae*: Insights from dairy phages. *Mol Microbiol,* 39, 213 - 222.

Brüssow, H., Canchaya, C. & Hardt, W.-D. 2004. Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev,* 68, 560 - 602.

Call, D. R., Orfe, L., Davis, M. A., Lafrentz, S. & Kang, M.-S. 2008. Impact of compounding error on strategies for subtyping pathogenic bacteria. *Foodborne Pathog Dis,* 5, 505-516.

Campbell, A. 1994. Comparative molecular biology of lambdoid phages. *Annu Rev Microbiol,* 48, 193-222.

Campbell, A. 2006. General aspect of lysogeny. *In:* Calender, R. & Abedon, S. T. (eds.) *The Bacteriophages.* New York: Oxford University Press.

Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M. A., Barrell, B. G. & Parkhill, J. 2005. ACT: the Artemis Comparison Tool. *Bioinformatics,* 21, 3422-3423.

Casjens, S. 2003. Prophages and bacterial genomes: What have we learned so far? *Mol Microbiol,* 49, 277 - 300.

Casjens, S. R., Gilcrease, E. B., Winn-Stapley, D. A., Schicklmaier, P., Schmieger, H., Pedulla, M. L., Ford, M. E., Houtz, J. M., Hatfull, G. F. & Hendrix, R. W. 2005. The generalized transducing *Salmonella* bacteriophage ES18: complete genome sequence and DNA packaging strategy. *J Bacteriol,* 187, 1091-104.

CDC 2008. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food -- 10 states, United States, 2007. *MMWR Morb Mortal Wkly Rep,* 57, 366-370.

CDC 2009. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food -- 10 states, United States, 2008. *MMWR Morb Mortal Wkly Rep,* 58, 333-337.

Centers of Disease Control and Prevention. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Reports, 2005-2008. Atlanta, Georgia, US: Department of Health and Human Services.

Chamber, R. M., McAdam, P., de Sa, J. D. H., Ward, L. R. & Rowe, B. 1987. A phage-typing scheme for *S. virchow*. *FEMS Microbiol Lett,* 40, 155-157.

Chart, H., Row, B., Threlfall, E. J. & Ward, L. R. 1989. Conversion of *Salmonella* Enteritidis phage type 4 to phage type 7 involes loss of lipopolysaccharide with concomitant loss of virulence. *FEMS Microbiol Lett,* 51, 37-40.

Cheng, H. H., Muhlrad, P. J., Hoyt, M. A. & Echols, H. 1988. Cleavage of the cII protein of phage lambda by purified HflA protease: control of the switch between lysis and lysogeny. *Proc Natl Acad Sci U S A,* 85, 7882-7886.

Chiu, T.-H., Pang, J.-C., Chen, M.-H. & Tsen, H.-Y. 2011. Improvement of strain discrimination by combination of RAPD with PFGE for the analysis of the swine isolates of *Salmonella enterica* serovar Choleraesuis. *World J Microbiol Biotechnol,* 27, 465 - 469.

Cho, S., Boxrud, D., Bartkus, J. M., Whittam, T. S. & Saeed, M. 2007. Multiple-locus variable-number tandem repeat analysis of *Salmonella* Enteritidis isolates from human and non-human sources using a single multiplex PCR. *FEMS Microbiol Lett,* 275, 16-23.

Cooke, F. J., Wain, J., Fookes, M., Ivens, A., Thomson, N., Brown, D. J., Threlfall, E. J., Gunn, G., Foster, G. & Dougan, G. 2007. Prophage sequences defining hot spots of genome variation in *Salmonella enterica* serovar Typhimurium can be used to discriminate between field isolates. *J Clin Microbiol,* 45, 2590 - 2598.

Coombes, B. K., Wickham, M. E., Brown, N. F., Lemire, S., Bossi, L., Hsiao, W. W., Brinkman, F. S. & Finlay, B. B. 2005. Genetic and molecular analysis of GogB, a phage-encoded type III-secreted substrate in *Salmonella enterica* serovar typhimurium with autonomous expression from its associated phage. *J Mol Biol,* 348, 817-830.

Cowan, L. S., Mosher, L., Diem, L., Massey, J. P. & Crawford, J. T. 2002. Variable-number tandem repeat typing of *Mycobacterium tuberculosis* isolates with low copy numbers of IS6110 by using mycobacterial interspersed repetitive units. *J Clin Microbiol,* 40, 1592-1602.

Crosa, J. H., Brenner, D. J., Ewing, W. H. & Falkow, S. 1973. Molecular relationships among the *Salmonellae*. *J Bacteriol,* 115, 307 - 315.

Cruickshank, R., Duguid, J., Marmion, B. P. & Swain, R. H. A. 1975. *Medical Microbiology,* London, England, W. B. Saunders and Co.

Daniels, D. L., Schroeder, J. L., Szybalski, W., Sanger, F. & Blattner, F. R. 1983. A molecular map of coliphage lambda. *In:* Hendrix, R. W., Roberts, J. W., Stahl, F. W. & Weisberg, R. A. (eds.) *Lambda II.* Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press.

Darwin, K. H. & Miller, V. L. 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. *Clin Microbiol Rev,* 12, 405-428.

Davis, M. A., Baker, K. N. K., Call, D. R., Warnick, L. D., Soyer, Y., Wiedmann, M., Gröhn, Y., McDonough, P. L., Hancock, D. D. & Besser, T. E. 2009. Multilocus variable-number tandem repeat method for typing *Salmonella enterica* serovar Newport. *J Clin Microbiol,* 47, 1934-1938.

Davison, J. 1999. Genetic exchange between bacteria in the environment. *Plasmid,* 42, 73-91.

Davos, D. 2005. *Salmonella*. *In:* McIver, C. L. (ed.) *A compendium of laboratory diagnostic methods for common and unusual enteric pathogens.* Australia: Australian Society of Microbiology Inc.

De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y. & Fang, F. C. 1997. Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc Natl Acad Sci U S A,* 94, 13997-4001.

Demczuk, W., Soule, G., Clark, C., Ackermann, H.-W., Easy, R., Khakria, R., Rodgers, F. & Ahmed, R. 2003. Phage-based typing scheme for Salmonella enterica serovar Heidelberg, a causative agent of food poisonings in Canada. *J Clin Microbiol,* 41, 4279-4284.

Denæud, F. & Vergnaud, G. 2004. Identification of polymorphic tandem repeats by direct comparison of genome sequence from difference bacterial strains: A web-based resource. *BMC Bioinformatics,* 5.

Deng, W., Liou, S.-R., Plunkett III, G., Mayhew, G. F., Rose, D. J., Burland, V., Kodoyianni, V., Schwartz, D. C. & Blattner, F. R. 2003. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. *J Bacteriol,* 185, 2330 - 2337.

Drahovská, H., Mikasová, E., Szemes, T., Ficek, A., Sásik, M., Majtán, V. & Turna, J. 2007. Variability in occurence of multiple prophage genes in *Salmonella* Typhimurium strains isolates in Slovak Republic. *FEMS Microbiol Lett,* 270, 237-244.

Echeita, M. A., Herrera, S., Garaizar, J. & Usera, M. 2002. Multiplex PCR-based detection and identification of the most common *Salmonella* second phase flagellar antigens. *Res Microbiol,* 153, 107-113.

Eklund, M., Poysky, F. T., Reed, S. M. & Smiith, C. A. 1971. Bacteriophage and the toxigenicity of *Clostridium botulinum* type C. *Science,* 172, 480-482.

Ezquerra, E., Burnens, A. P., Frith, K., Costas, M. & Stanley, J. 1993. Molecular genotype analysis of *Salmonella* Bovismorbificans. *Mol Cell Probes,* 7, 45-54.

Fakhr, M., Nolan, L. K. & Logue, C. M. 2005. Multilocus sequence typing lacks the discriminatory ability of pulsed-field gel electrophoresis for typing *Salmonella enterica* serovar Typhimurium. *J Clin Microbiol,* 43, 2215 - 2219.

Fang, N. X., Huang, B., Hiley, L., Bates, J. & Savill, J. 2012. A rapid multiplex DNA suspension array method for *Salmonella* typhimurium subtyping using prophage-related markers. *J Microbiol Methods,* 88, 19-27.

Farrant, J. L., Sansone, A., Canvin, J. R., Pallen, M. J., Langford, P. R., Wallis, T. S., Dougan, G. & Kroll, J. S. 1997. Bacterial copper-and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. *Mol Microbiol,* 25, 785-796.

Feavers, I. M., Gray, S. J., Urwin, R., Russell, J. E., Bygraves, J. A., Kaczmarski, E. B. & Maiden, M. C. 1999. Multilocus sequence typing and antigen gene sequencing in the investigation of a meningococcal disease outbreak. *J Clin Microbiol,* 37, 3883-3887.

Figueroa-Bossi, N., Coissac, E., Netter, P. & Bossi, L. 1997. Unsuspected prophage-like elements in *Salmonella* typhimurium. *Mol Microbiol,* 25, 161-173.

Figueroa-Bossi, N. & Bossi, L. 1999. Inducible prophages contribute to *Salmonella* virulence in mice. *Mol Microbiol,* 33.

Figueroa-Bossi, N., Uzzau, S., Maloriol, D. & Bossi, L. 2001. Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in *Salmonella*. *Mol Microbiol,* 39, 260 - 271.

Figueroa-Bossi, N. & Bossi, L. 2004. Resuscitation of a defective prophage in *Salmonella* cocultures. *J Bacteriol,* 186, 4038-4041.

Finnegan, J. & Egan, J. B. 1979. Physical map of the coliphage 186 chromosome. *Mol Gen Genet,* 172, 287-293.

Ford, M. E., Sarkis, G. J., Belanger, A. E., Hendrix, R. W. & Hatfull, G. F. 1998. Genome structure of mycobacteriophage D29: Implications for phage evolution. *J Mol Biol,* 279, 143-164.

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

Galanis, E., Wong, D. M. A., Patrick, M. E., Binztein, N., Cieslik, A., Chalermchaikit, T., Aidara-Kane, A., Ellis, A., Angulo, F. J. & Wegener, H. C. 2006. Web-based surveillance and global *Salmonella* distribution, 2000-2002. *Emerg Infect Dis,* 12, 381-388.

Gan, E., Smooker, P. M. & Coloe, P. J. 2010. Molecular typing of *Salmonella enterica* serovar Sofia in Australia by pulsed-field gel electrophoresis and repetitive element PCR typing. *J Appl Microbiol,* 109, 292-303.

Gardy, J. L., Johnston, J. C., Ho Sui, S. J., Cook, V. J., Shah, L., Brodkin, E., Rempel, S., Moore, R., Zhao, Y., Holt, R., Varhol, R., Birol, I., Lem, M., Sharma, M. K., Elwood, K., Jones, S. J., Brinkman, F. S., Brunham, R. C. & Tang, P. 2011. Whole-genome sequencing and social-network analysis of a tuberculosis outbreak. *N Engl J Med,* 364, 730-739.

Gatto, A. J., Peters, T. M., Green, J., Fisher, I. S. T., Gill, O. N., O' Brien, S. J., Maguire, C., Berghold, C., Lederer, I., Gerner-Smidt, P., Torpdahl, M., Siitonen, A., Lukinmaa, S., Tschäpe, H., Prager, R., Luzzi, I., Dionisi, A. M., van der Zwaluw, W. K., Heck, M., Coia, J., Brown, D., Usera, M., Echeita, A. & Threlfall, E. J. 2006. Distribution of molecular subtypes within Salmonella enterica serotype Enteritidis phage type 4 and S. Typhimurium definitive phage type 104 in nine European countries, 2000-2004: Results of an international multi-centre study. *Epidemiol Infect,* 134, 729-736.

Gemski, P., O.Brien, A. D. & Wohlhieter, J. A. 1978. Cellular release of heat-liable enterotoxin of *Escherichia coli* by bacteriophage induction. *Infect Immun,* 19, 1076-1082.

Giammanco, G. M., Mammina, C., Romani, C., Luzzi, I., Dionisi, A. M. & Nastasi, A. 2007. Evaluation of a modified single-enzyme amplified fragment length polymorphism (SE-AFLP) technique for subtyping *Salmonella enterica* serotype Enteritidis. *Res Microbiol,* 158, 10-17.

Gilbert, G. L. 2008. Using MLVA to type strains of *Salmonella* Typhimurium in New South Wales. *N S W Public Health Bull,* 19, 29-31.

Gilsdorf, A., Jansen, A., Alpers, K., Dieckmann, H., van Treeck, U., M. Hauri, A., Fell, G., Littmann, M., Rautenberg, P., Prager, R., Rabsch, W., Roggentin, P., Shroeter, A., Miko, A., Bartelt, E., Braunig, J. & Ammon, A. 2005. A nationwide outbreak of Salmonella Bovismorbificans PT24, Germany, December 2004-March 2005. *Euro Surveill,* 10, 2667.

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

Grimont, P. A. D. & Weill, F.-X. 2007. *Antigenic formulae of the Salmonella serovars,* Paris, France, World Health Organisation Collaborating Centre for Reference and Research on *Salmonella*, Pasteur Institute.

Groisman, E. A. & Ochman, H. 1996. Pathogenicity islands: Bacterial evolution in quantum leaps. *Cell,* 87, 791-794.

Groman, N. B. 1953. Evidence for the induced nature of the change from nontoxigenicity to toxigenicity in *Corynebacterium diphtheriae* as a result of exposure to specific bacteriophage. *J Bacteriol,* 66, 184-191.

Groman, N. B. & Eaton, M. 1955. Genetic factors in *Corynebacterium diphtheriae* conversion. *J Bacteriol,* 70.

Grundmann, H., Hori, S. & Tanner, G. 2001. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. *J Clin Microbiol,* 39, 4190-4192.

Guibourdenche, M., Roggentin, P., Mikoleit, M., Fields, P. I., Bockemühl, J., Grimont, P. A. D. & Weill, F.-X. 2010. Supplement 2003 - 2007 (No. 47) to the White-Kauffmann-Le Minor Scheme. *Res Microbiol,* 161, 26-29.

Gunn, J. S., Alpuche-Aranda, C. M., Loomis, W. P., Belden, W. J. & Miller, S. I. 1995. Characterization of the *Salmonella typhimurium pagC/pagD* chromosomal region. *J Bacteriol,* 177, 5040-5047.

Harbottle, H., White, D. G., McDermott, P. F., Walker, R. D. & Zhao, S. 2006. Comparison of multilocus sequence typing, pulsed-field gel electrophoresis, and anitimicrobial susceptibility typing for characterization of *Salmonella enterica* serotype Newport isolates. *J Clin Microbiol,* 44, 2449-2457.

Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R. & Galan, J. E. 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell,* 93, 815-826.

Harvey, D., Harrington, C., Heuzenroeder, M. W. & Murray, C. 1993. Lysogenic phage in *Salmonella enterica* serovar Heidelberg (*Salmonella heidelberg*): Implications for organism tracing. *FEMS Microbiol Lett,* 108, 291 - 293.

Hatfull, G. F. 2008. Bacteriophage genomics. *Curr Opin Microbiol,* 11, 447-453.

Hendriksen, R. S., Vieira, A. R., Karlsmose, S., Danilo, M. A., Wong, L. F., Jensen, A. B., Wegener, H. C. & Aarestrup, F. M. 2011. Global monitoring of Salmonella serovar distribution from the World Health Organization global foodborne infections network country data bank: Results of quality assured laboratories from 2001 to 2007. *Foodborne Pathog Dis,* 8, 887 - 900.

Hendrix, R. W., Smith, M. C. M., Burns, R. N., Ford, M. E. & Hatfull, G. F. 1999. Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage. *Proc Natl Acad Sci U S A,* 96, 2192-2197.

Hendrix, R. W., Hatfull, G. F. & Smith, M. C. M. 2003. Bacteriophages with tails: Chasing their origins and evolution. *Res Microbiol,* 154, 253-257.

Hendrix, R. W. & Casjens, S. R. 2006. Bacteriophge λ and its genetic neighorhood. *In:* Calender, R. & Abedon, S. T. (eds.) *The Bacteriophages.* 2nd ed. New York: Oxford University Press.

Hensel, M., Shea, J. E., Bäumler, A. J., Gleeson, C., Blattner, F. R. & Holden, D. W. 1997. Analysis of the boundaries of *Salmonella* pathogenicity island 2 and the corresponding chromosomal region of *Esherichia coli* K-12. *J Bacteriol,* 1105-1111.

Herikstad, H., Motarjemi, Y. & Tauxe, R. 2002. *Salmonella* surveillance: A global survey of public health serotyping. *Epidemiol Infect,* 129, 1-8.

Hermans, A. P. H. M., Abee, T., Zwietering, M. H. & Aarts, H. J. M. 2005. Identification of novel *Salmonella enterica* serovar Typhimurium DT104-specific prophage and nonprophage chromosomal

sequences among serovar typhimurium isolates by genomic substractive hybridisation. *Appl Environ Microbiol,* 71, 4979 - 4985.

Herrera-León, S., McQuiston, J. R., Usera, M., Fields, P. I., Garaizar, J. & Echeita, A. 2004. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp. *J Clin Microbiol,* 42, 2581-2586.

Herrera-León, S., Ramiro, R., Arroyo, M., Díez, R., Usera, M. & Echeita, M. A. 2007. Blind comparison of traditional serotyping with three multiplex PCRs for the identification of *Salmonella* serotypes. *Res Microbiol,* 158, 122-127.

Ho, T. D. & Slauch, J. M. 2001. Characterization of grvA, an antivirulence gene on the gifsy-2 phage in *Salmonella enterica* serovar Typhimurium. *J Bacteriol,* 183, 611-620.

Ho, T. D., Figueroa-Bossi, N., Wang, M., Uzzau, S., Bossi, L. & Slauch, J. M. 2002. Identification of GtgE, a novel virulence factor encoded on the Gifsy-2 bacteriophage of *Salmonella enterica* serovar Typhimurium. *J Bacteriol,* 184, 5234-5239.

Ho, Y. S., Mahoney, M. E., Wulff, D. L. & Rosenberg, M. 1988. Identification of the DNA binding domain of the phage lambda cII transcriptional activator and the direct correlation of cII protein stability with its oligomeric forms. *Genes Dev,* 2, 184-195.

Hofer, B., Ruge, M. & Dreiseikelmann, B. 1995. The superinfection exclusion gene (sieA) of bacteriophage P22: identification and overexpression of the gene and localization of the gene product. *J Bacteriol,* 177, 3080 - 3086.

Hopkins, K. L., Maguire, C., Best, E. L., Liebana, E. & Threlfall, E. J. 2007. Stability of multiple-locus variable-number tandem repeats in *Salmonella enterica* serovar Typhimurium. *J Clin Microbiol,* 45, 3058-3061.

Hu, H., Lan, R. & Reeves, P. R. 2002. Fluorescent amplified fragment length polymorphism analysis of *Salmonella enterica* serovar Typhimurium reveals phage-type- specific markers and potential for microarray typing *J Clin Microbiol,* 40, 3406-3415.

Hu, H., Lan, R. & Reeves, P. R. 2006. Adaptation of multilocus sequencing for studying variations within a major clone: Evolutionary relationships of *Salmonella enterica* serovar Typhimurium. *Genetics,* 172, 743-750.

Humphrey, T. 2000. Public-health aspects of *Salmonella* infection. *In:* Wray, C. & Wray, A. (eds.) *Salmonella in domestic animals.* United Kingdom: CABI publishing.

Hunter, P. R. & Gaston, M. A. 1988. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. *J Clin Microbiol,* 26, 2465 - 2466.

Ikeda, H. & Tomizawa, J.-I. 1968. Prophage P1, an extrachromosomal replication unit. *Cold Spring Harbor Symposia on Quantitative Biology,* 33, 791-798.

Irvine, W. N., Gillespie, I. A., Smyth, F. B., Rooney, N., McClenaghan, A., Devine, M. J. & Tohani, V. K. 2009. Investigation of an outbreak of *Salmonella enterica* serovar Newport infection. *Epidemiol Infect,* 137, 1449-1456.

Ispahani, P. & Slack, R. C. 2000. Enteric fever and other extraintestinal salmonellosis in University Hospital, Nottingham, UK, between 1980 and 1997. *Eur J Clin Microbiol Infect Dis,* 19, 679-687.

Jones, Y. E., McLaren, I. M. & Wray, C. 2000. Laboratory aspects of *Salmonella*. *In:* Wray, C. & Wray, A. (eds.) *Salmonella in Domestic Animals.* United kingdom: CABI Publishing.

Juhala, R. J., Ford, M. E., Duda, R. L., Youlton, A., Hatfull, G. F. & Hendrix, R. W. 2000. Genomic sequences of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid bacteriophages. *J Mol Biol,* 299, 27 - 51.

Kahl, B. C., Mellmann, A., Deiwick, S., Peters, G. & Harmsen, D. 2005. Variation of the polymorphic region X of the protein A gene during persistant airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in *Staphylococcus aureus*. *J Clin Microbiol,* 43, 502-505.

Kauffmann, F. 1966. *The bacteriology of Enterobacteriaceae,* Baltimore, Williams and Wilkins.

Kotetishvili, M., Stine, O. C., Kreger, A., Morris Jr., J. G. & Sulakvelidze, A. 2002. Multilocus sequence typing for characterization of clinical and environmental *Salmonella* strains. *J Clin Microbiol,* 40, 1626 - 1635.

Kuukasjarvi, T., Tanner, M., Pennanen, S., Karhu, R., Visakorpi, T. & Isola, J. 1997. Optimising DOP-PCR for universal amplification of small DNA samples in comparative genomic hybridisation. *Genes, Chromosomes Cancer,* 18, 94 - 101.

Laconcha, I., Baggesen, D. L., Rementeria, A. & Garaizar, J. 2000. Genotypic characterisation by PFGE of *Salmonella enterica* serotype Enteritidis phage types 1, 4, 6, and 8 ssolated from animal and human sources in three European countries. *Vet Microbiol,* 75, 155 - 165.

Lan, R., Davison, A. M., Reeves, P. R. & Ward, L. R. 2003. AFLP analysis of *Salmonella enterica* serovar Typhimurium isolates of phage types DT 9 and DT 135: Diversity within phage types and its epidemiological significance. *Microbes Infect,* 5, 841-850.

Lan, R., Stevenson, G., Donohoe, K., Ward, L. & Reeves, P. R. 2007. Molecular markers with potential to replace phage typing for *Salmonella enterica* serovar typhimurium. *J Microbiol Methods,* 68, 145-156.

Larsson, J. T., Torpdahl, M., Petersen, R. F., Sorensen, G., Lindstedt, B. A. & Nielsen, E. M. 2009. Development of a new nomenclature for *Salmonella* Typhimurium multilocus variable number of tandem repeats analysis (MLVA). *Euro Surveill,* 14.

Lawson, A. J., Desai, M., O' Brien, S. J., Davies, R. H., Ward, L. R. & Threlfall, E. J. 2004. Molecular characterisation of an outbreak strain of multiresistant Salmonella enterica serovar Typhimurium DT104 in the UK. *Clin Microbiol Infect,* 10, 143-147.

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

Le Minor, L. 1988. Typing of *Salmonella* species. *Eur J Clin Microbiol Infect Dis,* 7, 214-218.

Leekitcharoenphon, P., Lukjancenko, O., Friis, C., Aarestrup, F. M. & Ussery, D. W. 2012. Genomic variation in *Salmonella enterica* core genes for epidemiological typing. *BMC Genomics,* 13, 88.

Liebana, E., Garcia-Migura, L., Breslin, M. F., Davies, R. H. & Woodward, M. J. 2001. Diversity of strains of *Salmonella enterica* serotype Enteritidis from English poultry farms assessed by multiple genetic fingerprinting. *J Clin Microbiol,* 39, 154-161.

Liebana, E., Garcia-Migura, L., Clouting, C., Clifton-Hadley, F. A., Linsay, E., Threlfall, E. J., McDowell, S. W. J. & Davies, R. H. 2002. Multiple genetic typing of *Salmonella enterica* serotype Typhimurium isolates of different phage types (DT104, U302, DT204b, and DT49) from animals and humans in England, Wales, and Northern Ireland. *J Clin Microbiol,* 40, 4450-4456.

Lienau, E. K., Strain, E., Wang, C., Zheng, J., Ottesen, A. R., Keys, C. E., Hammack, T. S., Musser, S. M., Brown, E. W., Allard, M. W., Cao, G., Meng, J. & Stones, R. 2011. Identification of a salmonellosis outbreak by means of molecular sequencing. *N Engl J Med,* 364, 981-982.

Liesegang, A., Davos, D., Balzer, J. C., Rabsch, W., Prager, R., Lightfoot, D., Siitonen, A., Claus, H. & Tschäpe, H. 2002. Phage typing and PFGE pattern analysis as tools for epidemiological surveillance of *Salmonella enterica* serovar Bovismorbificans infections. *Epidemiol Infect,* 128, 119-130.

Lindstedt, B.-A., Heir, E., Vardund, T. & Kapperud, G. 2000. Fluorescent amplified-fragment length polymorphism genotyping of *Salmonella enterica* subsp. *enterica* serovars and comparison with pulsed-field gel electrophoresis. *J Clin Microbiol,* 38, 1623 - 1627.

Lindstedt, B.-A., Heir, E., Gjernes, E. & Kapperud, G. 2003. DNA fingerprinting of *Salmonella enterica* subsp. *enterica* serovar Typhimurium with emphasis on phage type DT104 based on variable number of tandem repeat loci. *J Clin Microbiol,* 41, 1469 - 1479.

Lindstedt, B.-A., Vardund, T., Aas, L. & Kapperud, G. 2004. Multiple-locus variable-number tandem-repeats analysis of *Salmonella enterica* subsp. enterica serovar Typhimurium using PCR multiplexing and multicolour capillary electrophoresis. *J Microbiol Methods,* 59, 163-172.

Lindstedt, B.-A. 2005. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis,* 26, 2567-2582.

Lindstedt, B.-A., Torpdahl, M., Nielsen, E. M., Vardund, T., Aas, L. & Kapperud, G. 2006. Harmonization of the multiple-locus variable-number tandem repeat analysis method between Denmark and Norway for typing *Salmonella* Typhimurium isolates and closer examination of the VNTR loci. *J Appl Microbiol,* 102, 728-735.

Litrup, E., Torpdahl, M., Malorny, B., Huehn, S., Christensen, H. & Nielsen, E. M. 2010. Association between phylogeny, virulence potential and serovars of *Salmonella enterica*. *Infect Genet Evol,* 10, 1132-1139.

Little, J. W. 2005. Lysogeny, prophage induction, and lysogenic conversion. *In:* Waldor, M. K., Friedman, D. I. & Adhya, S. L. (eds.) *Phages: Their role in bacterial pathogenesis and biotechnology.* Washington, D. C.: ASM Press.

Liu, Y., Lee, M.-A., Ooi, E.-E., Mavis, Y., Tan, A.-L. & Quek, H.-H. 2003. Molecular typing of *Salmonella enterica* serovar Typhi isolates from various countries in Asia by a multiplex PCR assay on variable-number tandem repeats. *J Clin Microbiol,* 41, 4388 - 4394.

Luk, J. M., Kongmuang, U., Reeves, M. W. & Lindberg, A. A. 1993. Selective amplification of abequose and paratose synthase genes (*rfb*) by polymerase chain reaction for identification of *Salmonella* major serogroups (A, B, C2 and D) *J Clin Microbiol,* 31, 2118-2123.

Lukinmaa, S., Nakari, U.-M., Eklund, M. & Siitonen, A. 2004. Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens. *APMIS,* 112, 908 - 929.

Lukinmaa, S., Nakari, U.-M., Liimatainen, A. & Siitonen, A. 2006. Genomic diversity within phage types of *Salmonella enterica* ssp. *enterica* serotypes Enteriditis and Typhimurium. *Foodborne Pathog Dis,* 3, 97-105.

Maguire, H., Pharoah, P., Walsh, B., Davison, C., Barrie, D., Threlfall, E. J. & Chamber, S. 2000. Hospital outbreak of *Salmonella virchow* possibly associated with a food handler. *J Hosp Infect,* 44, 261-266.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. & Spratt, B. G. 1998. Multilocus sequencing typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A,* 95, 3140 - 3145.

Majtánová, L. 1997. Occurrence of *Salmonella enterica* serotype Enteritidis phage types in the Slovak Republic. *Eur J Epidemiol,* 13, 243-245.

Malorny, B., Schroeter, A., Bunge, C., Hoog, B., Steinbeck, A. & Helmuth, R. 2001. Evaluation of molecular typing methods for *Salmonella enterica* serovar Typhimurium DT104 isolated in Germany from healthy pigs. *Vet Res,* 32, 119-129.

Malorny, B., Junker, E. & Helmuth, R. 2008. Multi-locus variable -number tandem repeat analysis for outbreak studies of *Salmonella enterica* serotype Enteriditis. *BMC Microbiol,* 8.

Maniloff, J. & Ackermann, H. W. 1998. Taxonomy of bacterial viruses: Establishment of tailed virus genera and the order *Caudovirales*. *Arch Virol,* 143, 2051-2063.

Martín, M. C., González-Hevia, M. A., Alvarez-Riesgo, J. A. & Mendoza, M. C. 2001. *Salmonella* serotype Virchow causing salmonellosis in a Spanish region. Characterization and survey of clones by DNA fingerprinting, phage typing and antimicrobial resistance. *Eur J Epidemiol,* 17, 31-40.

Maslow, J. N., Slutsky, A. M. & Arbeit, R. D. 1993. The application of pulsed-field gel electrophoresis to molecular epidemiology. *In:* Persing, D. H., Smith, T. F., Tenover, F. C. & White, T. J. (eds.) *Diagnostic Molecular Microbiology: Principles and Applications.* Washington, D. C.: American Society of Microbiology.

Maslow, J. N. & Mulligan, M. 1996. Epidemiologic typing systems. *Infect Control Hosp Epidemiol,* 17, 595-604.

McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, H., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R. & Wilson, R. K. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature,* 413, 852 - 856.

McDermott, P. F. 2006. Antimicrobial resistance in nontyphoidal *Salmonellae*. *In:* Aarestrup, F. M. (ed.) *Antimicrobial resistance in bacteria of animal origin.* Washington, D. C.: ASM Press.

Mellmann, A., Harmsen, D., Cummings, C. A., Zentz, E. B., Leopold, S. R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S. F., Henkhaus, J. K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P. M., Moore, R. L., Guenther, S., Rothberg, J. M. & Karch, H. 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS One,* 6, e22751.

Messer, R. D., Warnock, T. H., Heazlewood, R. J. & Hanna, J. N. 1997. *Salmonella* meningitis in children in far north Queensland. *J Paediatr Child Health,* 33, 535-538.

Miao, E. A., Scherer, C. A., Tsolis, R. M., Kingsley, R. A., Adams, L. G., Baumler, A. J. & Miller, S. I. 1999. *Salmonella typhimurium* leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Mol Microbiol,* 34, 850-864.

Mikasova, E., Drahovska, H., Szemes, T., Kuchta, T., Karpiskova, R., Sasik, M. & Turna, J. 2005. Characterization of *Salmonella enterica* serovar Typhimurium strains of veterinary origin by molecular typing methods. *Vet Microbiol,* 109, 113-120.

Miller, R. V. 1998. Bacterial gene swapping in nature. *Sci Am,* 278, 47-51.

Miller, R. V. & Day, M. J. 2008. Contribution of lysogeny, pseudolysogeny, and starvation to phage ecology. *In:* Abedon, S. T. (ed.) *Bacteriophage Ecology: Population growth, evolution, and impact of bacterial viruses.* The United Kingdom: Cambridge University Press.

Mirold, S., Rabsch, W., Stender, S., Ischape, H., Russmann, H., Igwa, E. & Hardt, W.-D. 1999. Isolation of a temperate bacteriophage encoding the Type III effector protein SopE from an epidemic *Salmonella typhimurium* strain. *Proc Natl Acad Sci U S A,* 96, 9845 - 9850.

Mirold, S., Rabsch, W., Tschäpe, H. & Hardt, W.-D. 2001. Transfer of the Salmonella type III effector *sopE* between unrelated phage families. *J Mol Biol,* 312, 7-16.

Mmolawa, P. T., Willmore, R., Thomas, C. J. & Heuzenroeder, M. W. 2002. Temperate phages in *Salmonella enterica* serovar Typhimurium: Implications for epidemiology. *Int J Med Microbiol,* 291, 633 - 644.

Mmolawa, P. T., Schmieger, H. & Heuzenroeder, M. W. 2003. Bacteriophage ST64B, a genetic mosaic of genes from diverse sources isolated from *Salmonella enterica* serovar Typhimurium DT 64. *J Bacteriol,* 185, 6481-6485.

Murchan, S., Kauffmann, M. E., Deplano, A., De Ryck, R., Struelens, M., Zinn, C. E., Fussing, V., Salmenlinna, S., vuopio-Varkila, J., El Solh, N., Cuny, C., Witte, W., Tassios, P., Legakis, N., van Leeuwen, W., van Belkum, A., Vindel, A., Laconcha, I., Garaizar, J., Haeggman, S., Olsson-Liljequist, B.,

Ransjo, U., Coombes, G. & Cookson, B. 2003. Harmonisation of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant staphylococcus aureus: A single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *J Clin Microbiol,* 41, 1574-1585.

Murray, C. 1994. *Salmonella* serovars and phage types in humans and animals in Australia 1987-1992. *Aust Vet J,* 71, 78-81.

Murray, P. R., Rosenthal, K. S. & Pfaller, M. A. 2005. *Enterobacteriaceae*. *Medical Microbiology.* 5th ed. Philadelphia: Elsevier Mosby.

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. & White, R. 1987. Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science,* 235, 1616-1622.

Nastasi, A., Mammina, C. & Aleo, A. 1994. Epidemic dissemination of *Salmonella enterica* spp. *enterica* serovar Bovismorbificans in southern Italy in the years 1989-1991. *Eur J Epidemiol,* 10, 81-84.

Noller, A. C., McEllistrem, M. C., Pacheco, A. G., Boxrud, D. J. & Harrison, L. H. 2003. Multilocus variable-number tandem repeat analysis distinguishes outbreak and sporadic *Escherichia coli* O157:H7 isolates. *J Clin Microbiol,* 41, 5389-5397.

Octavia, S. & Lan, R. 2007. Single-nucleotide-polymorphism typing and genetic relationships of *Salmonella enterica* serovar Typhi isolates. *J Clin Microbiol,* 45, 3795-3801.

Octavia, S. & Lan, R. 2009. Multiple-locus variable-number tandem-repeat analysis of *Salmonella enterica* serovar Typhi. *J Clin Microbiol,* 47, 2369-2376.

Oliver, D. M. & Bean, P. 1999. Minireview principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol,* 37, 1661 - 1669.

OzFoodNet 2006. Burden and causes of foodborne disease in Australia: Annual report of the OzFoodNet network, 2005. *Commun Dis Intell,* 30, 278 - 300.

OzFoodNet 2007. Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: Annual report of the OzfoodNet network, 2006. *Commun Dis Intell,* 31, 345-363.

OzFoodNet 2008. Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: Annual report of the OzfoodNet network, 2007. *Commun Dis Intell,* 32, 400-424.

Pang, S., Octavia, S., Reeves, P. R., Wang, Q., Gilbert, G. L., Sintchenko, V. & Lan, R. 2012. Genetic relationships of phage types and single nucleotide polymorphism typing of Salmonella enterica Serovar Typhimurium. *J Clin Microbiol,* 50, 727-34.

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

Pouhiniemi, R., Heiskanen, T. & Siitonen, A. 1997. Molecular epidemiology of two international sprout-borne *Salmonella* outbreaks. *J Clin Microbiol,* 35, 2487-2491.

Pourcel, C., André-Mazeaud, F., Neubauer, H., Ramisse, F. & Vergnaud, G. 2004. Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. *BMC Microbiol,* 4.

Price-Carter, M., Roy-Chowdhury, P., Pope, C. E., Paine, S., de Lisle, G. W., Collins, D. M., Nicol, C. & Carter, P. E. 2011. The evolution and distribution of phage ST160 within *Salmonella enterica* serotype Typhimurium. *Epidemiol Infect,* 139, 1262-1271.

Ptashne, M. 2004. *A genetic switch - Phage lambda revisited,* New York, Cold Spring Harbor Laboratory Press.

Public Health Agency of Canada. 2006. Laboratory Surveillance Data for Enteric Pathogens in Canada: annual summary 2006. The Canadian Science Centre for Hyman and Animal Health. Winnipeg, Manitoba, Canada.

Ramisse, V., Houssu, P., Hernandez, E., Denoeud, F., Hilaire, V., Lisanti, O., Ramisse, F., Cavallo, J.-D. & Vergnaud, G. 2004. Variable number of tandem repeats in *Salmonella enterica* subsp. enterica for typing purposes. *J Clin Microbiol,* 42, 5722 - 5730.

Ranade, K. & Poteete, A. R. 1993. Superinfection exclusion (sieB) genes of bacteriophages P22 and lambda. *J Bacteriol,* 175, 4712 - 4718.

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

Reeves, M. W., Evins, G. M., Heiba, A. A., Plikaytis, B. D. & Farmer III, J. J. 1989. Clonal nature of *Salmonella typhi* and its genetic relatedness to other *Salmonellae* as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. *J Clin Microbiol,* 27, 313 - 320.

Reissbrodt, R., Heier, H., Tschape, H., Kingsley, R. A. & Williams, P. H. 2000. Resuscitation by ferrioxamine E of stressed Salmonella enterica serovar typhimurium from soil and water microcosms. *Applied and Environmental Microbiology,* 66, 4128-30.

Ribot, E. M., Fair, M. A., Gautom, R., Cameron, D. N., Hunter, S. B., Swaminathan, B. & Barrett, T. J. 2006. Standardisation of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathog Dis,* 3, 59-67.

Ridley, A. M., Threlfall, E. J. & Rowe, B. 1998. Genotypic characterisation of *Salmonella enteritidis* phage types by plasmid analysis, ribotyping, and pulsed-field gel electrophoresis. *J Clin Microbiol,* 36, 2314 - 2321.

Riley, L. W. 2004. *Molecular epidemiology of infectious diseases: Principles and practices* Washington, D. C., ASM Press.

Rimhanen-Finne, R., Niskanen, T., Lienemann, T., Johansson, T., Sjoman, M., Korhonen, T., Guedes, S., Kuronen, H., Virtanen, M. J., Makinen, J., Jokinen, J., Siitonen, A. & Kuusi, M. 2011. A nationwide outbreak of *Salmonella* Bovismorbificans associated with sprouted alfalfa seeds in Finland, 2009. *Zoonoses Public Health,* 58, 589-596.

Roberts, J. W. & Devoret, R. 1983. Lysogenic induction. *In:* Hendrix, R. W., Roberts, J., Stahl, F. & Weisberg, R. (eds.) *Lambda II.* New York: Cold Spring Harbor Laboratory Press.

Ross, I. L. & Heuzenroeder, M. W. 2005a. Discrimination within phenotypically closely related definitive types of *Salmonella enterica* serovar Typhimurium by the multiple amplification of phage locus typing technique. *J Clin Microbiol,* 43, 1604 - 1611.

Ross, I. L. & Heuzenroeder, M. W. 2005b. Use of AFLP and PFGE to discriminate between *Salmonella enterica* serovar Typhimurium DT126 isolates from separate food-related outbreaks in Australia. *Epidemiol Infect,* 133, 635 - 644.

Ross, I. L. & Heuzenroeder, M. W. 2008. A comparison of three molecular typing methods for the discrimination of *Salmonella enterica* serovar Infantis. *FEMS Immunol Med Microbiol,* 53, 375-384.

Ross, I. L. & Heuzenroeder, M. W. 2009. A comparison of two PCR-based typing methods with pulsed-field gel electrophoresis in *Salmonella enterica* serovar Enteritidis. *Int J Med Microbiol,* 299, 410-420.

Ross, I. L., Parkinson, I. H. & Heuzenroeder, M. W. 2009. The use of MAPLT and MLVA analyses of phenotypically closely related isolates of Salmonella enterica serovar Typhimurium. *Int J Med Microbiol,* 299, 37-41

Ross, I. L., Davos, D. E., Mwanri, L., Raupach, J. & Heuzenroeder, M. W. 2011. MLVA and phage typing as complementary tools in the epidemiological investigation of *Salmonella enterica* serovar Typhimurium clusters. *Curr Microbiol,* 62, 1034-1038.

Ruiz, J., Nunez, M. L., Sempere, M. A., Diaz, J. & Gomez, J. 1995. Systemic infections in three infants due to a lactose-fermenting strain of *Salmonella virchow*. *Eur J Clin Microbiol Infect Dis,* 14, 454-456.

Rychlik, I., Hradecka, H. & Malcova, M. 2008. *Salmonella enterica* serovar Typhimurium typing by prophage-specific PCR. *Microbiology,* 154, 1384-1389.

Sabat, A., Malachowa, N., Miedzobrodzki, J. & Hryniewicz, W. 2006. Comparison of PCR-based methods for typing *Staphylococcus aureus*. *J Clin Microbiol,* 44, 3804-3807.

Sangal, V., Harbottle, H., Mazzoni, C. J., Helmuth, R., Guerra, B., Didelot, X., Paglietti, B., Rabsch, W., Brisse, S., Weill, F. X., Roumagnac, P. & Achtman, M. 2010. Evolution and population structure of *Salmonella enterica* serovar Newport. *J Bacteriol,* 192, 6465-6476.

Santos, S. B., Carvalho, C. M., Sillankorva, S., Nicolau, A., Ferreira, E. C. & Azeredo, J. 2009. The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. *BMC Microbiol,* 9, 148.

Schicklmaier, P. & Schmieger, H. 1995. Frequency of generalized transducing phages in natural isolates of the *Salmonella typhimurium* complex. *Appl Environ Microbiol,* 61, 1637-1640.

Schicklmaier, P., Moser, E., Wieland, T., Rabsch, W. & Schmieger, H. 1998. A comparative study on the frequency of prophagesamong natural isolates of *Salmonella* and *Escherichia coli* with emphasis on generalized transducers. *Antonie Van Leeuwenhoek,* 73, 49-54.

Schmieger, H. 1999. Molecular survey of the *Salmonella* phage typing system of Anderson. *J Bacteriol,* 181, 1630-1635.

Schmieger, H. & Schicklmaier, P. 1999. Transduction of multiple drug resistance of *Salmonella enterica* serovar typhimurium DT 104. *FEMS Microbiol Lett,* 170, 251-256.

Semple, A. B., Turner, G. C. & Lowry, D. M. 1968. Outbreak of food-poisoning caused by Salmonella virchow in spit-roasted chicken. *Br Med J,* 4, 801-803.

Seyfarth, A. M., Wong, D. M. A., Aarestrup, F. M. & Wegener, H. C. 2003. *The external quality assurance system of the WHO global Salmonella surveillance and laboratory support project* [Online]. Switzerland, Geneva: World Health Organisation. Available: http://www.who.int/salmsurv/activities/GSS_EQAS/salmsurveqas2003story/en/index.html [Accessed 30th September 2009].

Simon, M. N., Davis, R. W. & Davidson, N. 1971. Heteroduplexes of DNA molecules of lambdoid phages: Physical mapping of their base sequence relationships by electron microscopy. *In:* Hershey, A. D. (ed.) *The bacteriophage lambda.* Cold Spring Harbour, New York: Cold Spring Harbour Laboratory.

Singh, A., Georing, R. V., Simjee, S., Foley, S. L. & Zervos, M. J. 2006. Application of molecular techniques to the study of hospital infection *Clin Microbiol Rev,* 19, 512-530.

Stafford, R. J., McCall, B. J., Neill, A. S., Leon, D. S., Dorricott, G. J., Towner, C. D. & Micalizzi, G. J. 2002. A statewide outbreak of *Salmonella* bovismorbificans phage type 32 infection in Queensland. *Commun Dis Intell,* 26, 568-573.

Stanley, J., Jones, C. S. & Threlfall, E. J. 1991. Evolutionary Lines Among *Salmonella enteriditis* Phage Types are Identified by Insertion Sequence *IS* 200 Distribution. *FEMS Microbiol Lett,* 82, 83 - 90.

Stanley, T. L., Ellermeier, C. D. & Slauch, J. M. 2000. Tissue-specific gene expression identifies a gene in the lysogenic phage Gifsy-1 that affects *Salmonella enterica* serovar typhimurium survival in Peyer's patches. *J Bacteriol,* 182, 4406-13.

Struelens, M. 1998. Molecular epidemiologic typing systems for bacterial pathogens: Current issues and perspectives. *Mem Inst Oswaldo Cruz,* 93, 581 - 586.

Struelens, M., De Gheldre, Y. & Deplano, A. 1998. Comparative and library epidemiological typing systems: Outbreak investigations versus surveillance systems. *Infect Control Hosp Epidemiol,* 19, 565-569.

Sullivan, A. M., Ward, L. R., Rowe, B., Woolcock, J. B. & Cox, J. M. 1998. Phage types of Australian isolates of *Salmonella enterica* subsp. enterica serovar Virchow. *Lett Appl Microbiol,* 27, 216-218.

Susskind, M. M., Botstein, D. & Wright, A. 1974a. Superinfection exclusion by P22 prophage in lysogens of *Salmonella typhimurium*. III. Failure of superinfecting phage DNA to enter sieA+ lysogens. *Virology,* 62, 350 - 366.

Susskind, M. M., Wright, A. & Botstein, D. 1974b. Superinfection exclusion by P22 prophage in lysogens of *Salmonella typhimurium*. IV. Genetics and physiology of sieB exclusion. *Virology,* 62, 367 - 384.

Susskind, M. M. & Botstein, D. 1975. Mechanism of action of *Salmonella* phage P22 antirepressor. *J Mol Biol,* 98, 413 - 424.

Susskind, M. M. & Botstein, D. 1978. Molecular genetics of bacteriophage P22. *Microl Rev,* 42, 385 - 413.

Suttle, C. A. 2005. Viruses in the sea. *Nature,* 437, 356-361.

Tamada, Y., Nakaoka, Y., Nishimori, K., Doi, A., Kumaki, T., Uemura, N., Tanaka, K., Makino, S. I., Sameshima, T., Akiba, M., Nakazawa, M. & Uchida, I. 2001. Molecular typing and epidemiological study of *Salmonella enterica* serotype Typhimurium isolates from cattle by fluorescent amplified-fragment length polymorphism fingerprintiing and pulsed-field gel electrophoresis. *J Clin Microbiol,* 39 1057 - 1066.

Tan, S. 2010. *Temperate bacteriophages and the molecular epidemiology of antibiotic resistance in Salmonella enterica* PhD, University of Adelaide.

Taormina, P. J., Beuchat, L. R. & Slutsker, L. 1999. Infections associated with eating seed sprouts: An international concern. *Emerg Infect Dis,* 5, 626-634.

Telenius, H., Carter, N. P., Bebb, C. E., Nordenskjold, M., Ponder, B. A. & Tunnacliffe, A. 1992. Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics,* 13, 718-725.

Tenover, F. C., Arbeit, R. D., Archer, G., Bibble, J., Byrne, S., Georing, R., Hancock, G., Hebert, G., Hill, G. A., Hollis, R., Jarvis, R. W., Kreisworth, B., Eisner, W., Maslow, J., McDougal, L. K., Miller, J. M., Milligan, M. & Pfaller, M. A. 1994. Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *J Clin Microbiol,* 32, 407 - 415.

Tenover, F. C., Arbeit, R. D., Georing, R., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. *J Clin Microbiol,* 33, 2233-2239.

Tenover, F. C., Arbeit, R. D. & Georing, R. V. 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: A review for healthcare epidemiologists. *Infect Control Hosp Epidemiol,* 18, 426 - 439.

Thomson, N., Baker, S., Pickard, D., Fookes, M., Anjum, M., Hamlin, N., Wain, J., House, D., Bhutta, Z., Chan, K., Falkow, S., Parkhill, J., Woodward, M., Ivens, A. & Dougan, G. 2004. The role of prophage-like elements in the diversity of *Salmonella enterica* serovars. *J Mol Biol,* 339, 279-300.

Threlfall, E. J. & Frost, J. A. 1990. The identification, typing and fingerprinting of *Salmonella*: Laboratory aspects and epidemiological applications *J Appl Bacteriol,* 68, 5 - 16.

Threlfall, E. J., Hampton, M. D., Chart, H. & Rowe, B. 1994. Use of plasmid profile typing for surveillance of *Salmonella enteritidis* phage type 4 from humans, poultry and eggs. *Epidemiol Infect,* 112, 25 - 31.

Threlfall, E. J., Hampton, M. D., Ward, L. R. & Rowe, B. 1996. Application of pulsed-field gel electrophoresis to an international outbreak of *Salmonella agona*. *Emerg Infect Dis,* 2, 130-132.

Threlfall, E. J. 2002. Antimicrobial drug resistance in *Salmonella*: Problems and prospectives in food- and water-borne infections *FEMS Microbiol Rev,* 26, 141-148.

Tindall, B. J., Grimont, P. A. D., Garrity, G. M. & Euzéby, J. P. 2005. Nomenclature and taxonomy of the genus *Salmonella*. *Int J Syst Evol Microbiol,* 55, 521-524.

Todar, K. 2008. *Salmonella and Salmonellosis* [Online]. Available: http://www.textbookofbacteriology.net/salmonella.html [Accessed 17/09/2009].

Torre, E., Threlfall, E. J., Hampton, M. D., Ward, L. R., Gilbert, I. & Rowe, B. 1993. Characterization of *Salmonella virchow* phage types by plasmid profile and IS*200* distribution. *J Appl Bacteriol,* 75, 435-440.

Usera, M. A., Rodriguez, A., Echeita, A. & Cano, R. 1998. Multiple analysis of a foodborne outbreak caused by infant formula contaminated by an atypical *Salmonella virchow* strain. *Eur J Clin Microbiol Infect Dis,* 17, 551-555.

Uzzau, S., Brown, D. J., Wallis, T., Rubino, S., Leori, G., Bernard, S., Casadesus, J., Platt, D. J. & Olsen, J. E. 2000. Host adapted serotypes of *Salmonella enterica*. *Epidemiol Infect,* 125, 229-255.

van Belkum, A., Scherer, S., van Alphen, L. & verbrugh, H. 1998a. Short-sequence DNA repeats in prokaryotic genomes. *Microbiol Mol Biol Rev,* 62, 275-293.

van Belkum, A., van Leeuwen, W., Kauffmann, M. E., Cookson, B., Forey, F., Etienne, J., Georing, R., Tenover, F., Steward, C., O' Brien, F., Grubb, W., Tassios, P., Legakis, N., Morvan, A., Sohl, N. E., De Ryck, R., Struelens, M., Salmenlinna, A., Vuopio-Varkila, J., Kooistra, M., Talens, A., Witte, W. & verbrugh, H. 1998b. Assessment of resolution and intercenter reproducibility of results of genotyping *Staphylococcus aureus* by pulsed-field gel electrophoresis of SmaI macrorestriction fragments: A multicenter study. *J Clin Microbiol,* 36, 1653-1659.

van Belkum, A., Struelens, M., De Visser, A., Verbrugh, H. & Tibayrenc, M. 2001. Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. *Clin Microbiol Rev,* 14, 545 - 560.

van Belkum, A., Tassios, P. T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N. K., Fussing, V., Green, J., Feil, E., Gerner-Smidt, P., Brisse, S. & Struelens, M. 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin Microbiol Infect,* Suppl. 3, 1-46.

Vander Byl, C. & Kropinski, A. M. 2000. Sequence of the genome of *Salmonella* bacteriophage P22. *J Bacteriol,* 182, 6472 - 6481.

Voetsch, A. C., Van Gilder, T. J., Angulo, F. J., Farley, M. M., Shallow, S., Marcus, R., Cieslak, P. R., Deneen, V. C. & Tauxe, R. 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clin Infect Dis,* 38 (Supplement 3), S127-134.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res,* 23, 4407-4414.

Walker, J. M. & Rapley, R. 2000. *Molecular biology and biotechnology,* Cambridge, Royal Society of Chemistry.

Wang, Q., Kong, F., Jelfs, P. & Gilbert, G. L. 2008. Extended phage locus typing of Salmonella enterica serovar Typhimurium, using multiplex PCR-based reverse line blot hybridization. *J Med Microbiol,* 57, 827-38.

Ward, L. R., De Sa, J. D. & Rowe, B. 1987. A phage-typing scheme for *Salmonella* Enteriditis. *Epidemiol Infect,* 99, 291 - 294.

Weinberger, M., Andorn, N., Agmon, V., Cohen, D., Shohat, T. & Pitlik, S. D. 2004. Blood invasiveness of *Salmonella enterica* as a function of age and serotype. *Epidemiol Infect,* 132, 1023-1028.

Weinberger, M., Solnik-Isaac, H., Shachar, D., Reisfeld, A., Valinsky, L., Andorn, N., Agmon, V., Yishai, R., Bassal, R., Fraser, A., Yaron, S. & Cohen, D. 2006. *Salmonella enterica* serotype Virchow: epidemiology, resistance patterns and molecular characterisation of an invasive *Salmonella* serotype in Israel. *Clin Microbiol Infect,* 12, 999-1005.

Weller, T. M. A. 2000. Methicillin-resistant *Staphylococcus aureus* typing methods: Which should be the international standard? *J Hosp Infect,* 44, 160 - 172.

WHO. 2003. *Drug-resistant Salmonella* [Online]. Available: http://www.who.int/mediacentre/factsheets/fs139/en/print.html [Accessed 23/09/2009].

Willcocks, L. J., Morgan, D., Sufi, F., Ward, L. R. & Patrick, H. E. 1996. *Salmonella* Virchow PT 26 infection in England and Wales: A case control study investigating an increase in cases during 1994. *Epidemiol Infect,* 117, 35-41.

Witonski, D., Stefanova, R., Ranganathan, A., Schutze, G. E., Eisenach, K. D. & Cave, M. D. 2006. Variable-number tandem repeats that are useful in genotyping isolates of *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Newport. *J Clin Microbiol,* 44, 3849-3854.

Wood, M. W., Rosqvist, R., Mullan, P. B., Edwards, M. H. & Galyov, E. E. 1996. SopE, a secreted protein of *Salmonella dublin*, is translocated into the target eukaryotic cell via a sip-dependent mechanism and promotes bacterial entry. *Mol Microbiol,* 22, 327-338.

Worley, M. J., Ching, K. H. & Heffron, F. 2000. *Salmonella SsrB* activates a global regulon of horizontally acquired genes. *Mol Microbiol,* 36, 749-761.

Wright, A. 1971. Mechanism of conversion of the *Salmonella* O antigen by bacteriophage epsilon 34. *J Bacteriol,* 105, 927-936.

Yan, S. S., Pendrak, M. L., Abela-Ridder, B., Punderson, J. W., Fedorko, D. P. & Foley, S. L. 2003. An overview of *Salmonella* typing public health perspectives. *Clin Appl Immunol Rev,* 4, 189 - 204.

Yee, A. J., De Grandis, S. & Gyles, C. L. 1993. Mitomycin-induced synthesis of a Shiga-like toxin from Enteropathogenic *Escherichia coli* H:I:8 *Infect Immun,* 61, 4510 - 4513.

Yoshida, C., Franklin, K., Konczy, P., McQuiston, J. R., Fields, P. I., Nash, J. H., Taboada, E. N. & Rahn, K. 2007. Methodologies towards the development of an oligonucleotide microarray for determination of *Salmonella* serotypes. *J Microbiol Methods,* 70, 261-271.

Zar, J. H. 1984. *Biostatistical Analysis,* Englewood Cliffs, N. J., Prentice Hall.

Zhao, S., McDermott, P. F., Friedman, S., Abbott, J., Ayers, S., Glenn, A., Hall-Robinson, E., Hubert, S. K., Harbottle, H., Walker, R. D., Chiller, T. M. & White, D. G. 2006. Antimicrobial resistance and genetic relatedness among Salmonella from retail foods of animal origin: NARMS retail meat surveillance. *Foodborne Pathog Dis,* 3, 106-117.

Zhao, S., White, D. G., Friedman, S. L., Glenn, A., Blickenstaff, K., Ayers, S. L., Abbott, J. W., Hall-Robinson, E. & McDermott, P. F. 2008. Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006. *Appl Environ Microbiol,* 74, 6656-6662.

Zheng, J., Keys, C. E., Zhao, S., Meng, J. & Brown, E. W. 2007. Enhanced subtyping scheme for *Salmonella* Enteritidis. *Emerg Infect Dis,* 13, 1932-1935.

Appendix 1.1 The complete list of 62 *S.* Virchow isolates used in Chapter 3

Isolates	Time	Serovar	Source	Location	Phage type
V01	2007	*S.* Virchow	Chicken meat	QLD	19
V02	2007	*S.* Virchow	Human	QLD	33
V03	2007	*S.* Virchow	Human	NT	8
V04	2007	*S.* Virchow	Broiler litter	VIC	RDNC
V05	2007	*S.* Virchow	Broiler litter	VIC	25
V06	2007	*S.* Virchow	Chicken meat	QLD	Untypable
V07	2007	*S.* Virchow	Broiler litter	NSW	31
V08	2006	*S.* Virchow	Layer litter	NSW	17
V09	2006	*S.* Virchow	Broiler litter	VIC	34
V10	2006	*S.* Virchow	Chicken meat	QLD	25a
V11	2006	*S.* Virchow	Human	O/S	RDNC
V12	2006	*S.* Virchow	Human	O/S	RDNC
V14	2006	*S.* Virchow	Chicken meat	QLD	21
V15	2006	*S.* Virchow	Human	NSW	8
V16	2005	*S.* Virchow	Broiler litter	Not known	31
V17	2005	*S.* Virchow	Human	NSW	36 var 1
V18	2006	*S.* Virchow	Broiler litter	VIC	36 var 1
V19	2006	*S.* Virchow	Human	SA	11
V20	2005	*S.* Virchow	Chicken meat	Unknown	19
V21	2005	*S.* Virchow	Human	SA	17
V23	2005	*S.* Virchow	Chicken meat	NSW	34
V24	2006	*S.* Virchow	Chicken meat	QLD	8
V25	2006	*S.* Virchow	Human	NT	8
V26	2006	*S.* Virchow	Human Blood	NSW	8
V29	2006	*S.* Virchow	Broiler litter	NSW	8
V30	2006	*S.* Virchow	Layer litter	NSW	8
V34	2006	*S.* Virchow	Chicken meat	QLD	8
V37	2006	*S.* Virchow	Broiler litter	VIC	8
V39	2006	*S.* Virchow	Bovine	SA	8
V44	2005	*S.* Virchow	Human	NT	8
V47	2007	*S.* Virchow	Broiler litter	QLD	8
V48	2007	*S.* Virchow	Human Blood	SA	8
V49	2007	*S.* Virchow	Bovine	SA	8
V53	2007	*S.* Virchow	Human	NT	8
V56	2007	*S.* Virchow	Human	NT	8
V57	2007	*S.* Virchow	Broiler litter	NSW	8
V58	2007	*S.* Virchow	Broiler litter	NSW	8
V59	2007	*S.* Virchow	Human	O/S	8
V60	2007	*S.* Virchow	Human	NSW	8

Isolates	Time	Serovar	Source	Location	Phage type
V64	2008	*S.* Virchow	Human	SA	8
V68	2008	*S.* Virchow	Human Blood	NT	8
V69	2008	*S.* Virchow	Broiler litter	NSW	8
V70	2008	*S.* Virchow	Human Blood	NT	8
98-V01	1998	*S.* Virchow	Human	VIC	8
98-V02	1998	*S.* Virchow	Human (O/B)	VIC	8
98-V03	1998	*S.* Virchow	Human (O/B)	VIC	8
98-V04	1998	*S.* Virchow	Human (O/B)	VIC	8
98-V05	1998	*S.* Virchow	Human (O/B)	VIC	8
98-V06	1998	*S.* Virchow	Human (O/B)	VIC	8
98-V07	1998	*S.* Virchow	Human	VIC	8
98-V08	1998	*S.* Virchow	Human (O/B)	SA	8
98-V09	1998	*S.* Virchow	Human (O/B)	SA	8
98-V10	1998	*S.* Virchow	Human (O/B)	SA	8
98-V11	1998	*S.* Virchow	Human (O/B)	SA	8
98-V12	1998	*S.* Virchow	Human (O/B)	SA	8
98-V13	1998	*S.* Virchow	Sun-dried tomato (O/B)	SA	8
98-V14	1998	*S.* Virchow	Human	SA	8
98-V15	1998	*S.* Virchow	Human	NSW	8
98-V16	1998	*S.* Virchow	Beef meat	QLD	8
98-V17	1997	*S.* Virchow	Human	QLD	8
98-V18	1996	*S.* Virchow	Chicken meat	QLD	8
98-V19	1997	*S.* Virchow	Human	SA	8

O/B = isolates associated in the *S.* Virchow PT 8 outbreak in 1998 (Bennett *et al.,* 2003)

Appendix 1.2 The complete list of 73 *S.* Bovismorbificans isolates used in Chapter 4

Isolates	Time	Serovar	Source	Location	Phage type
B01	2007	*S.* Bovismorbicans	Human	QLD	RDNC
B02	2007	*S.* Bovismorbicans	Human	NSW	40
B03	2007	*S.* Bovismorbicans	Human	NSW	11
B04	2007	*S.* Bovismorbicans	Broiler litter	QLD	32
B05	2007	*S.* Bovismorbicans	Bovine	SA	Untypable
B06	2006	*S.* Bovismorbicans	Pork meat	WA	Untypable
B07	2006	*S.* Bovismorbicans	Bovine	WA	Untypable
B08	2006	*S.* Bovismorbicans	Chicken meat	NSW	Untypable
B09	2006	*S.* Bovismorbicans	Canine	VIC	Untypable
B10	2006	*S.* Bovismorbicans	Canine	SA	14
B11	2006	*S.* Bovismorbicans	Chicken meat	VIC	14
B12	2006	*S.* Bovismorbicans	Human	WA	24
B13	2006	*S.* Bovismorbicans	Bovine	TAS	32
B14	2006	*S.* Bovismorbicans	Chicken meat	NSW	35
B15	2005	*S.* Bovismorbicans	Human	SA	RDNC
B16	2005	*S.* Bovismorbicans	Human	NSW	35
B17	2005	*S.* Bovismorbicans	Bovine	VIC	RDNC
B18	2005	*S.* Bovismorbicans	Broiler litter	VIC	Untypable
B19	2005	*S.* Bovismorbicans	Bovine	QLD	35
B20	2005	*S.* Bovismorbicans	Human	NSW	16
B21	2005	*S.* Bovismorbicans	Bovine	SA	24
B22	2005	*S.* Bovismorbicans	Human	NT	13
B23	2005	*S.* Bovismorbicans	Bovine	SA	38
B24	2005	*S.* Bovismorbicans	Bovine	VIC	39
B25	2006	*S.* Bovismorbicans	Human	SA	24
B26	2006	*S.* Bovismorbicans	Bovine	WA	24
B27	2006	*S.* Bovismorbicans	Human	NSW	24
B28	2006	*S.* Bovismorbicans	Canine	QLD	24
B29	2007	*S.* Bovismorbicans	Bovine	SA	24
B30	2006	*S.* Bovismorbicans	Human	NSW	14
B31	2006	*S.* Bovismorbicans	Layer litter	NSW	14
B32	2006	*S.* Bovismorbicans	Chicken meat	QLD	14
B33	2007	*S.* Bovismorbicans	Human	QLD	14
B34	2005	*S.* Bovismorbicans	Egg Pulp	SA	13
B35	2006	*S.* Bovismorbicans	Bovine	NSW	13
B36	2007	*S.* Bovismorbicans	Human	SA	13
B37	2007	*S.* Bovismorbicans	Chicken meat	NSW	13
B38	2007	*S.* Bovismorbicans	Chicken meat	NSW	13
B39	2008	*S.* Bovismorbicans	Human Blood	VIC	24
B40	2008	*S.* Bovismorbicans	Human	SA	24

Isolates	Time	Serovar	Source	Location	Phage type
B41	2008	*S.* Bovismorbicans	Human	NSW	24
B42	2008	*S.* Bovismorbicans	Egg Pulp	SA	24
B43	2008	*S.* Bovismorbicans	Bovine	NSW	24
B44	2007	*S.* Bovismorbicans	Human	SA	24
B45	2007	*S.* Bovismorbicans	Human	WA	24
B46	2008	*S.* Bovismorbicans	Bovine	WA	24
B47	2007	*S.* Bovismorbicans	Sheep	SA	24
B48	2007	*S.* Bovismorbicans	Sheep	SA	24
B49	2007	*S.* Bovismorbicans	Human	WA	24
B50	2007	*S.* Bovismorbicans	Human	WA	24
B51	2007	*S.* Bovismorbicans	Beef meat	VIC	24
B52	2007	*S.* Bovismorbicans	Human	VIC	24
B53	2008	*S.* Bovismorbicans	Human	TAS	14
B54	2008	*S.* Bovismorbicans	Chicken meat	NSW	14
B55	2007	*S.* Bovismorbicans	Human	QLD	14
B56	2008	*S.* Bovismorbicans	Canine	VIC	13
B57	2008	*S.* Bovismorbicans	Human	NT	13
B58	2006	*S.* Bovismorbicans	Feed sheep	SA	24
B59	2006	*S.* Bovismorbicans	Human	VIC	24
B60	2006	*S.* Bovismorbicans	Human	WA	24
B61	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B62	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B63	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B64	2006	*S.* Bovismorbicans	Salami (O/B)	VIC	11
B65	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B66	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B67	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B68	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B69	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B70	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B71	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B72	2006	*S.* Bovismorbicans	Human (O/B)	VIC	11
B73	2006	*S.* Bovismorbicans	Salami (O/B)	VIC	11

O/B = isolates associated in the *S.* Bovismorbificans outbreak in 2006 (ASRC Annual Report 2006)

Appendix 1.3 The complete list of 64 *S.* Heidelberg isolates used in Chapter 5

Isolates	Time	Serovar	Source	Location	Phage type
H01	2008	*S.* Heidelberg	Human	VIC	1
H02	2008	*S.* Heidelberg	Human Blood	VIC	1
H03	2008	*S.* Heidelberg	Human	QLD	1
H04	2008	*S.* Heidelberg	Goat meat	VIC	1
H05	2008	*S.* Heidelberg	Human	NSW	1
H06	2008	*S.* Heidelberg	Human	QLD	1
H07	2008	*S.* Heidelberg	Goat meat	QLD	1
H08	2008	*S.* Heidelberg	Human	VIC	1
H09	2007	*S.* Heidelberg	Human Urine	QLD	1
H10	2007	*S.* Heidelberg	Human	QLD	1
H11	2007	*S.* Heidelberg	Human	WA	1
H12	2007	*S.* Heidelberg	Human	O/S	1
H13	2007	*S.* Heidelberg	Human	NSW	1
H14	2006	*S.* Heidelberg	Goat meat	SA	1
H15	2006	*S.* Heidelberg	Human	SA	1
H16	2006	*S.* Heidelberg	Human	VIC	1
H17	2006	*S.* Heidelberg	Soy flour	NSW	1
H18	2008	*S.* Heidelberg	Human	NSW	2
H19	2007	*S.* Heidelberg	Human Urine	NSW	2
H20	2007	*S.* Heidelberg	Human	VIC	2
H21	2007	*S.* Heidelberg	Porcine intestine	QLD	2
H22	2007	*S.* Heidelberg	Human	QLD	2
H23	2008	*S.* Heidelberg	Human	NSW	3
H24	2007	*S.* Heidelberg	Human	SA	3
H25	2007	*S.* Heidelberg	Human Blood	O/S	3
H26	2006	*S.* Heidelberg	Human	SA	3
H27	2007	*S.* Heidelberg	Human	QLD	4
H28	2006	*S.* Heidelberg	Human Blood	QLD	4
H29	2007	*S.* Heidelberg	Human	NSW	5
H30	2007	*S.* Heidelberg	Human	SA	7
H31	2008	*S.* Heidelberg	Human	NSW	8
H32	2008	*S.* Heidelberg	Feed Chicken	VIC	8
H33	2008	*S.* Heidelberg	Goat meat	QLD	8
H35	2007	*S.* Heidelberg	Human	SA	8
H36	2007	*S.* Heidelberg	Human	SA	8
H37	2006	*S.* Heidelberg	Goat meat	SA	8
H38	2006	*S.* Heidelberg	Human	VIC	8
H39	2007	*S.* Heidelberg	Human	NSW	9
H40	2006	*S.* Heidelberg	Human	WA	9
H41	2006	*S.* Heidelberg	Human	O/S	23

Isolates	Time	Serovar	Source	Location	Phage type
H42	2006	*S.* Heidelberg	Human	WA	23
H43	2006	*S.* Heidelberg	Human	O/S	25
H44	2007	*S.* Heidelberg	Human	QLD	26
H45	2007	*S.* Heidelberg	Human	O/S	27
H46	2006	*S.* Heidelberg	Human	VIC	6a
H47	2008	*S.* Heidelberg	Human	O/S	RDNC
H48	2008	*S.* Heidelberg	Goat meat	QLD	Untypable
H49	2006	*S.* Heidelberg	Pork meat	QLD	Untypable
H50	2007	*S.* Heidelberg	Human	VIC	1
H51	2007	*S.* Heidelberg	Human	NSW	1
H52	2007	*S.* Heidelberg	Human	QLD	4
H53	2007	*S.* Heidelberg	Human	QLD	4
H54	2007	*S.* Heidelberg	Human Blood	NSW	1
H55	2007	*S.* Heidelberg	Human	NSW	2
H56	2006	*S.* Heidelberg	Goat meat	VIC	2
H57	2006	*S.* Heidelberg	Human	NSW	4
H58	2006	*S.* Heidelberg	Human	NSW	1
H59	2006	*S.* Heidelberg	Human	NSW	2
H60	2007	*S.* Heidelberg	Human	QLD	4
H61	2008	*S.* Heidelberg	Meat beef	NSW	1
H62	2009	*S.* Heidelberg	Goat meat	QLD	1
H63	2009	*S.* Heidelberg	Goat meat	QLD	1
H64	2008	*S.* Heidelberg	Canine	NT	1
H65	2008	*S.* Heidelberg	Goat meat	QLD	1

Appendix 2.1 The complete positive MAPLT reactions for the 43 *S.* Virchow isolates

Isolates	ninBF1R1	SB04	P186P	P186BP	P186G	P186cII	Fels2late	Fels2G	Fels2LysB	Fels2Ter	Fels2cII	Fels2c1	Fels2 Int	Isolates
V01	-	-	-	-	-	-	-	-	-	-	-	-	-	V01
V02	-	-	+	+	-	-	-	-	-	-	-	-	-	V02
V03	-	-	-	-	-	-	-	-	-	-	-	-	-	V03
V04	-	-	+	+	+	-	-	-	-	-	-	-	-	V04
V05	-	-	-	-	-	+	+	+	+	+	+	+	+	V05
V06	-	-	-	-	-	-	-	-	-	-	-	-	-	V06
V07	-	-	-	-	+	-	+	+	+	+	+	+	+	V07
V08	-	-	-	-	-	+	+	+	+	+	+	+	+	V08
V09	-	-	+	+	+	+	-	-	-	-	-	-	-	V09
V10	-	-	+	+	+	-	-	-	-	-	-	-	-	V10
V11	-	-	-	-	-	+	+	+	-	+	+	-	+	V11
V12	-	-	-	-	-	+	+	+	-	+	+	-	+	V12
V14	+	-	-	-	-	-	-	-	-	-	-	-	-	V14
V15	-	-	-	-	-	-	-	-	-	-	-	-	-	V15
V16	-	-	+	+	-	-	-	-	-	-	-	-	-	V16
V17	-	-	+	+	+	+	-	-	-	-	-	-	-	V17
V18	-	-	-	-	-	-	-	-	-	-	-	-	-	V18
V19	-	-	+	+	+	+	-	-	-	-	-	-	-	V19
V20	-	-	+	+	+	+	-	-	-	-	-	-	-	V20
V21	-	-	+	+	+	+	-	-	-	-	-	-	-	V21
V23	-	-	+	+	+	+	-	-	-	-	-	-	-	V23
V24	-	-	+	+	-	-	-	-	-	-	-	-	-	V24
V25	-	-	-	-	-	-	-	-	-	-	-	-	-	V25
V26	-	-	-	-	-	-	-	-	-	-	-	-	-	V26
V29	-	-	+	+	-	-	-	-	-	-	-	-	-	V29
V30	-	-	-	-	-	+	+	+	+	+	+	+	+	V30
V34	-	-	-	-	-	-	-	-	-	-	-	-	-	V34
V37	-	-	-	-	-	-	-	-	-	-	-	-	-	V37
V39	-	-	+	+	+	+	-	-	-	-	-	-	-	V39
V44	-	-	-	-	-	-	-	-	-	-	-	-	-	V44
V47	-	-	-	-	-	-	-	-	-	-	-	-	-	V47
V48	-	-	-	-	-	-	-	-	-	-	-	-	-	V48
V49	-	-	+	+	+	+	-	-	-	-	-	-	-	V49
V53	-	-	-	-	-	-	-	-	-	-	-	-	-	V53
V56	-	-	-	-	-	-	-	-	-	-	-	-	-	V56
V57	-	-	-	-	-	+	+	+	+	+	+	+	+	V57
V58	-	+	-	-	-	-	-	-	-	-	-	-	-	V58
V59	+	-	-	-	-	-	-	-	-	-	-	-	-	V59
V60	-	-	-	-	-	-	-	-	-	-	-	-	-	V60
V64	-	-	-	-	-	-	-	-	-	-	-	-	-	V64
V68	-	-	-	-	-	-	-	-	-	-	-	-	-	V68
V69	-	-	-	-	-	-	-	-	-	-	-	-	-	V69
V70	-	-	-	-	-	-	-	-	-	-	-	-	-	V70

Isolates	Gifsy1A	Gifsy2Cap	GifsyninG	GifsyH	SopE	ES18 PCP	SfV05	SfV11	SfV16	SfV26	SfV34	P7sit	P1sit	DOP13.7	Isolates
V01	-	-	+	+	+	-	-	-	-	-	-	+	-	-	V01
V02	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V02
V03	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V03
V04	-	-	+	-	-	-	-	-	-	-	-	-	-	-	V04
V05	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V05
V06	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V06
V07	+	-	+	-	+	-	-	-	-	-	-	+	+	-	V07
V08	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V08
V09	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V09
V10	+	-	+	+	+	-	-	-	-	-	-	-	-	+	V10
V11	-	+	+	+	+	-	-	+	-	-	-	-	-	-	V11
V12	-	+	+	+	+	-	-	+	-	-	-	-	-	-	V12
V14	+	-	+	+	+	+	-	-	-	-	-	-	-	-	V14
V15	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V15
V16	+	-	+	+	+	-	-	-	-	-	-	-	-	+	V16
V17	+	-	+	-	-	-	-	-	-	-	-	+	-	-	V17
V18	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V18
V19	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V19
V20	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V20
V21	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V21
V23	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V23
V24	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V24
V25	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V25
V26	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V26
V29	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V29
V30	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V30
V34	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V34
V37	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V37
V39	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V39
V44	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V44
V47	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V47
V48	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V48
V49	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V49
V53	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V53
V56	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V56
V57	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V57
V58	+	-	+	+	+	-	+	+	+	+	+	-	-	-	V58
V59	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V59
V60	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V60
V64	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V64
V68	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V68
V69	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V69
V70	+	-	+	+	+	-	-	-	-	-	-	-	-	-	V70

Appendix 2.2 The complete positive MAPLT reactions for the 60 *S.* Bovismorbificans isolates

Isolates	PTc1	Tc2	ninB	SB04	SB06	SB21	SB28	BIM1	P186G	P186BP	P186cII	Isolates
B01	-	+	+	-	-	-	-	-	+	+	-	B01
B02	-	-	-	-	-	-	-	-	-	+	+	B02
B03	-	-	-	-	-	-	-	-	-	+	-	B03
B04	+	+	+	-	-	-	-	-	-	-	-	B04
B05	+	+	+	-	-	-	-	-	-	-	+	B05
B06	+	+	+	-	-	-	-	-	-	+	+	B06
B07	+	+	+	-	-	-	-	-	+	+	+	B07
B08	-	-	-	-	-	-	-	-	-	-	-	B08
B09	+	+	+	+	+	+	+	+	-	-	-	B09
B10	-	-	-	-	-	-	-	-	-	-	-	B10
B11	-	-	-	-	-	-	-	-	-	-	-	B11
B12	+	+	+	-	-	-	-	-	+	+	+	B12
B13	+	+	+	-	-	-	-	-	-	-	-	B13
B14	-	-	-	-	-	-	-	-	-	-	-	B14
B15	-	-	-	+	+	+	+	+	-	-	-	B15
B16	-	-	-	+	-	-	-	-	-	-	-	B16
B17	-	-	+	-	-	-	-	-	-	-	-	B17
B18	-	-	-	-	-	-	-	-	-	-	-	B18
B19	-	-	-	-	-	-	-	-	-	-	-	B19
B20	-	-	-	-	-	-	-	-	-	+	+	B20
B21	+	+	+	-	-	-	-	-	-	-	-	B21
B22	-	-	-	-	-	-	-	-	+	+	-	B22
B23	-	-	+	-	-	-	-	-	-	-	-	B23
B24	-	-	-	-	-	-	-	-	-	-	+	B24
B25	+	+	+	-	-	-	-	-	-	-	+	B25
B26	+	+	+	-	-	-	-	-	+	+	+	B26
B27	+	+	+	-	-	-	-	-	-	-	+	B27
B28	+	+	+	-	-	-	-	-	-	-	+	B28
B29	+	+	+	-	-	-	-	-	-	-	+	B29
B30	-	-	-	-	-	-	-	-	-	-	-	B30

Isolates	PTc1	Tc2	ninB	SB04	SB06	SB21	SB28	BIM1	P186G	P186BP	P186cII	Isolates
B31	-	-	-	-	-	-	-	-	+	+	-	B31
B32	-	-	-	-	-	-	-	-	-	-	-	B32
B33	-	-	-	-	-	-	-	-	-	-	-	B33
B34	-	-	-	-	-	-	-	-	-	-	-	B34
B35	-	-	-	-	-	-	-	-	-	-	-	B35
B36	-	-	-	-	-	-	-	-	-	-	-	B36
B37	-	-	-	-	-	-	-	-	-	-	-	B37
B38	-	-	-	-	-	-	-	-	-	-	-	B38
B39	+	+	+	+	+	+	+	+	-	-	-	B39
B40	+	+	+	-	-	-	-	-	-	-	+	B40
B41	+	+	+	-	-	-	-	-	+	+	+	B41
B42	+	+	+	-	-	-	-	-	-	-	+	B42
B43	+	+	+	-	-	-	-	-	-	-	-	B43
B44	-	-	-	-	-	-	-	-	-	-	-	B44
B45	-	-	-	-	-	-	-	-	+	+	-	B45
B46	+	+	+	-	-	-	-	-	+	+	+	B46
B47	+	+	+	-	-	-	-	-	-	-	+	B47
B48	+	+	+	-	-	-	-	-	-	+	+	B48
B49	+	+	+	-	-	-	-	-	-	+	+	B49
B50	-	-	-	-	-	-	-	-	+	+	-	B50
B51	+	+	+	-	-	-	-	-	-	-	-	B51
B52	+	+	+	-	-	-	-	-	-	-	-	B52
B53	-	-	-	-	-	-	-	-	+	+	-	B53
B54	-	-	-	-	-	-	-	-	-	-	-	B54
B55	-	+	+	-	-	-	-	-	+	+	-	B55
B56	-	-	-	-	-	-	-	-	+	+	+	B56
B57	-	-	-	-	-	-	-	-	+	+	-	B57
B58	+	+	+	-	-	-	-	-	-	-	+	B58
B59	-	-	-	-	-	-	-	-	-	+	+	B59
B60	+	+	+	-	-	-	-	-	+	+	+	B60

Isolates	Fels2late	Fels2G	Fels2lysB	Fels2int	Fels2c1	Gifsy1gogB	Gifsy1A	Gifsy2int	Isolates
B01	-	-	-	-	-	-	-	+	B01
B02	-	-	-	-	-	-	-	+	B02
B03	-	-	-	-	-	+	+	+	B03
B04	-	+	-	+	-	-	-	+	B04
B05	+	+	+	+	+	+	+	+	B05
B06	+	+	+	+	+	+	+	+	B06
B07	+	+	+	+	+	+	+	+	B07
B08	-	-	-	-	-	-	-	+	B08
B09	-	+	-	+	-	-	-	+	B09
B10	-	-	-	-	-	-	-	+	B10
B11	-	-	-	-	-	-	-	+	B11
B12	+	+	+	+	+	+	+	+	B12
B13	-	+	-	+	-	-	-	+	B13
B14	-	-	-	-	-	-	-	+	B14
B15	-	+	-	+	-	-	-	+	B15
B16	-	+	-	+	-	-	-	+	B16
B17	-	+	-	+	-	-	-	+	B17
B18	-	+	-	+	-	-	-	+	B18
B19	-	+	-	+	-	-	-	+	B19
B20	+	+	+	+	+	+	+	+	B20
B21	-	+	-	+	-	-	-	+	B21
B22	-	+	-	+	-	-	-	+	B22
B23	-	+	-	+	-	-	-	+	B23
B24	+	+	+	+	+	+	+	+	B24
B25	+	+	+	+	+	+	+	+	B25
B26	+	+	+	+	+	+	+	+	B26
B27	+	+	+	+	+	+	+	+	B27
B28	+	+	+	+	+	+	+	+	B28
B29	+	+	+	+	+	+	+	+	B29
B30	-	-	-	-	-	-	-	+	B30

Isolates	Fels2late	Fels2G	Fels2lysB	Fels2int	Fels2c1	Gifsy1gogB	Gifsy1A	Gifsy2int	Isolates
B31	-	-	-	-	-	-	-	+	B31
B32	-	-	-	-	-	-	-	+	B32
B33	-	-	-	-	-	-	-	+	B33
B34	-	+	-	+	-	-	-	+	B34
B35	-	+	-	+	-	-	-	+	B35
B36	-	+	-	+	-	-	-	+	B36
B37	-	+	-	+	-	-	-	+	B37
B38	-	+	-	+	-	-	-	+	B38
B39	-	+	-	+	-	-	-	+	B39
B40	+	+	+	+	+	+	+	+	B40
B41	+	+	+	+	+	+	+	+	B41
B42	+	+	+	+	+	+	+	+	B42
B43	-	-	-	-	-	-	-	+	B43
B44	-	+	-	+	-	-	-	+	B44
B45	-	+	-	+	-	-	-	+	B45
B46	+	+	+	+	+	+	+	+	B46
B47	+	+	+	+	+	+	+	+	B47
B48	+	+	+	+	+	+	+	+	B48
B49	+	+	+	+	+	+	+	+	B49
B50	-	+	-	+	-	-	-	+	B50
B51	-	-	-	-	-	-	-	+	B51
B52	-	+	-	+	-	-	-	+	B52
B53	-	-	-	-	-	-	-	+	B53
B54	-	-	-	-	-	-	-	+	B54
B55	-	-	-	-	-	-	-	-	B55
B56	-	+	-	+	-	-	-	+	B56
B57	-	+	-	+	-	-	-	+	B57
B58	+	+	+	+	+	+	+	+	B58
B59	+	+	+	+	+	+	+	+	B59
B60	+	+	+	+	+	+	+	+	B60

Isolates	Gifsy2Cap	GifsyninG	GifsyRec	GifsyH	SopE	SL254-tail	M13-222	Isolates
B01	-	-	-	-	-	+	+	B01
B02	-	+	+	+	-	-	-	B02
B03	+	+	-	+	-	-	-	B03
B04	-	-	-	+	-	-	-	B04
B05	-	+	-	+	-	+	-	B05
B06	-	+	-	+	-	+	-	B06
B07	-	+	-	+	-	+	-	B07
B08	-	+	-	+	+	+	+	B08
B09	-	-	-	+	-	-	-	B09
B10	-	+	-	+	+	-	-	B10
B11	-	+	-	+	+	+	+	B11
B12	-	+	-	+	-	+	-	B12
B13	-	-	-	+	-	-	-	B13
B14	-	+	-	+	+	+	+	B14
B15	-	-	-	+	-	-	-	B15
B16	-	-	-	+	-	-	-	B16
B17	-	-	-	+	-	-	-	B17
B18	-	-	-	+	-	-	-	B18
B19	-	-	-	+	-	-	-	B19
B20	-	+	-	+	-	-	-	B20
B21	-	-	-	+	-	-	-	B21
B22	-	-	-	+	-	-	-	B22
B23	-	-	-	+	-	-	-	B23
B24	-	+	-	+	-	+	-	B24
B25	-	+	-	+	-	+	-	B25
B26	-	+	-	+	-	+	-	B26
B27	-	+	-	+	-	+	-	B27
B28	-	+	-	+	-	+	-	B28
B29	-	+	-	+	-	+	-	B29
B30	-	+	-	+	+	+	+	B30

Isolates	Gifsy2Cap	GifsyninG	GifsyRec	GifsyH	SopE	SL254-tail	M13-222	Isolates
B31	-	+	-	+	+	+	+	B31
B32	-	+	-	+	-	+	+	B32
B33	-	+	-	+	-	+	+	B33
B34	-	-	-	+	-	-	-	B34
B35	-	-	-	+	-	-	-	B35
B36	-	-	-	+	-	-	-	B36
B37	-	-	-	+	-	-	-	B37
B38	-	-	-	+	-	-	-	B38
B39	-	-	-	+	-	-	-	B39
B40	-	+	-	+	-	+	-	B40
B41	-	+	-	+	-	+	-	B41
B42	-	+	-	+	-	+	-	B42
B43	-	-	-	+	-	+	-	B43
B44	-	-	-	+	-	-	-	B44
B45	-	-	-	+	-	-	-	B45
B46	-	+	-	+	-	+	-	B46
B47	-	+	-	+	-	+	-	B47
B48	-	+	-	+	-	+	-	B48
B49	-	+	-	+	-	-	-	B49
B50	-	-	-	+	-	-	-	B50
B51	-	-	-	+	-	+	-	B51
B52	-	-	-	+	-	-	-	B52
B53	-	+	-	+	+	+	+	B53
B54	-	+	-	+	-	+	+	B54
B55	-	-	-	-	-	+	+	B55
B56	-	-	-	+	-	-	-	B56
B57	-	-	-	+	-	-	-	B57
B58	-	+	-	+	-	+	-	B58
B59	-	+	-	+	-	-	-	B59
B60	-	+	-	+	-	+	-	B60

Appendix 2.3 The complete positive MAPLT reactions for the 64 *S.* Heidelberg isolates

Isolates	PTcI	PTg17	PTgtrC	Pint	sieB	ninB	SB06	SB21	SB28	BIM1	Isolates
H01	-	-	-	-	-	-	-	-	-	-	H01
H02	-	-	-	-	-	-	-	-	-	-	H02
H03	-	-	-	-	-	-	-	-	-	-	H03
H04	-	-	-	-	-	-	-	-	-	-	H04
H05	-	-	-	-	-	-	-	-	-	-	H05
H06	-	-	-	-	-	-	-	-	-	-	H06
H07	-	-	-	-	-	-	-	-	-	-	H07
H08	-	-	-	-	-	-	-	-	-	-	H08
H09	-	-	-	-	-	-	-	-	-	-	H09
H10	-	-	-	-	-	-	-	-	-	-	H10
H11	-	-	-	-		-	-	-	-	-	H11
H12	-	-	-	-	-	-	-	-	-	-	H12
H13	-	-	-	-	-	-	-	-	-	-	H13
H14	-	-	-	-	-	-	-	-	-	-	H14
H15	-	-	-	-	-	-	-	-	-	+	H15
H16	-	-	-	-	-	-	-	-	-	-	H16
H17	-	-	-	-	-	-	-	-	-	-	H17
H18	+	+	+	-	-	-	-	-	-	-	H18
H19	-	-	-	-	-	-	-	-	-	-	H19
H20	+	+	+	-	-	-	-	-	-	-	H20
H21	+	+	+	+	-	-	-	+	-	+	H21
H22	-	-	-	-	-	-	-	-	-	-	H22
H23	-	-	-	-	-	+	-	-	-	-	H23
H24	-	-	-	-	-	-	-	-	-	-	H24
H25	-	-	-	-	-	-	-	-	-	-	H25
H26	-	-	-	-	-	-	-	-	-	-	H26
H27	-	+	+	-	-	+	-	-	-	-	H27
H28	-	+	+	-	-	+	-	-	-	-	H28
H29	+	+	+	+	-	-	+	+	-	-	H29
H30	-	-	-	-	-	-	-	-	-	-	H30
H31	-	-	-	-	-	-	-	-	-	-	H31
H32	-	-	-	-	-	-	-	-	-	-	H32
H33	-	-	-	-	-	-	-	-	-	-	H33

Isolates	PTcI	PTg17	PTgtrC	Pint	sieB	ninB	SB06	SB21	SB28	BIM1	Isolates
H35	-	-	-	-	-	-	-	-	-	-	H35
H36	-	-	-	-	-	-	-	-	-	-	H36
H37	-	-	-	-	-	-	-	-	-	-	H37
H38	-	-	-	-	-	-	-	-	-	-	H38
H39	+	-	+	+	-	-	-	+	+	-	H39
H40	+	-	+	-	-	-	-	-	-	-	H40
H41	-	-	-	+	-	-	+	+	+	-	H41
H42	-	-	-	-	-	-	-	-	-	-	H42
H43	-	-	-	-	-	-	-	-	-	+	H43
H44	-	+	+	-	-	-	-	-	-	-	H44
H45	-	-	-	+	-	-	-	+	+	-	H45
H46	-	-	-	-	-	-	-	-	-	+	H46
H47	+	-	+	-	+	-	-	-	-	-	H47
H48	-	-	-	-	-	-	-	-	-	-	H48
H49	+	+	+	-	-	-	-	-	-	-	H49
H50	+	+	+	-	-	-	-	-	-	-	H50
H51	-	-	-	-	-	-	-	-	-	-	H51
H52	-	+	+	-	-	+	-	-	-	-	H52
H53	-	+	+	-	-	+	-	-	-	-	H53
H54	-	+	+	-	-	-	-	-	-	-	H54
H55	+	+	+	-	-	-	-	-	-	-	H55
H56	-	-	-	-	-	-	-	-	-	-	H56
H57	-	+	+	-	-	+	-	-	-	-	H57
H58	-	-	-	-	-	-	-	-	-	-	H58
H59	+	+	+	-	-	-	-	-	-	-	H59
H60	-	+	+	-	-	+	-	-	-	-	H60
H61	-	-	-	-	-	-	-	-	-	-	H61
H62	-	-	-	-	-	-	-	-	-	-	H62
H63	-	-	-	-	-	-	-	-	-	-	H63
H64	-	-	-	-	-	-	-	-	-	-	H64
H65	-	-	-	-	-	-	-	-	-	-	H65

Isolates	Fels2G	Fels2lysB	Fels2ter	Fels2port	Gifsy1A	Gifsy2cap	Gifsy2J	GifsyRec	GifsyninG	SopE	SL254-tail	Isolates
H01	-	-	-	-	-	+	+	+	-	+	-	H01
H02	-	-	-	-	-	+	+	+	-	+	-	H02
H03	-	-	-	-	-	+	+	+	-	+	-	H03
H04	+	+	+	+	-	+	+	+	-	+	+	H04
H05	-	-	-	-	-	+	+	+	-	+	-	H05
H06	-	-	-	-	-	+	+	+	-	+	-	H06
H07	+	+	+	+	-	+	+	+	-	+	+	H07
H08	+	+	+	+	-	+	+	+	-	+	+	H08
H09	-	-	-	-	-	+	+	+	-	+	-	H09
H10	-	-	-	-	-	+	+	+	-	+	-	H10
H11	-	-	-	-	-	+	+	+	-	+	-	H11
H12	-	-	-	-	-	+	+	+	-	+	-	H12
H13	-	-	-	-	-	+	+	+	-	+	-	H13
H14	+	+	+	+	-	+	+	+	-	+	-	H14
H15	-	-	-	-	+	+	+	+	-	+	-	H15
H16	-	-	-	-	-	+	+	+	-	+	-	H16
H17	-	-	-	-	-	+	+	+	-	+	-	H17
H18	-	-	-	-	-	+	+	+	-	+	-	H18
H19	-	-	-	-	-	+	+	+	-	+	+	H19
H20	-	-	-	-	-	+	+	+	-	+	-	H20
H21	-	-	-	-	-	+	+	+	-	+	-	H21
H22	-	-	-	-	-	+	+	+	-	+	+	H22
H23	-	-	-	-	-	+	+	-	+	+	-	H23
H24	-	-	-	-	-	+	+	-	+	+	+	H24
H25	-	-	-	-	-	+	+	-	+	+	-	H25
H26	-	-	-	-	-	+	+	-	+	+	+	H26
H27	-	-	-	-	-	+	+	+	-	+	-	H27
H28	-	-	-	-	-	+	+	+	-	+	-	H28
H29	-	-	-	-	-	+	+	+	-	+	-	H29
H30	-	-	-	-	-	+	+	+	-	+	-	H30
H31	-	-	-	-	-	+	+	+	-	+	+	H31
H32	-	-	-	-	-	+	+	+	-	+	+	H32
H33	-	-	-	-	-	+	+	+	-	+	+	H33

Isolates	Fels2G	Fels2lysB	Fels2ter	Fels2port	Gifsy1A	Gifsy2cap	Gifsy2J	GifsyRec	GifsyninG	SopE	SL254 tail	Isolates
H35	+	+	+	+	-	+	+	+	-	+	+	H35
H36	+	+	+	+	-	+	+	+	-	+	+	H36
H37	+	+	+	+	-	+	+	+	-	+	+	H37
H38	-	-	-	-	-	+	+	+	-	+	+	H38
H39	-	-	-	-	-	+	+	-	+	+	-	H39
H40	-	-	-	-	-	+	+	-	+	+	-	H40
H41	-	-	-	-	-	+	+	-	+	+	-	H41
H42	-	-	-	-	-	+	+	+	-	+	+	H42
H43	-	-	-	-	-	+	+	+	+	+	-	H43
H44	-	-	-	-	-	+	+	+	-	+	-	H44
H45	-	-	-	-	-	+	+	-	+	+	+	H45
H46	-	-	-	-	+	+	+	+	-	+	-	H46
H47	-	-	-	-	-	+	+	-	+	+	-	H47
H48	+	+	+	+	-	+	+	+	-	+	-	H48
H49	-	-	-	-	-	+	+	+	-	+	-	H49
H50	-	-	-	-	-	+	+	+	-	+	-	H50
H51	-	-	-	-	-	+	+	+	-	+	-	H51
H52	-	-	-	-	-	+	+	+	-	+	-	H52
H53	-	-	-	-	-	+	+	+	-	+	-	H53
H54	-	-	-	-	-	+	+	+	-	+	-	H54
H55	-	-	-	-	-	+	+	+	-	+	-	H55
H56	+	+	+	+	-	+	+	+	-	+	-	H56
H57	-	-	-	-	-	+	+	+	-	+	-	H57
H58	-	-	-	-	-	+	+	+	-	+	-	H58
H59	-	-	-	-	-	+	+	+	-	+	-	H59
H60	-	-	-	-	-	+	+	+	-	+	-	H60
H61	-	-	-	-	-	+	+	+	-	+	-	H61
H62	+	+	+	+	-	+	+	+	-	+	+	H62
H63	+	+	+	+	-	+	+	+	-	+	+	H63
H64	-	-	-	-	-	+	+	+	-	+	-	H64
H65	+	+	+	+	-	+	+	+	-	+	+	H65