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Discrimination within Phenotypically Closely Related Definitive Types of *Salmonella enterica* Serovar Typhimurium by the Multiple Amplification of Phage Locus Typing Technique

Ian L. Ross and Michael W. Heuzenroeder*

Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia

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Multilocus sequence typing (MLST) is a relatively new high-resolution typing system employed for epidemiological studies of bacteria, including *Salmonella*. Discrimination based on MLST of housekeeping genes may be problematical, due to the high identity of gene sequences of closely related *Salmonella* species. The presence of genomic sequences derived from stable temperate phages in *Salmonella* offers an alternative for MLST of *Salmonella*. We have used MLST of prophage loci in *Salmonella enterica* serovar Typhimurium to discriminate closely related isolates of serovar Typhimurium. We have compared these results to MLST of five housekeeping genes, as well as pulsed-field gel electrophoresis (PFGE). The presence or absence of prophage loci in the 73 serovar Typhimurium isolates tested, as well as allelic variation as detected by sequencing, provided greater discrimination between isolates than either MLST of housekeeping genes or PFGE. Amplification of prophage loci alone separated serovar Typhimurium isolates into 27 groups comprising multiple isolates or individual strains. Sequencing of isolates found within the clusters separated isolates even further. By contrast, PFGE could only divide the 73 isolates into five distinct groups. MLST using housekeeping genes did not provide any significant separation of isolates in comparison to amplification or MLST of prophage loci. The results demonstrate that the amplification and sequencing of prophage loci provides a high-resolution, objective method for the discrimination of closely related isolates of serovar Typhimurium. It is proposed that multiple amplification of phage locus typing may provide sufficient discrimination for epidemiological purposes without recourse to MLST.

Nontyphoidal *Salmonella* species are a common causative agent of bacterial food-borne disease worldwide (4, 12). Classical typing methods for identification of isolates involves serotyping based on the Kauffmann-White serological scheme that targets the cell surface O and H antigens. This system currently identifies more than 2,000 serotypes worldwide (19). Phage typing systems have been developed for further discrimination of serovars commonly associated with disease such as *Salmonella enterica* serovar Typhimurium (1) and *Salmonella enterica* serovar Enteritidis (35). More recently, molecular typing methods have been assessed and adopted for further discrimination of *Salmonella* isolates (for a review of methodologies for typing *Salmonella*, see reference 36). Of the molecular-based systems, pulsed-field gel electrophoresis (PFGE) is currently considered by many to be the "gold standard" for molecular typing of *Salmonella* (21). More recently, amplified-fragment length polymorphism (AFLP) has been developed and adopted by researchers because of its potential to discriminate between closely related isolates, flexibility with variable selective primers, and choices of restriction enzyme systems (9, 28, 33).

There are currently a number of potential problems with both classical and most molecular-based methods for typing *Salmonella*. Phage typing requires a well-maintained phage library, precise methodology, and experience in interpretation of results (8, 30). Interpretation of plaque patterns is often subjective and prone to variation between laboratories, especially between phage types with similar reaction patterns. For example, serovar Typhimurium definitive type 12 (DT12) and DT108 are phenotypically similar phage types with regard to lysis patterns of serovar Typhimurium and are separated on the Anderson typing panel by the sensitivity of DT108 with bacteriophages A21, A27, and A35. In many Australian isolates of DT108, there is no observed reaction with phage A21. Furthermore, there are cases where there are very weak reactions with phages A27 and A35.

Perceived problems with phage typing are further compounded, as a significant number of isolates can be untypable by this method. Often, one phage type may predominate in a geographical area, making discrimination between outbreak and nonoutbreak isolates difficult (21, 30). Molecular methods such as PFGE, although less subjective than phage typing, at times do not provide clear discrimination between isolates. Furthermore, some isolates are not typable by PFGE, for example, *Salmonella enterica* serovar Kentucky strains and *Salmonella enterica* subsp. *salamae* serovar Sofia (8, 22).

Multilocus sequence typing (MLST) is a relatively new high-resolution typing system developed for both evolutionary and epidemiological studies of microorganisms (6, 8, 10). MLST involves the nucleotide sequencing of approximately 400-bp regions of at least seven genes, usually housekeeping genes. Although multilocus enzyme electrophoresis (MLEE) is similar to MLST in that both systems target enzymes or their genes, MLST has advantages over MLEE. MLST will detect single-nucleotide polymorphisms, especially third-base redundancy within a codon, whereas MLEE relies on nonconservative changes in the amino

* Corresponding author. Mailing address: Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Box 14, Rundle Mall, P.O. Adelaide, S.A. 5000, Australia. Phone: 618 8222 3275. Fax: 618 8222 3543. E-mail: heuzenroeder@imvs.sa.gov.au.

acid sequence of a target enzyme that result in a change of its electrophoretic mobility on a starch gel (28). For example, Kotetishvili et al. (8) found that the ratio of nonsynonymous (amino acid substitution) to synonymous base changes (no change in amino acids) in three *Salmonella* housekeeping genes was >1. This meant that there was a low incidence of amino acid substitutions that would have affected enzyme mobility in MLEE studies.

Sequencing of *Salmonella* genes has generally been restricted to comparing sequences of housekeeping genes between the major groups of *Salmonella* or serovars of *S. enterica* subsp. *enterica*, including serovars Typhimurium and Enteritidis. Sequence variation can be demonstrated for geographically and epidemiologically unrelated isolates (5, 8, 16, 34). Consequently, MLST is a useful tool for studying evolution and global epidemiology of the salmonellae. However, for investigations of outbreaks, the fine discrimination of closely related isolates based on MLST of housekeeping genes may be not be possible, due to the high identity of the housekeeping gene sequences.

The presence of genomic sequences derived from temperate phages in *Salmonella* may offer an alternative target for MLST of *Salmonella* (25, 26). Although a typical prophage genome only constitutes approximately 1% of the bacterial chromosome, these loci are subject to mutational events at a higher rate than housekeeping genes. Furthermore, recombination between phages has resulted in an array of mosaic genetic structures of many bacteriophages, both within individual genes and in the overall genome organization (3, 17, 24, 32). Indeed, it has been stated that prophage genes account for most of the genetic diversity among closely related *Salmonella* strains (7). The presence of immunity exclusion genes such as the *cI* repressor gene of the *immC* region of some phages may confer a selective advantage for the host cell, rendering the cell immune to superinfection by lytic phage (20). This suggests that prophage genes, both in their presence or absence in a host cell and in their nucleotide sequence, vary considerably within a serovar. Consequently, heterogenicity of prophage genes is likely to be greater than that of host housekeeping genes, making them superior targets for MLST.

Previous studies by our laboratory have identified two bacteriophages (ST64T and ST64B) induced from a strain of serovar Typhimurium DT64. These two temperate phages were separated from each other by a cesium chloride gradient. The two bacteriophages were fully sequenced, and the sequences were deposited in GenBank (www.ncbi.nlm.nih.gov) under accession numbers AY052766 (ST64T) and AY055382 (ST64B) (14, 15). Southern hybridization studies have suggested that these bacteriophages are present in a number of serovar Typhimurium phage types as well as other *S. enterica* subsp. *enterica* serovars, including Enteritidis, Virchow, Heidelberg, and Hadar (13, 31). Based on the widespread nature of these phages, we used this sequence data to design a number of primer sets to analyze sequence variations of prophage loci. As well as using locus sequences from bacteriophages ST64B and ST64T, we also examined a range of similar loci from the well-characterized bacteriophage P22 (18, 32). A number of *Salmonella* housekeeping genes were also assessed to compare their level of sequence identity to the level of sequence identity of the prophage loci. We used this data to discriminate DT12 and DT108 strains of serovar Typhimurium phenotypically that were closely related by lysis pattern, in addition to DT126-related food poisoning by MLST and by amplification of phage loci alone.

MATERIALS AND METHODS

Strains and culture conditions. A total of 73 serovar Typhimurium isolates were used in this study. Thirty-six isolates were a selection of mostly epidemiologically unrelated DT108 (18 isolates, including 2 isolates [03-108-022 and 03-108-023] from an outbreak in New South Wales in 2003), DT12 (9 isolates), and DT12a (3 isolates). Six isolates comprised serovar Typhimurium isolates of various phage types (one isolate each of DT64, DT9, DT135, and DT185 and two DT170 isolates).

Thirty-seven DT126 isolates were also included in the study. This group comprised 13 isolates from a 2001 restaurant outbreak in New South Wales, Australia. Another 10 DT126 isolates were obtained during an outbreak in 2003. Four of these isolates were designated DT126 var, due to a variation in reaction to the Anderson typing panel. A further 14 DT126 isolates were epidemiologically unrelated.

All serovar Typhimurium isolates used in this study were provided by the Australian *Salmonella* Reference Centre, Institute of Medical and Veterinary Science, Adelaide, South Australia. Serotyping had previously been undertaken using the Kaufmann-White scheme, and bacteriophage typing was performed using the Anderson scheme of 31 bacteriophages (1), both by the Australian *Salmonella* Reference Centre.

Amplification of phage loci. PCR of *Salmonella* was performed directly from cell lysates. Isolates were grown overnight in Luria-Bertani broth at 37°C with gentle shaking. A 30-μl PCR mixture was prepared as follows: 3.0 μl of 10× $MgCl_2$-free buffer, 1.0 μM each forward and reverse primer, 3.0 μl of 200 μM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1.0 U of *Taq* polymerase, and 2.0 μl of overnight cell culture. The volume was made up to 30 μl with H_2O. Buffer, $MgCl_2$, and *Taq* polymerase were supplied by Roche. Primers were designed based on published sequences (Table 1.). All primers were supplied by Geneworks, Adelaide, South Australia. The deoxynucleoside triphosphates were supplied by Amersham Biosciences, Piscataway, N.J..

Touchdown PCR was performed in a Corbett Research (Sydney, Australia) PC-960G gradient thermal cycler as follows: 94°C (10 min), then 40 cycles comprising 94°C double-stranded DNA melting for 30 s and 72°C elongation for 60 s. Primer annealing temperatures were as follows: 59°C (1 cycle), 58°C (1 cycle), 57°C (2 cycles), 56°C (3 cycles), 55°C (5 cycles), 54°C (8 cycles), 53°C (10 cycles), and 52°C (10 cycles). All annealing steps were for 30-s duration each. A final elongation step at 72°C for 5 min was also performed.

The amplification product was detected and prepared for sequencing by running 5.0 μl of PCR product on a 2.0% agarose gel (Progen, Darra, Queensland) in 1.0× TBE buffer (23) at 5.0 V/cm with pUC19 digested with HpaII (Biotech, Belmont, Western Australia) as a marker. Bands were visualized with UV light after being stained with ethidium bromide. The remaining 25-μl PCR product of positive samples was prepared for sequencing by passage through a QIAquick PCR purification column (QIAGEN, Hilden, Germany) and collection in 30 μl of elution buffer per the manufacturer's instructions. Amplicons were stored at −20°C prior to sequencing.

MLST. Sequencing was performed in both directions with Big Dye Terminator, version 3-1 (Applied Biosystems, Foster City, Calif.). A 20-μl reaction mixture comprised 4.0 μl of BD3-1 master mix, 1.5 μl of 3.0 mM either forward or reverse primer, 12.5 μl of H_2O, and 2.0 μl of template. Sequencing was performed with a Corbett Research PC-960G gradient thermal cycler and comprised 25 cycles with the following parameters: 96°C (30 s), 50°C (15 s), and 60°C (4.0 min). The sequence product was precipitated, washed with 75% isopropanol, and then dried. Sequencing was performed with an Applied Biosystems 3700 DNA analyzer.

PFGE. The protocol for PFGE followed that of Maslow et al. (11). Briefly, cells grown overnight in brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) were embedded in agarose and lysed by incubation of the plug in 4 ml of lysis buffer supplemented with 4 mg of lysozyme (Roche, Mannheim, Germany) and 80 μg of RNase/ml. Plugs were then digested with proteinase K and washed, and the DNA was digested overnight with XbaI restriction endonuclease (New England BioLabs, Beverley, Mass.). The plugs were placed into the wells of a 1% agarose gel prepared with PFGE-grade agarose (Bio-Rad Laboratories, Hercules, Calif.) in 0.5× TBE buffer. *Staphylococcus aureus* strain NCTC 8325 digested with SmaI was used as a molecular size marker (29). The PFGE was run on a Bio-Rad CHEF-DR III system for 19 h at 6.0 V cm^{-1} at 4°C, with an initial switch time of 2.0 s and a final switch time of 50 s. After being run, the gel was stained in ethidium bromide, destained in water, and photographed under UV light.

TABLE 1. Primers used in this study

Source of sequence[a]	Locus	Primer name	Primer sequence (5′–3′)[b]	Size of sequence (bp)	Location in GenBank[c]
Bacteriophage ST64B	*immC cI* (5′) (BIM1)	Forward: BIM1F1	ATGGTGGCCTTGTCGACGC	433	28028–28502
		Reverse: BIM1R1	GCTAACGTGAAGGATTTGTTCCG		
	immC cI (3′) (BIM2)	Forward: BIM2F1	CCATTACCGGCGCTTGCAC	410	28441–28893
		Reverse: BIM2R1	TAACGTATAACCATGCGATTTCCG		
	immC cro (BIM3)	Forward: BIM3F1	GCGATATACGCAAAAGAAGGAGG	475	28819–29336
		Reverse: BIM3R1	TGGCTACTGAATGTGCCAGG		
	*imm*C put *c2* (BIM4)	Forward: BIM4F1	GCTGGTACTGCAACGTGCC	526	29284–29851
		Reverse: BIM4R1	CGAATGACATGGACATAAAGTCC		
	ORF SB6	Forward: SB6F1	ACGACAAGCGCGTTGAGGC	512	4394–494
		Reverse: SB6R1	GCTTCCACGTTGAAGAAGGC		
	ORF SB26	Forward: SB26F1	GACACCATCAATGTATGGATCGC	477	19466–19989
		Reverse: SB26R1	AGGTTATCTATAATTCCGACCTGG		
	ORF SB28	Forward: SB28F1	TGCAGTCAAGAGGACGTCC	589	21366–21994
		Reverse: SB28R1	TGCCGATATGCTGATCTGGC		
	ORF SB37	Forward: SB37F1	TGGTAGTGAATTGGTTAGCTGCG	439	26841–27322
		Reverse: SB37R1	CGGAAAGCTGTTACAGCAGG		
	ORF SB46	Forward: SB46F1	CATTGATGGTATCGAAGTTCGCC	448	33640–34130
		Reverse: SB46R1	CCTGGAGTTTCTGGCACGC		
Bacteriophage ST64T	gene *9* (5′)[d]	Forward: G9F1	GCRATTCCTTGCATCTGGAGC	562	38606–39208
		Reverse: G9R1	GCAATGCGGGAACCTTTGCC		
	gene *9* (3′)[d]	Forward: G9F2	TACCGTAGAAGATTGCGCTGG	464	39995–40501
		Reverse: G9R2	GGRACAAATGGTATCTCTGCCC		
	gene *17*[d]	Forward: F17A	GGCTGTYGTTTCTTCTTTCAGGC	264	10609–10921
		Reverse: R17A	AGGAAATATGAAATTACGTGTCTGGC		
	gtrA[d]	Forward: GTRAF1	AGACCTTTCCGAATCCGCTG	292	2449–2784
		Reverse: GTRAR1	TAATTGCCGAGAAAGTGATAAGGG		
	gtrB[d]	Forward: GTRBF1	CTTTCTCGGCAATTAGCCTG	383	2041–2463
		Reverse: GTRBR1	TTAGCCAGCACCATATCCGC		
	gtrC[d]	Forward: GTRCF1	CTACTACTCGCTATTCTTTGCGC	492	152–689
		Reverse: GTRCR1	CATTAACACCTCTGACCACATCC		
	mnt	Forward: TMNTF1	GAGTAAAGCCCGGTTCGCC	280	38228–38552
		Reverse: TMNTR1	TATAACCAGTAGATCATATGATGCCG		
	c2	Forward: TC2F1	GGAATTGTTAGAGGCCTTGCC	362	12886–13287
		Reverse: TC2R1	GATTTCCCCTGATTAGCTGGG		
	cro	Forward: TCROF1	CCATCCTGAGGAGATATACCG	222	13653–13917
		Reverse: TCROR1	GGTTCAGATTGGTAAAGAGCGG		
	c1[c]	Forward: PTC1F1	CTTTACCAATCTGAACCGCCG	385	13901–14332
		Reverse: PTC1R1	CTGAGTTGTTTTGGCATAATTACTCC		
Bacteriophage P22	*mnt*	Forward: PMNTF1	TTATAAGTAGTCAATATGGCCCAGG	275	37946–38269
		Reverse: PMNTR1	AATACACTAACTTGGAGTGATGGC		
	int	Forward: INTPF1	CATTTCCTGCAATACCGAAATCGG	461	3257–3717
		Reverse: INTPR1	GCTGGCTTGAGCCTCACG		
	cro	Forward: PCROF1	AGTGTTCTTTAATTTCGGAGCGAG	195	13468–13708
		Reverse: PCROR1	CGGTTCAGATTGGTAAAGAGCG		
	c3	Forward: PC3F1	CTGCACAAGGATGGTTCCG	251	9902–10193
		Reverse: PC3R1	ATTGATTGGTATAGCGAGTGCC		
	sieA	Forward: SIEAF1	GCGCTATAAGCCAAGGACGG	462	36788–37292
		Reverse: SIEAR1	TGAGTTATGCTGTGCTAGTTGCC		
	sieB	Forward: SIEBF1	CGATGAACAACTCATGGTGGC	568	11507–12117
		Reverse: SIEBR1	AGCGAGGTAAGGTATTTGTCG		
Salmonella housekeeping genes	*fhuA*	Forward: FHUAF1	AGAAGAAACCATTACCGTAACCG	402	223851–224296
		Reverse: FHUAR1	TGCTAACCATCGAAATGATACCG		
	sucA	Forward: AROCF1	GCACCGAAGAGAAACGCTG	600	802274–802915
		Reverse: AROCR1	GGTTGTTGATAACGATACGTAC		
	tonB	Forward: TONBF1	AGAATCTGTACATTTTCCACTCGC	433	1831399–1831867
		Reverse: TONBR1	CAGCGCAGCCTATCACGG		
	manB[e]	Forward: MANBF1	GGCAGCTACAGACAAATCAGC	508	2187413–2187961
		Reverse: MANBR1	GCCATAAATGGCATCCTCCG		
	glnA	Forward: FHUAF1	GTTATCGACCCGTTCTTCGC	579	4216166–4216783
		Reverse: FHUAR1	GTTGGTGCCGTTCTTCGCC		

[a] Primer sequences from bacteriophages and *Salmonella* housekeeping genes derived from the following GenBank accession numbers ST64B (AY055382), ST64T (AY052766), P22 (NC_002371), and *Salmonella* housekeeping genes (NC_003197) (www.ncbi.nlm.nih.gov).
[b] Y, C or T; R, A or G.
[c] Location in published GenBank sequence includes primers.
[d] The primers derived from bacteriophage ST64T are also used to detect similar regions in bacteriophage P22.
[e] The phosphomannomutase gene *manB* is designated *cpsG* in the referred GenBank accession number NC_003197.

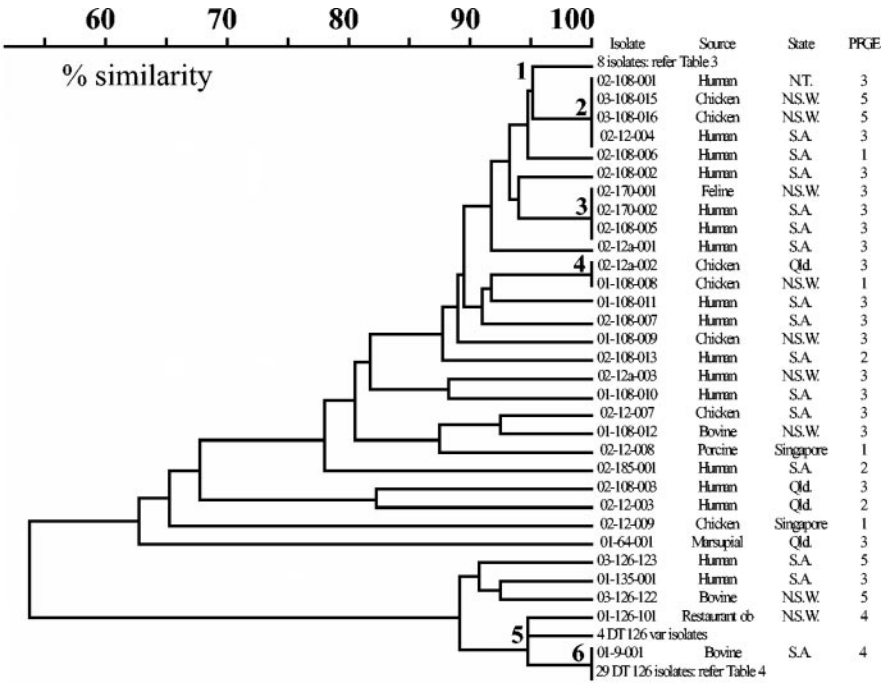


FIG. 1. Dendrogram of PCR profiles for separation of serovar Typhimurium isolates based on MAPLT. The dendrogram was generated by Dice coefficient with clustering by UPGMA, based on the presence or absence of amplified product. A total of six clusters of isolates with identical PCR profiles were generated, as well as a total of 21 isolates with unique profiles. Boldface numbers in the dendrogram refer to cluster numbers. Cluster 1 comprises the eight non-DT126 isolates, and cluster 5 comprises the four DT126 var isolates. Abbreviations: N.S.W., New South Wales; N.T., Northern Territory; Qld., Queensland; S.A., South Australia; Vic., Victoria.

Data analysis. Isolates were initially separated based on PCR results. Each primer set was given a number, and data were entered into a spreadsheet consisting of all positive results for each strain. The spreadsheet was imported into GelCompar IV (Applied Maths, Kortrijk, Belgium) with appropriate formatting. A dendrogram of isolate PCR profiles was generated by a Dice coefficient and clustering by unweighted-pair group method using average linkages (UPGMA) (Fig. 1).

Nucleotide sequences were analyzed in both directions with GeneBase, version 1.0 software (Applied Maths).

RESULTS

Multiple amplification of phage locus typing (MAPLT). Twenty-two of the 25 prophage-derived primer sets generated amplified product from at least one of the 73 serovar Typhimurium isolates (Table 2). The most frequently amplified regions were the loci from bacteriophage ST64B. A range of P22 and ST64T prophage loci were routinely found in non-DT126 isolates; these included the 5′ and 3′ regions of the $9_{P22/ST64T}$, $sieB_{P22/ST64T}$, $gtrC_{P22/ST64T}$, $int_{P22/ST64T}$, and mnt_{ST64T} genes. Amplified product from other primer sets $gtrA_{P22/ST64T}$, $gtrB_{P22/ST64T}$, and mnt_{P22} was obtained with only a small number of isolates. The three prophage primer sets that failed to amplify with any isolate were $sieA_{P22/ST64T}$, cro_{ST64T}, and $c3_{P22}$. Only one isolate (01-126-114) failed to generate amplified product with any of the 25 prophage primer sets.

Analysis of the PCR profiles of the 72 isolates that tested positive for prophage loci is summarized in Fig. 1. Six separate clusters, each containing isolates with identical amplification profiles, were identified (Fig. 1). Four separate clusters of non-DT126 isolates with identical PCR profiles were observed. Different phage types were represented in each of these four clusters. Eighteen non-DT126 isolates (including the DT135 isolate) were all separated from each other by differences in their PCR profiles. Cluster 6 contained 29 DT126 isolates plus the DT9 isolate and included 12 of the 13 2001 outbreak isolates and the 6 DT126 isolates from the 2003 outbreak. A further cluster (cluster 5) consisted of the four DT126 var isolates from the 2003 outbreak. These four isolates were separated from the other DT126 isolates from the same outbreak, as well as the other DT126 isolates as they did not contain the $SB28_{ST64B}$ loci (putative *int* gene). The DT126 isolates, as well as the DT9 and DT135 isolates, had PCR profiles significantly different from the other serovar Typhimurium phage types tested. All but three DT126 isolates (01-126-101, 02-126-122, and 02-126-123) failed to produce a PCR product with primers from P22 and ST64T.

MLST analysis. All PCR products for all relevant primer sets were sequenced with both forward and reverse primers, and the number of different alleles for each primer set was determined (Table 2). Most bacteriophage-derived primer sets produced at least two different alleles when sequenced, except those primer sets where only a small number of strains generating a PCR product were observed. Some primer sets produced only one allele even when a significant number of isolates produced PCR product, for example, $SB28_{ST64B}$ and the mnt_{ST64T} gene. In a few cases where significant numbers of isolates produced product, only one or two isolates contained an allele distinct from the majority of isolates. For example, a

TABLE 2. The incidence of loci, number of alleles, and their distribution in 73 *S. enterica* serovar Typhimurium isolates

Prophage or gene group	Locus	No. positive[a]	No. of alleles[b]	No. with most-common allele[c]
ST64B	*c1*: 5′	72	3	56
	c1: 3′	70	2	62
	SB39 *cro*	71	3	62
	SB40 *c2*	70	5	63
	SB6	68	3	59
	SB26	55	2	54
	SB28	63	1	63
	SB37	68	3	53
	SB46	62	5	49
ST64T and P22	gene *9*: 5′	33	3	30
	gene *9*: 3′	36	3	25
	gene *17*	9	2	6
	gtrA	4	1	4
	gtrB	7	2	6
	gtrC	31	4	25
	c1	1	1	1
ST64T	*mnt*	29	1	29
	c2	1	1	1
	cro	0	0	NA
P22	*mnt*	4	1	1
	cro	1	1	1
	int	34	4	30
	c3	0	0	NA
	sieA	0	0	NA
	sieB	33	2	30
Housekeeping genes	*fhuA*	73	1	73
	glnA	73	2	63
	manB	46	2	45
	tonB	15	1	15
	sucA	73	1	73

[a] Number of isolates with amplified product for each primer set.
[b] Number of different alleles as detected by sequencing of amplified product.
[c] Number of isolates containing the most commonly observed allele. NA, not applicable.

second $SB26_{ST64B}$ allele was present in only one isolate, 01-9-001.

The housekeeping genes *fhuA*, *glnA*, and *sucA* amplified in all 73 isolates. In contrast, 46 and 15 isolates yielded product for *manB* and *tonB*, respectively. More than one allele was present only in *glnA* and *manB*. Isolate 01-126-114 possessed a *manB* allele different from that of the other 45 that were positive with *manB* primers. Six DT126 and four DT126 var isolates from the 2003 outbreak had a characteristic *glnA* allele.

MLST of phage loci was undertaken to further discriminate between isolates that clustered as depicted in Fig. 1. Separation of the eight isolates in cluster 1 produced three subgroups (Table 3). Separation was based on a single allele in each case, with the two-outbreak strains possessing a $SB37_{ST64B}$ allele different from that of the other six isolates. Isolate 02-12-002 could be separated from the other seven isolates in this cluster based upon the $immC_{ST64B}$ *c1* 5′ sequence.

In cluster 2, isolate 02-108-001 could be separated from the other three isolates based upon the $immC_{ST64B}$ region. In cluster 3, no further separation could be achieved. Cluster 4 comprised two isolates, which could be separated based upon different alleles within the ST64B *immC* region and $SB37_{ST64B}$.

MLST of the DT126 isolates indicated a lower degree of variation than observed above (Table 4). No allelic variation

TABLE 3. The separation of MAPLT-clustered non-DT126 isolates by sequencing

Cluster[a]	Isolate	Differing allele(s)[b]	PFGE[c]
1	02-108-004		3
	03-108-014		3
	02-12-001		3
	02-12-005		3
	02-12-006		2
	02-12-002	$c1_{ST64B}$ (5′)	3
	03-108-022	$SB37_{ST64B}$	3
	03-108-023	$SB37_{ST64B}$	3
2	02-108-001	$immC_{ST64B}$	3
	03-108-015		5
	03-108-016		5
	02-12-004		3
3	02-170-001		3
	02-170-002		3
	02-108-005		3
4	01-108-008		1
	02-12a-002	$immC_{ST64B}$, $SB37_{ST64B}$	3

[a] Based on clusters as depicted in Fig. 1.
[b] Unique allele(s) which separate an isolate(s) within each cluster.
[c] PFGE clusters as depicted in Fig. 2.

was observed for the 13 isolates from the 2001 restaurant outbreak or the 10 2003 outbreak isolates for prophage loci. However, separation of the 2003 isolates could be achieved based upon *glnA*. Of the remaining DT126 isolates only one

TABLE 4. Separation of DT126 and DT126 var isolates by MAPLT and sequencing

Isolate	Variable prophage locus[a]	Housekeeping allele no.[b]				
		manB	*glnA*	*manB*	*tonB*	*sucA*
12 × 2001 RO[c]	0	1	1	—	—	1
01-126-101	$gtrA_{P22/ST64T}$ + ve	1	1	—	—	1
6 × 2003 RO[d]	0	1	2	—	—	1
4 × 2003 RO[e]	No $SB28_{ST64B}$	1	2	—	—	1
01-126-114	No locus amplified by PCR	1	1	2	1	1
02-126-115	0	1	1	1	—	1
02-126-116	0	1	1	—	1	1
02-126-117	0	1	1	1	1	1
02-126-118	0	1	1	1	1	1
03-126-119	0	1	1	1	—	1
03-126-120	0	1	1	1	1	1
03-126-121	0	1	1	1	1	1
03-126-122	int_{P22} + ve	1	1	1	1	1
03-126-123	cro_{P22} + ve, unique SB6 allele	1	1	1	1	1
03-126-124	0	1	1	—	—	1
03-126-125	0	1	1	—	—	1
03-126-126	0	1	1	—	—	1
03-126-127	0	1	1	—	—	1
01-9-001[f]	Unique SB6 allele	1	1	1	—	1
01-135-001[f]	$gtrB_{P22/ST64T}$ + ve	1	1	1	—	1

[a] Variation from the prophage PCR profile of the 29 DT126 isolates (see cluster 6 in Fig. 1) where only the nine ST64B loci were detected. 0, no variation detected.
[b] Allelic number as determined by sequencing, allelic numbers are assigned for comparative purposes only. —, no amplification product detected.
[c] Twelve isolates out of thirteen from the 2001 restaurant outbreak (RO). Isolate 01-126-101 is the thirteenth isolate.
[d] Six DT126 isolates from the 2003 restaurant outbreak (RO).
[e] Four DT126 var isolates from the 2003 restaurant outbreak (RO).
[f] Single DT9 and DT135 isolates were included as phenotypic outliers with regard to DT.

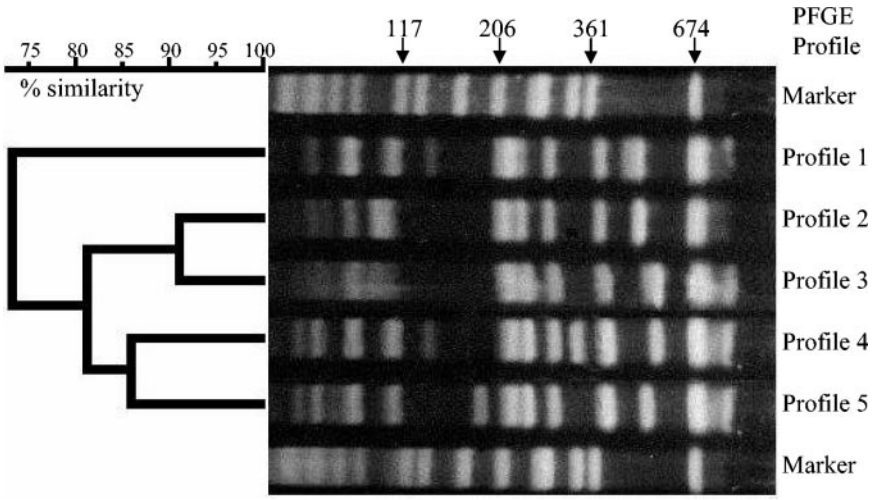


FIG. 2. Separation of serovar Typhimurium isolates based on PFGE. Dendrogram generated by Dice coefficient with clustering by UPGMA. Five different profiles were generated. Profiles 2 and 3 had >90% similarity, suggesting isolates with these profiles are genetically closely related. Serovar Typhimurium DT126 isolates were either profile 4 or 5; the remaining serovar Typhimurium isolates were found in all five profiles. Marker sizes are in kilobases $\times 10^3$.

isolate, 01-126-123, exhibited different prophage alleles from the other DT126 isolates.

PFGE. The 73 serovar Typhimurium isolates generated five different PFGE profiles (Fig. 2). PFGE profiles 2 and 3 displayed >90% similarity, suggesting that isolates within these profiles are closely related. All DT126 isolates were exclusively in profiles 4 or 5. The remaining isolates could be found in all profiles, although profile 3 contained the majority of isolates (25 of 36 non-DT126 isolates). No relationship was observed between phage type and pulsed-field profile.

DISCUSSION

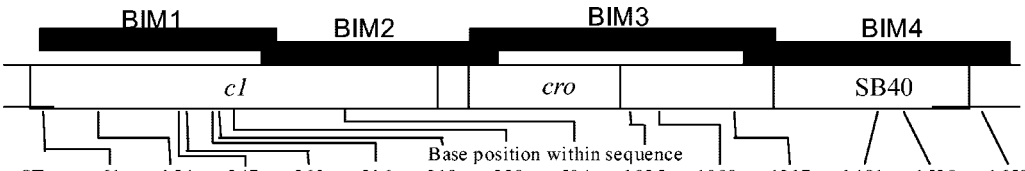
Phage typing of *Salmonella* can be subjective and may fail to provide sufficient discrimination between isolates. Discrimination by molecular methods such as PFGE is often unsatisfactory, due to the clonal nature of *Salmonella*. Although MLST of *Salmonella* housekeeping genes provides a satisfactory level of discrimination for diverse isolates of *Salmonella* (8), this method may not be suitable for closely related isolates within a serovar, due to sequence identity of their housekeeping genes. Prophages are genetically variable and are widespread within the genus *Salmonella* (7). The primary aim of this paper was to investigate the potential of prophage loci as suitable targets for the discrimination of serovar Typhimurium phage types closely related by lysis pattern.

Amplification of prophage loci provided the first level of typing of the serovar Typhimurium isolates based on the presence or absence of PCR product. We term this method multiple amplification of phage locus typing (MAPLT). PCR of prophage loci in serovar Typhimurium generated 27 distinct profiles (Fig. 1), compared to only 5 profiles generated by PFGE (Fig. 2). The MAPLT profiles comprised either single isolates or clusters of isolates with identical PCR profiles within each cluster. Some MAPLT profiles were closely related with the only product from a single primer set separating them; for example, clusters 1 and were separated by the absence of the $SB26_{ST64B}$ gene amplification in isolates of cluster 2.

DT126 isolates (as well as the single DT9 and DT135 isolates) were clearly different from the other phage types analyzed. There were six MAPLT profiles for DT126, in comparison to PFGE, which only generated two profiles, even though the isolates were from diverse sources. These results demonstrate the limitations of PFGE and the potential of MAPLT as an epidemiological tool when typing closely related strains.

It was also observed that most DT126 isolates contained only ST64B sequences. The apparent lack of other phage-related sequences in this phage type reduced the ability of MAPLT to discriminate between isolates. This observation may partially explain the susceptibility of this phage type to almost all (except A6 and A8) of the bacteriophages in the Anderson typing panel, since no P22- and ST64T-like immunity genes were amplified. In contrast, DT12 and DT108 contain a mosaic of sequences that are related to phages other than ST64B, including genes related to immunity. It is significant that DT12 and DT108 are susceptible to only three and six members of the Anderson panel, respectively. It is this lack of diversity of susceptibility of certain phage types of serovar Typhimurium to the typing panel that can prove problematical in classical phage typing. Typing is then dependent on the more subjective assessment of lysis intensity. The likely presence of a mosaic of phage loci in these DTs makes MAPLT a suitable method for fine discrimination between isolates, as a high number of suitable targets will be present for PCR.

MLST was undertaken with all PCR products, to separate clustered isolates as illustrated in Fig. 1 and to determine which prophage loci exhibit the greatest sequence variability. This information is important, since nucleotide sequence may sometimes be required for further discrimination of isolates. It was observed that the more unrelated isolates by MAPLT showed greater variability of prophage sequences (data not shown). Interestingly, DT170 and DT108 in cluster 3 could not



ST	61	154	247	262	316	319	330	504	1035	1098	1217	1481	1528	1659
1	A	T	G	T	A	G	G	G	T	C	G	C	C	A
2	-	C	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-		-	-	-	-	A	-	-	A	-	-	-
4	-	C	-	-	-	-	-	A	A	T	A	-	-	-
5	-	-	-	-	-	-	-	-	A	T	A	-	-	-
6	-	C	-	-	-	-	-	A	-	-	A	A	-	T
7	G	C	C	G	G	A	A	A	A	T	A	A	T	T
8	G	C	C	G	G	A	A	A	A	T	A	-	-	-

FIG. 3. Sequence types (ST) within the *immC* region of phage ST64B. The base position is the position within the sequenced region; hence, position 1 is position 28047 in GenBank accession number AY055382, which includes ST1. Eight different sequence types including ST1 were detected in the 73 serovar Typhimurium isolates. Where there was no nucleotide difference to ST1, this is indicated by a −. The filled boxes indicate regions covered by primers BIM1 to BIM4.

be separated further (Table 3); these DTs can be difficult to separate using the Anderson typing panel with only variable reactions, with phages A14 and A21 providing separation of the two phage types. It could be postulated that these three isolates do indeed represent the same phage type.

Limited separation of the DT126 isolates was achieved by sequencing of prophage loci (Table 4). In addition, PFGE and MAPLT suggest that DT126 was more clonal than the other DTs tested. In 2001, DT126 became the most frequently isolated phage type in Australia and was associated with a number of food-associated outbreaks (2). After 2001, the frequency of DT126 isolation declined to pre-2001 numbers. It is possible that the DT126 isolates isolated during and after 2001 were from a common clone from within Australia or an imported clone. This clone found its way into the food chain, resulting in the increased number of outbreak incidences.

The sequences derived from bacteriophage ST64B have been found to be highly variable in the non-DT126 isolates tested in both amplification and nucleotide sequence analyses (Table 2) (Fig. 3). Genomic sequences of ST64B are widespread in serovars of *S. enterica* subsp. *enterica*, including serovar Typhimurium (13, 31). The apparent widespread incidence of ST64B genomic material in serovar Typhimurium observed with MAPLT and sequence data suggests that loci from ST64B will make excellent targets for isolate discrimination within serovar Typhimurium. It is also likely that these sequences could be of use in other *Salmonella* serovars (C. P. Tucker and M. W. Heuzenroeder, unpublished data). In contrast, the incidence of ST64T and P22 loci was generally restricted to the non-DT126 isolates (Tables 2 and 4). Although discrimination of some isolates was enhanced by targeting ST64T and P22 loci, it should be noted that these loci (a) occur at lower frequency or (b) have limited allelic variation. For example, mnt_{ST64T} and $sieB_{P22}$, both of which occur in high frequency in non-DT126 isolates, were found to have little sequence variation (Table 2). While these loci provided less discrimination between isolates tested in this study than those derived from ST64B, they may be potentially useful for isolate discrimination in other DTs or serovars that are yet to be tested.

MLST of the five *Salmonella* housekeeping genes provided little discrimination between isolates, either by amplification or sequencing (Table 2). For three of five loci, all isolates that generated PCR product had identical sequences. A different *glnA* allele was found only in the 10 DT126 isolates from the 2003 outbreak, while only a single isolate contained a different *manB* allele. In addition to three of the genes described by Kotetishvili et al. (8) used in this study, other genes have been described in the literature for MLST, including the 6-phosphophogluconate dehydrogenase (*gnd*) (16), malate dehydrogenase (*mdh*) (5), and isocitrate dehydrogenase (*icd*) (34) genes. In these studies, strains were representative of all the described *Salmonella* subspecies and some serovars. As a consequence of the broad range of strains employed, allelic variation was not unexpected and demonstrated that sequencing of *Salmonella* housekeeping genes is a useful taxonomic tool. However, MLST of housekeeping genes may not be sufficient for separation within a serovar or phage type in real outbreak scenarios; the data presented here reinforce this concept.

Like PFGE, MAPLT and subsequent sequencing of loci did not separate the DT12 and DT108 isolates into groups based on phage type. MAPLT provided a greater level of separation of isolates than PFGE, regardless of phage type. It can be difficult to discriminate between DT108 and DT170, since they react with six and eight members of the typing panel, respectively, and share five reactions, with three being variable or weak. These results suggest that DT170 isolates may be distinguished from the majority of DT108 isolates by MAPLT when phage typing delivers ambiguous results. Further testing with more DT170 isolates will be required to confirm this initial observation.

Ten DT126 isolates were obtained from an outbreak in a restaurant in 2003. MAPLT and sequencing analyses indicated that these isolates were similar to all the other DT126 isolates tested, with the exception of the *glnA* sequence. When phage typed, four of the isolates did not react with phages A12 and A13, which normally produce semiconfluent lysis on DT126. These four isolates were designated DT126 var, based upon this difference. MAPLT separated DT126 var from the other 2003 outbreak isolates. It could be postulated that DT126 var is the result of phage type conversion or some other genetic event by a mobile element rendering the DT126 var isolates resistant to A12 and A13 typing phages. This demonstrates that when multiple phage-derived primers are used, MAPLT

has the power to discern underlying genetic relationships between strains, even when a phage type conversion or acquisition of a mobile element occurs during an outbreak.

In conclusion, this pilot study has shown the potential of utilizing prophage loci for MLST of phenotypically closely related phage types by lysis patterns of serovar Typhimurium. It is clear from the data that phage type does not necessarily reflect a close genetic relationship between isolates. The results show that the genetic variation of prophage genomes makes them excellent targets for PCR amplification and sequencing, compared to housekeeping genes. The variation of frequency of occurrence of many of the loci selected indicates that discrimination of isolates can often be achieved by MAPLT alone, without the need to determine the nucleotide sequence. As this is the first report of the utilization of prophage loci for typing, further work is required to obtain a minimal number of primer sets that will give the maximum level of isolate discrimination. This will mean the examination of genes from other *Salmonella* phages as a source of new primer sets, for example, A12 and A13, which are genetically distinct from the P22- and ES18-related phages in the Anderson typing panel (27).

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REFERENCES

1. **Anderson, E. S., L. R. Ward, M. J. de Saxe, and J. D. de Sa.** 1977. Bacteriophage-typing designations of *Salmonella typhimurium.* J. Hyg. **78:**297–300.
2. **Australian *Salmonella* Reference Centre.** 2001. 2001 annual report. Institute of Medical and Veterinary Science, Adelaide, South Australia.
3. **Baker, J., R. Limberger, S. J. Schneider, and A. Campbell.** 1991. Recombination and modular exchanges in the genesis of new lamboid phages. New Biol. **3:**297–308.
4. **Bell, C., and A. Kyriakides.** 2002. *Salmonella*—a practical approach to the organism and its control in foods. Blackwell Science, Ltd., London, United Kingdom.
5. **Boyd, E. F., K. Nelson, F.-S. Wang, T. S. Whittam, and R. K. Selander.** 1994. Molecular genetic basis of allelic polymorphism in malate dehydrogenase (*mdh*) in natural populations of *Escherichia coli* and *Salmonella enterica.* Proc. Natl. Acad. Sci. USA **91:**1280–1284.
6. **de Boer, P., B. Duim, A. Rigter, J. van der Plas, W. Jacobs-Reitsma, and J. Wagenaar.** 2000. Computer-assisted analysis and epidemiological value of genotyping methods for *Campylobacter jejuni* and *Campylobacter coli.* J. Clin. Microbiol. **38:**1940–1946.
7. **Figueroa-Bossi, N., and L. Bossi.** 2004. Resuscitation of a defective prophage in *Salmonella* cocultures. J. Bacteriol. **186:**4038–4041.
8. **Kotetishvili, M., O. C. Stine, A. Kreger, J. G. Morris, Jr., and A. Sulakvelidze.** 2002. Multilocus sequence typing for characterization of clinical and environmental *Salmonella* strains. J. Clin. Microbiol. **40:**1626–1635.
9. **Lindstedt, B.-A., E. Heir, T. Varund, and G. Kapperud.** 2000. A variation of the amplified-fragment length polymorphism (AFLP) technique using three restriction endonucleases, and assessment of the enzyme combination *Bgl*II-*Mfe*I for AFLP analysis of *Salmonella enterica* subsp. *enterica* isolates. FEMS Microbiol. Lett. **189:**19–24.
10. **Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA **95:**3140–3145.
11. **Maslow, J. N., A. M. Slutsky, and R. D. Arbeit.** 1993. Application of pulsed-field electrophoresis to molecular epidemiology, p. 563–572. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
12. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffen, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5**:607–625.
13. **Mmolawa, P. T., R. Willmore, C. J. Thomas, and M. W. Heuzenroeder.** 2002. Temperate phages in *Salmonella enterica* serovar Typhimurium: implications for epidemiology. Int. J. Med. Microbiol. **291:**633–644.
14. **Mmolawa, P. T., H. Schmieger, C. P. Tucker, and M. W. Heuzenroeder.** 2003. Genomic structure of the *Salmonella enterica* serovar Typhimurium DT 64 bacteriophage ST64T: evidence for modular genetic architecture. J. Bacteriol. **185:**3473–3475.
15. **Mmolawa, P. T., H. Schmieger, and M. W. Heuzenroeder.** 2003. Bacteriophage ST64B, a genetic mosaic of genes from diverse sources isolated from *Salmonella enterica* serovar Typhimurium DT 64. J. Bacteriol. **185:**6841–6845.
16. **Nelson, K., and R. K. Selander.** 1994. Intergeneric transfer and recombination of the 6-phosphogluconate dehydrogenase gene (*gnd*) in enteric bacteria. Proc. Natl. Acad. Sci. USA **91:**10227–10231.
17. **Oberto, J., S. B. Sloan, and R. A. Weisberg.** 1994. A segment of the phage HK022 chromosome is a mosaic of other lamboid chromosomes. Nucleic Acids Res. **22:**354–356.
18. **Pedulla, M. L., M. E. Ford, T. Karthikeyan, J. M. Houtz, R. W. Hendrix, G. F. Hatfull, A. R. Poteete, E. B. Gilcrease, D. A. Winn-Stapley, and S. R. Casjens.** 2003. Corrected sequence of the bacteriophage P22 genome. J. Bacteriol. **185:**1475–1477.
19. **Popoff, M. Y.** 2001. Antigenic formulas of the *Salmonella* serovars, 8th rev. World Health Organization Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France.
20. **Ptashne, M., A. Jeffrey, A. D. Johnson, R. Maurer, B. J. Meyer, C. O. Pabo, T. M. Roberts, and R. T. Sauer.** 1980. How the lambda repressor and Cro work. Cell **19:**1–11.
21. **Ridley, A. M., E. J. Threlfall, and B.Rowe.** 1998. Genotypic characterization of *Salmonella enteritidis* phage types by plasmid analysis, ribotyping, and pulsed-field gel electrophoresis. J. Clin. Microbiol. **36:**2314–2321.
22. **Ross, I. L., R. Willmore, and M. H. Heuzenroeder.** 2003. A fluorescent amplified fragment length polymorphism study of *Salmonella enterica* serovar Sofia, the major *Salmonella* serovar isolated from chickens in Australia. Int. J. Med. Microbiol. **293:**371–375.
23. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Schicklmaier, P., and H. Schmieger.** 1997. Sequence comparison of the genes for immunity, DNA relication, and cell lysis of the P22-related *Salmonella* phages ES18 and L. Gene **195:**93–100.
25. **Schicklmaier, P., E. Moser, T. Weiland, W. Rabsch, and H. Schmieger.** 1998. A comparative study on the frequency of prophages among natural isolates of *Salmonella* and *Escherichia coli* with emphasis on the generalized transducers. Antonie Leeuwenhoek **73:**49–54.
26. **Schicklmaier, P., and H. Schmieger.** 1995. Frequency of generalized transducing phages in natural isolates of the *Salmonella typhimurium* complex. Appl. Environ. Microbiol. **61:**1637–1640.
27. **Schmieger, H.** 1999. Molecular survey of the *Salmonella* phage typing system of Anderson. J. Bacteriol. **181:**1630–1635.
28. **Scott, F., J. Threlfall, and C. Arnold.** 2002. Genetic structure of *Salmonella* revealed by fragment analysis. Int. J. Syst. Evol. Microbiol. **52:**1701–1713.
29. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
30. **Threlfall, E. J., and J. A. Frost.** 1990. The identification, typing and fingerprinting of *Salmonella*: laboratory aspects and epidemiological applications. J. Appl. Bacteriol. **68:**5–16.
31. **Tucker, C. P., and M. W. Heuzenroeder.** 2004. ST64B is a defective bacteriophage in *Salmonella enterica* serovar Typhimurium DT64 that encodes a functional immunity region capable of mediating phage-type conversion. Int. J. Med. Microbiol. **294:**59–63.
32. **Vander Byl, C., and A. M. Kropinski.** 2000. Sequence of the genome of *Salmonella* bacteriophage P22. J. Bacteriol. **182:**6472–6481.
33. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, M. Zabeau.** 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. **23:**4407–4424.
34. **Wang, F-S., T. W. Whittam, and R. K. Selander.** 1997. Evolutionary genetics if the isocitrate dehydrogenase gene (*icd*) in *Escherichia coli* and *Salmonella enterica.* J. Bacteriol. **179:**6551–6559.
35. **Ward, L. R., J. D. H. de Sa, and B. Rowe.** 1987. A phage-typing scheme for *Salmonella enteritidis.* Epidemiol. Infect. **99:**291–294.
36. **Winokur, P. L.** 2003. Molecular epidemiolgical techniques for *Salmonella* strain discrimination. Front. Biosci. **8:**14–24.