Genetic structure of *Salmonella* revealed by fragment analysis

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Recently, the genus *Salmonella* has been studied by multilocus enzyme electrophoresis (MLEE) and three collections of strains defined by this method, SARA, SARB and SARC, have been assembled to represent the genetic diversity of *Salmonella choleraesuis* (commonly known as *Salmonella enterica*) subspecies I and of the genus as a whole. The novel technique fluorescent amplified-fragment length polymorphism (FAFLP) analysis was applied to these collections to determine the genetic diversity of *Salmonella*. FAFLP broadly confirmed the MLEE findings but added precision to them, successfully distinguishing between the subspecies of *S. enterica*. It revealed the clonal nature of some serotypes of *S. enterica* subspecies I and the diversity of others. The enteric *Salmonella* Paratyphi B strains clustered separately from those associated with gastroenteritis. FAFLP is a powerful, highly flexible, whole-genome method that can be used to provide an unweighted view of genetic variation within *Salmonella*.

Keywords: FAFLP, *Salmonella*, fragment analysis

INTRODUCTION

Salmonella are routinely classified by serotype on the basis of expression of three surface antigens (Ag), the somatic O Ag, the flagella H1 and H2 Ags and the capsular Vi Ag, according to the Kauffmann–White scheme. This scheme recognizes in excess of 2400 serotypes (Rowe & Hall, 1989). The majority of serotypes, including those responsible for disease in humans, are found in *Salmonella choleraesuis* (commonly known as *Salmonella enterica*) subspecies I (Beltran *et al.*, 1988). A number of subtyping schemes have been described for the most common and medically most important serotypes, including phage typing (Ward *et al.*, 1987; Briggs & Fratamico, 1999; Kariuki *et al.*, 1999b), pulsed-field gel electrophoresis (Powell *et al.*, 1994; Thong *et al.*, 1995; Ridley *et al.*, 1996; Kariuki *et al.*, 1999a, b; Laconcha *et al.*, 2000),

plasmid profile analysis (Threlfall *et al.*, 1989, 1994; Ridley *et al.*, 1998; Kariuki *et al.*, 1999a), ribotyping (Olsen *et al.*, 1997; Ridley *et al.*, 1998; Old *et al.*, 1999, 2000) and IS200 fingerprinting (Stanley *et al.*, 1991, 1992; Ezquerro *et al.*, 1993; Olsen *et al.*, 1994, 1997; Pelkonen *et al.*, 1994; Weide-Botjes *et al.*, 1998; Old *et al.*, 1999, 2000; Millemann *et al.*, 2000). However, neither these methods nor serotyping provides an adequate basis for determining the overall levels of genetic relatedness between strains and they therefore have no application in population genetics (Selander *et al.*, 1986; Beltran *et al.*, 1988).

Multi-locus enzyme electrophoresis (MLEE) has been used to measure genetic variation within bacterial populations, and the degree of overall genetic relatedness among isolates, populations and species, of a number of genera including *Escherichia*, *Salmonella*, *Legionella*, *Neisseria* and *Haemophilus* (Selander & Musser, 1990). MLEE analysis characterizes isolates on the basis of the relative electrophoretic mobilities of various water-soluble cellular enzymes. As the net electrostatic charge (and therefore the rate of migration) of a protein during electrophoresis is determined by its amino acid sequence, the mobility variants (electromorphs) of an enzyme can be equated directly with alleles at a corresponding structural gene locus (Selander *et al.*, 1986).

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Versions of the trees in Figs 2–4 showing various serotypes identified in colour are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Abbreviations: ET, electrophoretic type; FAFLP, fluorescent amplified-fragment length polymorphism; MLEE, multilocus enzyme electrophoresis; MLST, multilocus sequence typing.

MLEE studies of populations of *Salmonella* have described their basically clonal structure, with a few predominant clones achieving widespread if not global distribution (Selander *et al.*, 1986; Beltran *et al.*, 1988, 1991; Boyd *et al.*, 1993). Three *Salmonella* reference collections (SARA, SARB and SARC), which include 160 *Salmonella* strains, have been chosen by MLEE to represent the scope of genetic diversity within natural populations of *Salmonella*. These collections include representatives of the *Salmonella* species *S. choleraesuis*, *Salmonella bongori*, *Salmonella typhi*, *Salmonella enteritidis* and *Salmonella typhimurium* and the six subspecies of *S. choleraesuis* (Beltran *et al.*, 1991; Boyd *et al.*, 1993, 1996).

For a number of organisms, such as *Neisseria meningitidis* (Maiden *et al.*, 1998), *Streptococcus pneumoniae* (Enright & Spratt, 1998) and *Staphylococcus aureus* (Enright *et al.*, 2000), MLEE analysis is now being superseded by multilocus sequence typing (MLST), the partial sequencing of some of the housekeeping genes analysed in MLEE. Although sequencing of a few individual genes of *Salmonella* has also been described, including the isocitrate dehydrogenase gene *icd* (Wang *et al.*, 1997), the 6-phosphogluconate hydrogenase gene *gnd* (Nelson & Selander 1994), the malate dehydrogenase gene *mdh* (Boyd *et al.*, 1994) and the invasion genes (Li *et al.*, 1995; Boyd *et al.*, 1996), this multilocus sequencing approach has yet to be fully evaluated and therefore adopted for *Salmonella*.

Fluorescent amplified-fragment length polymorphism (FAFLP) is an accurate, rapid and reproducible method that permits simultaneous sampling of multiple loci throughout the bacterial genome (Arnold *et al.*, 1999a). It is a flexible technique where the degree of resolution can easily be modified by choice of primers, thereby allowing a range of applications from high-resolution genotyping for outbreak recognition to lower-resolution population structure investigations. AFLP has been used in studies of the higher eukaryotes to determine the genetic structure of populations (Sanchez *et al.*, 1999; Katiyar *et al.*, 2000; Keiper & McConchie, 2000) and, for prokaryotes, FAFLP data have correlated well with those generated by MLEE, e.g. for the EcoR collection of *Escherichia coli* (Ochman & Selander, 1984; Arnold *et al.*, 1999b).

Five species are recognised in classical *Salmonella* taxonomy: *S. choleraesuis*, *S. bongori*, *S. typhimurium*, *S. enteritidis* and *S. typhi*. *S. choleraesuis* is further divided into six subspecies: I, *choleraesuis*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *hautenae*; and VI, *indica* (Beltran *et al.*, 1988; Uzzau *et al.*, 2000).

Medical microbiology uses a different taxonomic system for *Salmonella*, based on MLEE, DNA hybridization and sequencing studies. There are two recognized species of the genus *Salmonella*: *S. choleraesuis* (commonly known as *S. enterica*) and *S. bongori* (Uzzau *et al.*, 2000). *S. choleraesuis* is again divided into six subspecies. *S. bongori* was previously con-

sidered to be subspecies V of *S. choleraesuis*; however, MLEE and DNA hybridization studies have revealed that it is sufficiently different from the other subspecies to be recognised as a separate species (Reeves *et al.*, 1989). The most recent studies have further subdivided subspecies IV into subspecies IV and VII (Boyd *et al.*, 1996). For the purposes of this paper, the classical *Salmonella* taxonomic nomenclature has been used and, where serotype names are used (shown with a capital letter and not italicized, e.g. *S. Paratyphi* B), these refer to serotypes of *S. choleraesuis* subspecies I.

In this study, we have used FAFLP to determine the genetic structure of the salmonellae as represented by the three *Salmonella* reference collections.

METHODS

Bacterial strains and growth conditions. *Salmonella* reference collections A, B and C (Table 1) were received from the *Salmonella* Genetic Stock Centre, University of Calgary, Canada. These strains have been characterized by MLEE; the strain designations used in this study are the same as in the original papers (Beltran *et al.*, 1991; Boyd *et al.*, 1993, 1996). Collections SARA and SARB consist entirely of *S. choleraesuis* subspecies I. SARA is composed of 72 strains of five serotypes representing the *S. typhimurium* clonal complex of *S. choleraesuis* (Beltran *et al.*, 1991) and SARB strains represent the genomic variation among the common serotypes of subspecies I. SARB consists of 72 strains of 37 serotypes including 18 serotypes represented by one electrophoretic type (ET) and 19 serotypes of multiple electrophoretic types, eight of which were apparently monophyletic (i.e. thought to be derived from a recent common ancestor through mutational divergence). The ETs of these eight serotypes were genotypically similar by MLEE, while the other 11 were polyphyletic (Boyd *et al.*, 1993). SARC consists of two representative strains of *S. bongori* and each of the seven *Salmonella* subspecies recognised by MLEE (Boyd *et al.*, 1996).

All strains were cultured aerobically on nutrient agar plates for 16 h. Stock cultures were stored on Protect bacterial preservers (Technical Service Consultants) at -70°C .

FAFLP. Genomic DNA was extracted by using the Qiagen DNeasy tissue kit. DNA was eluted into a final volume of 50 μl distilled water. FAFLP was performed as described by Desai *et al.* (1998). Briefly, *Mse*I and *Eco*RI were used to digest approximately 500 ng genomic DNA for 2 h. To the digested DNA was added 5.0 μl 10 $\times$ T4 DNA ligase buffer, 400 U T4 DNA ligase (New England Biolabs), 16.8 μl sterile distilled water and final concentrations of 1 nM *Eco*+0 adapter (5'-CTCGTAGACTGCGTACC-3' and 3'-CTGACGATGGTTAA-5') and 5 nM *Mse* adapter (5'-GACGATGAGTCCTGAG-3' and 3'-CTACTCAGGACTCAT-5') (both from Sigma-Genosys), to give a final reaction volume of 50 μl . Decreased adapter concentrations were used to reduce the production of $n+3$ FAFLP fragments (data not shown).

PCR was performed in a total reaction volume of 25 μl consisting of 2.5 μl of the above digestion/ligation reaction, 2.5 μl 10 $\times$ PCR buffer, 2.5 mM MgCl_2 , 0.2 mM dNTPs (all from Life Technologies), 0.2 μM 6-carboxyfluorescein (6-FAM) fluorescently labelled *Eco*+0 primer (5'-6-FAM-GACTGCGTACCAATTC-3'), 1 μM *Mse*+TA primer (5'-GATGAGTCCGAGTAATA-3') (both primers from

Table 1. SARA, SARB and SARC strains

SARA consists of 72 strains of the *S. typhimurium* complex (Beltran *et al.*, 1991), SARB consists of 72 strains of the common serotypes of *S. choleraesuis* (Boyd *et al.*, 1993) and SARC consists of 16 strains representing the *S. choleraesuis* subspecies and *S. bongori* (Boyd *et al.*, 1996).

Strain	Subsp.	Serotype	Antigenic formula	ET	Biotype	Locality	Source
SARA							
A01	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 1	1 f	Mexico	Human
A02	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 1		Lab strain	
A03	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 1	11 di	Rhode Island, USA	Horse
A04	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 1	25 e	Indiana, USA	Rabbit
A05	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 1	9 fi	Mongolia	
A06	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 2	17 a	Ohio, USA	Human
A07	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 3	1 a	Norway	
A08	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 5	1 a	Finland	
A09	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 7	1 a	California, USA	Parrot
A10	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 9	1 b	California, USA	Opossum
A11	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 10	2 a	Thailand	
A12	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 11	32 begi	Louisiana, USA	Horse
A13	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 12	29 b	France	
A14	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 13	1 a	Panama	
A15	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 14	17 a	Texas, USA	Dog
A16	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 15	3 d	N. Carolina, USA	Human
A17	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 16	3 d	Yugoslavia	
A18	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 17	3 d	Iowa, USA	Horse
A19	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 21	1 f	Mexico	Human
A20	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 22	1 a	France	
A21	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 23		Oregon, USA	Heron
A22	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 1		Massachusetts, USA	Human
A23	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 2		Pennsylvania, USA	Human
A24	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 3		Texas, USA	Human
A25	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 3		France	
A26	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 3		France	Human
A27	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 3		France	Human
A28	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 3		France	Human
A29	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 4		Florida, USA	Human
A30	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 1	9 i	Pennsylvania, USA	Chicken
A31	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 1	9 i	Maryland, USA	Swine
A32	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 1	9 i	Texas, USA	Dog
A33	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 1	9 gi	Mexico	Human
A34	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 1	9 i	Israel	
A35	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 2	9 i	Brazil	
A36	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 3	1 a	Thailand	
A37	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 4	9 i	Colorado, USA	Turkey
A38	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 5	9 i	Arizona, USA	Turkey
A39	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 7	9 gi	N. Carolina, USA	Human
A40	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 8	1 gi	USA	
A41**	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 1	3 gh	France	Human
A42*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 1	27 bg	Scotland, UK	Human
A43*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 1	11 ghi	Africa	Human
A44*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 1	19 gh	Middle East	Human
A45*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 1		France	Cow
A46*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 1a	11 bgi	Europe	Human
A47*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 2	9 a	Scotland, UK	Sewage
A48*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 2a	1 dh	Scotland, UK	Human
A49*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 2b	1 b	UK	Sewage
A50*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 3	2 a	Middle East	Food
A51*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 3	2 bg	France	Human
A52*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 3		France	Cow
A53*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 3		France	Human
A54*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 3		France	Human
A55*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 3a	2 bg	France	Human
A56*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 4	13 i	France	Human
A57*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 5	11 i	UK	Water
A58*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 5a	26 i	France	Human
A59*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 5b	10 i	France	Human
A60*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 5c	9 bi	France	Food
A61*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 6	1 a	Scotland, UK	Water
A62*	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:1,2	Pb 7	1 g	Africa	Human
A63	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 1		France	Human
A64	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 1		Kentucky, USA	Cow
A65	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 1		Florida, USA	Chicken
A66	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 1		Massachusetts, USA	Human
A67	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 1		Mexico	Human
A68	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 1a		France	Human

Table 1 (cont.)

Strain	Subsp.	Serotype	Antigenic formula	ET	Biotype	Locality	Source
A69	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 2		France	Human
A70	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 3		France	Human
A71	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 4		N. Carolina, USA	Human
A72	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 4a		France	Human
SARB							
B01	I	<i>S. Agona</i>	1,4,12:f,g,s:--	Ag 1		Peru	
B02	I	<i>S. Anatum</i>	3:10:e,h:1,6	An 1		Washington, USA	Human
B03	I	<i>S. Brandenburg</i>	1,4,12:1,v:e,n,z15	Ba 2	10 i	Scotland, UK	
B04	I	<i>S. Choleraesuis</i>	6,7:[c]:1,5	Cs 1		Minnesota, USA	Swine
B05	I	<i>S. Choleraesuis</i>	6,7:[c]:1,5	Cs 6		Switzerland	
B06	I	<i>S. Choleraesuis</i>	6,7:[c]:1,5	Cs 11		Thailand	
B07	I	<i>S. Choleraesuis</i>	6,7:[c]:1,5s	Cs 13		Australia	
B08	I	<i>S. Decatur</i>	6,7:c:1,5	Dt 1		France	
B09	I	<i>S. Derby</i>	1,4,[5],12:f,g:[1,2]	De 1		Oklaahoma, USA	Avian
B10	I	<i>S. Derby</i>	1,4,[5],12:f,g:[1,2]	De 13		Minnesota, USA	Swine
B11	I	<i>S. Derby</i>	1,4,[5],12:f,g:[1,2]	De 31		Pennsylvania, USA	Turkey
B12	I	<i>S. Dublin</i>	1,9,12:[Vi]:g,p:--	Du 1		Idaho, USA	Cattle
B13	I	<i>S. Dublin</i>	1,9,12:[Vi]:g,p:--	Du 3		France	Bovine
B14	I	<i>S. Dublin</i>	1,9,12:[Vi]:g,p:--	Du 2		Thailand	
B15	I	<i>S. Duisburg</i>	1,4,12,27:d:e,n,z,15	Di 1	9 i	Scotland, UK	
B16	I	<i>S. enteritidis</i>	1,9,12:g,m:[1,7]	En 1		Rhode Island, USA	
B17	I	<i>S. enteritidis</i>	1,9,12:g,m:[1,7]	En 2		Brazil	
B18	I	<i>S. enteritidis</i>	1,9,12:g,m:[1,7]	En 3		Connecticut, USA	
B19	I	<i>S. enteritidis</i>	1,9,12:g,m:[1,7]	En 7		Switzerland	
B20	I	<i>S. Emek</i>	8,20:g,m,s:--	Em 1		Israel	
B21	I	<i>S. Gallinarum</i>	1,9,12:--	Ga 2		Connecticut, USA	Human
B22	I	<i>S. Haifa</i>	1,4,[5],12:z10:1,2	Ha 1	1 a	Scotland, UK	
B23	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 1	9 i	Pennsylvania, USA	Chicken
B24	I	<i>S. Heidelberg</i>	1,4,[5],12:r:1,2	He 3	1 a	Thailand	
B25	I	<i>S. Indiana</i>	1,4,12:z:1,7	Id 1	1 a	Scotland, UK	
B26	I	<i>S. Infantis</i>	6,7:r:1,5	In 1		N. Carolina, USA	Human
B27	I	<i>S. Infantis</i>	6,7:r:1,6	In 3		Senegal	
B28	I	<i>S. Miami</i>	1,9,12:a:1,5	Mi 1		Georgia, USA	Human
B29	I	<i>S. Miami</i>	1,9,12:a:1,6	Mi 5		French Guyana	Human
B30	I	<i>S. Montevideo</i>	6,7:g,m,[p],s:[1,2,7]	Mo 1		Georgia, USA	Human
B31	I	<i>S. Montevideo</i>	6,7:g,m,[p],s:[1,2,7]	Mo 6		Florida, USA	Human
B32	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 1		Lab strain	
B33	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 2		France	Human
B34	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 3		France	Human
B35	I	<i>S. Muenchen</i>	6,8:d:1,2	Mu 4		N. Carolina, USA	Human
B36	I	<i>S. Newport</i>	6,8:e,h:1,2	Np 8		N. Carolina, USA	Human
B37	I	<i>S. Newport</i>	6,8:e,h:1,3	Np 11		Mexico	Human
B38	I	<i>S. Newport</i>	6,8:e,h:1,4	Np 15		Massachusetts, USA	Snake
B39	I	<i>S. Panama</i>	1,9,12:1,v:1,5	Pn 1		Italy	
B40	I	<i>S. Panama</i>	1,9,12:1,v:1,6	Pn 2		N. Carolina, USA	Human
B41	I	<i>S. Panama</i>	1,9,12:1,v:1,7	Pn 12		N. Carolina, USA	Human
B42	I	<i>S. Paratyphi A</i>	1,2,12:a:[1,5]	Pa 1		Lab strain	
B43 ^a	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:[1,2]	Pb 1	3 gh	France	Human
B44 ^b	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:[1,2]	Pb 3	2 a	Middle East	Food
B45 ^c	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:[1,2]	Pb 4	3 I	France	Human
B46 ^d	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:[1,2]	Pb 5	11 i	UK	Water
B47 ^e	I	<i>S. Paratyphi B</i>	1,4,[5],12:b:[1,2]	Pb 7	1 g	Africa	Human
B48	I	<i>S. Paratyphi C</i>	6,7,[Vi]:c:1,5	Pc 1		France	
B49	I	<i>S. Paratyphi C</i>	6,7,[Vi]:c:1,6	Pc 2		France	Human
B50	I	<i>S. Paratyphi C</i>	6,7,[Vi]:c:1,7	Pc 4		France	Human
B51	I	<i>S. Pullorum</i>	1,9,12:--	Pu 3		Germany	
B52	I	<i>S. Pullorum</i>	1,9,12:--	Pu 4		Germany	
B53	I	<i>S. Reading</i>	1,4,[5],12:e,h:1,5	Re 1	2 j	Scotland, UK	
B54	I	<i>S. Rubislaw</i>	11:r:e,n,x	Ru 1		Lab strain	
B55	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 3		Texas, USA	Human
B56	I	<i>S. Saintpaul</i>	1,4,[5],12:e,h:1,2	Sp 4		Florida, USA	Human
B57	I	<i>S. Schwarzengrund</i>	1,4,12,27:d:1,7	Sw 1	10 i	Scotland, UK	
B58	I	<i>S. Sendai</i>	1,9,12:a:1,5	Se 1		California, USA	Human
B59	I	<i>S. Senftenberg</i>	1,3,19:g,[s],t:--	Sf 1		Maryland, USA	Chicken
B60	I	<i>S. Stanley</i>	1,4,[5],12,27:d:1,2	St 1	26 bei	Scotland, UK	
B61	I	<i>S. Stanleyville</i>	1,4,[5],12,27:z4,z12:[1,2]	Sv 2	9 i	Scotland, UK	
B62	I	<i>S. Thompson</i>	6,7:k:1,5	Th 1		Florida, USA	Human
B63	I	<i>S. typhi</i>	9,12,[Vi]:d:[Z66]	Tp 1		Dakar, Senegal	
B64	I	<i>S. typhi</i>	9,12,[Vi]:d:[Z66]	Tp 2		Dakar, Senegal	
B65	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 1	1 f	Mexico	Human
B66	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 7	1 a	California, USA	Parrot
B67	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 12	29 b	France	
B68	I	<i>S. typhimurium</i>	1,4,[5],12:i:1,2	Tm 23		Oregon, USA	Heron
B69	I	<i>S. Typhisuis</i>	6,7:c:1,5	Ts 1		California, USA	Swine

Table 1 (cont.)

Strain	Subsp.	Serotype	Antigenic formula	ET	Biotype	Locality	Source
B70	I	<i>S. Typhisuis</i>	6,7:c:1,6	Ts 3		Iowa, USA	Swine
B71	I	<i>S. Wien</i>	1,4,12,27:b:1:w	Wi 1		France	Human
B72	I	<i>S. Wien</i>	1,4,12,27:b:1:w	Wi 2		France	Human
SARC							
C01	I	<i>S. typhimurium</i>	1,4[5],12:I:1,2	Tp 1		England, UK	Human
C02	I	<i>S. typhi</i>	9,12,[Vi]:d:[Z66]			Dakar, Senegal	
C03	II	<i>S. Unnamed</i>	58:d:z ₆			Massachusetts, USA	Human
C04	II	<i>S. Unnamed</i>	42:f:g,t:-				
C05	IIIa		62:z ₄ :z ₂₃ -			Oregon, USA	Cornsnake
C06	IIIa		62:z:36			California, USA	Human
C07	IIIb		50 _{1,2,3} :k:z			Oregon, USA	Human
C08	IIIb		35[k]:z ₃₅ -			California, USA	Human
C09	IV		45a,b:g:z ₅₁ -			Panama Canal Zone	Animal
C10	IV	<i>S. Chameleon</i>	16:z ₄ :z ₃₂ -			Illinois, USA	Human
C11	V	<i>S. Brookfield</i>	66:z ₄₁ -				Frog
C12	V	<i>S. Balboa</i>	48:z ₄₁ -			USA	Parakeet
C13	VI	<i>S. Vrindaban</i>	45:a:e,n,x			India	
C14	VI	<i>S. Srinagar</i>	11:b:e,n,x				
C15	VII	<i>S. Seminole</i>	1,40:g:z ₅₁			Tonga	
C16	VII	<i>S. Unnamed</i>	40:z ₄ :z ₂₄ -			Florida, USA	Human

* *S. Paratyphi* B biotypes are indicated as: *a*, Paratyphi B; *b*, Java; *c*, unknown.

Sigma-Genosys) and 0.65 U *Taq* DNA polymerase (Life Technologies). Touchdown PCR was performed as described by Desai *et al.* (1998) with the exception that the final extension step at 60 °C for 30 min was omitted to limit the addition of an *n*+1 adenine to FAFLP fragments by *Taq* DNA polymerase.

Fragment analysis. Gel separation and fragment analyses were performed as described previously (Grady *et al.*, 1999). Fragments were sized with GeneScan version 3.1 software (Applied Biosystems) and these data were transferred to GenoTyper 2.5 (Applied Biosystems).

Electrophoregrams were screened visually for the presence or absence of polymorphic fragments, which were scored in a binary matrix and recorded as a text (tab-delimited) file in Excel 98 (Microsoft). Dice coefficients of similarity (Dice, 1945) were calculated using in-house software. Cluster analysis was performed using the neighbour-joining method (Saitou & Nei, 1987) in the NEIGHBOR program of PHYLIP (Felsenstein, 1993) and then displayed in TREEVIEW version 1.5 (Page, 1996). A bootstrap tree (i.e. the generation of multiple datasets that are resampled versions of the input dataset) was produced in PAUP* (Swofford, 2000) using Nei-Li distances (Nei & Li, 1979) and neighbour-joining cluster analysis (Saitou & Nei, 1987) (data not shown). Where clusters and groupings of strains similar by FAFLP were observed, a 'cluster' was defined as those strains that both clustered by neighbour-joining and had high bootstrap support (i.e. > 75%) in Fig. 4; a 'group' was defined as those strains that clustered by neighbour-joining but had less than 75% bootstrap support in Fig. 4.

In silico FAFLP. *In silico* FAFLP analysis was performed as described by Arnold *et al.* (1999b) on the *inv/spa* genes of strains C09, C10, C11 and C12, respectively GenBank accession numbers U29357, U29358, U29355 and U29356 (Li *et al.*, 1995; Boyd *et al.*, 1996).

Fig. 1. Neighbour-joining tree of SARC strains. Bootstrap values were calculated in PAUP* using Nei-Li distances and neighbour-joining cluster analysis. The tree is rooted at *S. bongori* strains C11 and C12. Bar, 10% divergence.

Fig. 2. Neighbour-joining tree of SARB strains. Bootstrap values were calculated as described for Fig. 1. See text for definition of clusters and groups. Bar, 10% divergence. A version of this tree showing various serotypes identified in colour is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Fig. 3. Neighbour-joining tree of SARA strains. The tree is rooted at A30. Bootstrap values were calculated as described for Fig. 1. Clusters and groupings shown are defined in the text. Bar, 10% divergence. A version of this tree showing various serotypes identified in colour is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

RESULTS

We analysed 183 polymorphic fragments, in the size range 101–350 bp, of the 155 SARA, B and C strains,

and we found that no amplified fragment was common to all strains. There were 14 strain duplications between the collections and duplicates showed on average 99.2% similarity (range 98–100%). Figs 1–3 respect-

Fig. 4. Neighbour-joining tree of SARA, SARB and SARC strains. The tree is rooted at *S. bongori* strains C11 and C12. Bootstrap values were calculated as described for Fig. 1. SARC strains are underlined. Clusters and groupings shown are defined in the text. Bar, 10% divergence. A version of this tree showing various serotypes identified in colour is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

ively show FAFLP trees of SARC, SARB and SARA and Fig. 4 shows an FAFLP tree of all three reference collections combined.

FAFLP analysis of the *Salmonella* subspecies (SARC)

The strains from SARC fell into eight distinct pairs, consisting of two strains representing each of the seven MLEE defined subspecies and *S. bongori* (Fig. 1). There was high bootstrap support for these pairs, i.e. 99% for C13 and C14 (subspecies VI), 100% for C15 and C16 (subspecies VII), 100% for C03 and C04 (subspecies II), 95% for C09 and C10 (subspecies IV), 97% for C01, C02 (subspecies I) and 87% for C07 and C08 (subspecies IIIb). There was some bootstrap support (68%) for the separation of the subspecies that exhibit predominantly or exclusively diphasic expression of the flagella Ag (subspecies I, C01 and C02; II, C03 and C04; IIIb, C07 and C08; and VI, C13 and C14) from the monophasic subspecies (IIIa, C05 and C06; IV, C09 and C10; *S. bongori* C11 and C12; and VII, C15 and C16).

The two *S. bongori* strains, C11 and C12, were the most divergent by FAFLP, followed by the two strains of subspecies IIIa (C05 and C06). The strains of subspecies VII were divergent from those of subspecies IV, with 38 amplified-fragment differences between C10 (subspecies IV) and C15 (subspecies VII). Only 18 amplified fragments were common to all four strains. However, the two subspecies clustered to each other more than with other subspecies, with 78% bootstrap support.

FAFLP analysis of *Salmonella* subspecies I (SARB)

Both the strains of *S. typhi* were found in Cluster A, with high bootstrap support (100%) (Fig. 2). Cluster B consisted of two strains of *Salmonella* Choleraesuis (B04 and B06) and one of *Salmonella* Typhisu (B69), all three of which were epidemiologically unrelated strains from swine, as well as two strains of *Salmonella* Paratyphi C (B48 and B49). The latter two strains had identical FAFLP profiles. There were seven amplified-fragment differences between the cluster B strains and this cluster had 88% bootstrap support. The third strains of *S. Choleraesuis* (B05) and of *S. Paratyphi* C (B50) were very divergent from the two strains found in cluster B, as was the second strain of *S. Typhisu* (B70).

FAFLP analysis of the *S. typhimurium* complex (SARA)

Six of the ten strains of *Salmonella* Muenchen (A63–A68) clustered together (cluster C) and were divergent from the others of this serotype (Fig. 3). This cluster had 97% bootstrap support and there were 12 amplified fragments different between the six strains. Three of the remaining four strains of *S. Muenchen* also grouped with cluster C, but without bootstrap support. The strains of *S. Paratyphi* B were found to be diverse by FAFLP, although tight clustering was observed for

some groups of these strains. Fifteen of the 22 *S. Paratyphi* B strains in SARA were found in Group 1, four in Group 3 and one in Group 4. The remaining two strains of this serotype were very diverse by FAFLP from the clusters and groups described above. Cluster D in group 1 contained five *S. Paratyphi* B strains (A51–A55) that were identical by FAFLP, and cluster D had 76% bootstrap support. There were a further four *S. Paratyphi* B strains (A41–A44) that were identical by FAFLP in group 1; however, these strains had no bootstrap support and were therefore not considered a cluster. The four strains of *S. Paratyphi* B (A57–A60) in Group 3 had 85% bootstrap support. There were six amplified fragments different between the four strains in group 3.

Group 2 was composed of nine strains of *Salmonella* Heidelberg (A30–A35, A37, A39 and A40); however, there was no bootstrap support for the clustering of these isolates. The remaining strain of *S. Heidelberg* (A36) was found near group 2. Six of the seven strains of serotype *Salmonella* Saintpaul in SARA formed Group 4 (A22–A28), together with one strain of *S. Muenchen* (A70), though there was no bootstrap support for clustering of these strains. The remaining strain of *S. Saintpaul* (A29) clustered away from this group. The strains of *S. typhimurium* (A01–21) were found near each other; however, there was no bootstrap support for clustering or grouping of these strains.

SARA, B and C combined

The dendrogram of SARA, B and C strains is rooted at C11 and C12 (*S. bongori*) (Fig. 4). Cluster A contained all three *S. typhi* strains, with only seven amplified fragments different between them. This cluster, supported by a bootstrap value of 99%, also appeared very divergent from other serotypes by FAFLP. Cluster B, also seen in Fig. 2, remained in Fig. 4 when data from all three collections were combined, but with slightly lower bootstrap support than described previously (83%). Cluster C consisted of *S. Muenchen* strains and is also seen in Fig. 3. Found near cluster C, but not supported by bootstrap analysis, were a further five strains of *S. Muenchen* (A69, A71, B33, B35 and B36). The only remaining strain of this serotype (B34) was very divergent from this cluster.

Seventeen *S. Paratyphi* B strains and one *S. Paratyphi* A strain formed group 1 (also seen in Figs 2 and 3). Within this group was cluster D (also seen in Figs 2 and 3).

Group 2 consisted of nine of the 13 *S. Heidelberg* strains. One strain of *S. Heidelberg* (represented by A36 and B24) was found near this group, while the two remaining strains (A30 and B23) now clustered with *S. enteritidis* B18 and *Salmonella* Newport B37, with

80% bootstrap support. Five strains of *S. Paratyphi B* (A57–A60 and B46) and one of *Salmonella* Stanley (B60) constituted Group 3. This group had 62% bootstrap support and there were 13 amplified fragments different between these strains.

Twelve strains of serotypes *S. Saintpaul*, *Salmonella* Haifa, *S. Paratyphi B* and *Salmonella* Panama formed Group 4, but there was no bootstrap support for clustering of these strains. Two strains of *S. Saintpaul* (A29 and B56) and *Salmonella* Sendai B58 clustered separately from this group, with 100% bootstrap support. These strains had identical *Eco*+0 *Mse*+TA FAFLP profiles, but B58 had an extra amplified *Eco*+0 *Mse*+T fragment at 200 bp (data not shown), not present in A29 or B56. Strains of *S. Paratyphi B* were found to be very diverse by FAFLP. As well as the 17 strains found in Group 1, there were five in Group 3 and two in Group 4. The remaining two strains of this serotype were very diverse by FAFLP from the groups and clusters described above. *S. enteritidis* strains (B16–B19) were also very diverse by FAFLP, with strains being found throughout the tree.

In silico FAFLP

In silico FAFLP analysis demonstrated that no *Eco*RI or *Mse*+TA restriction sites were present in the *inv/spa* genes of C09 (subspecies IV), C15 and C16 (both subspecies VII). One *Mse*+TA site was present in C10 (subspecies IV) at –180 bp from the 3' end. However, there were no *Eco*+0 *Mse*+TA amplified fragments above this size present only in this strain, and not in the others of subspecies IV or VII. It is therefore unlikely that there is an *Eco*RI restriction site in close proximity to give an amplified fragment that would be detected in this analysis.

DISCUSSION

The results shown here correspond more closely with the genetic structure of *Salmonella* using the medical microbiology system of taxonomic definition. This consists of two *Salmonella* species, *S. bongori* and *S. choleraesuis* (or *S. enterica*), the latter of which is further subdivided into six subspecies. Classical *Salmonella* taxonomy recognizes these two species, but also includes *S. enteritidis*, *S. typhimurium* and *S. typhi* as *Salmonella* species. FAFLP shows that *S. enteritidis* and *S. typhimurium* are found within the main cluster of subspecies I strains and do not appear to be sufficiently divergent to be regarded as separate *Salmonella* species. *S. typhi* was more diverse by FAFLP, but still not necessarily sufficiently divergent to be considered a separate species. These FAFLP results are supported by MLEE, which also includes these serotypes in subspecies I (Boyd *et al.*, 1993).

MLEE and sequencing studies have been used to establish the population genetic frameworks (i.e. macro-variation) for *Salmonella*. Many MLEE studies have been performed, but there is no consensus among

researchers regarding which enzymes should be chosen, with the result that over 40 housekeeping enzymes have been used in various studies (Selander *et al.*, 1986, 1990a, b, 1992; Beltran *et al.*, 1988, 1991; Reeves *et al.*, 1989; Boyd *et al.*, 1993, 1996; Osaka *et al.*, 1999). Sequencing studies have been based on only a fraction of the genes encoding these proteins (Nelson & Selander, 1992, 1994; Boyd *et al.*, 1994, 1996; Nelson *et al.*, 1997; Wang *et al.*, 1997). Accurate comparison of the FAFLP results with those obtained using MLEE is therefore difficult, especially as the three reference collections have never been brought together before. However, we find that our FAFLP results, while supporting a number of findings from MLEE studies, are more closely related to the earlier hybridization studies and more recent sequencing studies of housekeeping genes and invasion genes (Boyd *et al.*, 1996).

SARC

FAFLP has been successful in distinguishing the MLEE-defined subspecies of *Salmonella*, each pair being found clustered together. These FAFLP results also support MLEE and DNA hybridization results in confirming that *S. bongori* is the most divergent form of *Salmonella* and should be regarded as a separate species (Reeves *et al.*, 1989; Boyd *et al.*, 1996). In addition, FAFLP analysis shows that the two strains of subspecies IIIa are the second most divergent form of *Salmonella*. This finding corresponds to sequencing studies of housekeeping and invasion genes, where this subspecies is second only to *S. bongori* in its divergence from the other subspecies. This is not, however, reflected in the MLEE findings, where subspecies IIIa is not divergent and is found clustering with subspecies I, IIIb and VI (Boyd *et al.*, 1996).

FAFLP supports the MLEE data in separating the strains of subspecies VII from those of subspecies IV, which are grouped together by biotype. Sequence analysis of five housekeeping genes revealed that the degree of nucleotide divergence between subspecies IV and VII was at least as great as that between subspecies I and VI or II and IIIb. However, little difference was observed in the sequence of their invasion genes (Boyd *et al.*, 1996). On the basis of these findings, Boyd *et al.* (1996) proposed a model of evolution based on a mosaic genome structure for strains of subspecies VII. They proposed that the genome of the group VII strains is a mosaic of large chromosomal segments with different evolutionary histories, the major part of the chromosome originating from an old lineage that has achieved a level of genetic differentiation similar to the other subspecies of *S. choleraesuis*. Presumably, it is this segment of the genome that is being assayed by MLEE and includes the five housekeeping genes. In this study, we have identified 18 polymorphic amplified fragments common to all four strains of these two subspecies. Recent horizontal transfer of gene segments including the *inv/spa* region from subspecies IV

to VII could account for the similarity in this region between these two groups. *In silico* FAFLP analysis of the *inv/spa* genes of the two strains of subspecies IV and VII showed that no amplified fragments would be detected from these loci using the primer combination *Eco*+0 and *Mse*+TA, indicating that FAFLP distinguishes between groups IV and VII in a manner similar to that by MLEE and the study involving the housekeeping genes.

The mono- and diphasic subspecies of *Salmonella* clustered separately. This pattern was also observed in the clustering of *Salmonella* based on the nucleotide sequences of housekeeping and invasion genes (Boyd *et al.*, 1996). Selander & Nelson (1996) proposed an evolutionary model to explain these findings: approximately 160–180 million years ago, *E. coli* and *S. choleraesuis* evolved from a common ancestor. *E. coli* developed as a commensal and opportunistic pathogen of mammals and birds, while *Salmonella* was associated with reptiles. Subsequently, members of *Salmonella* evolved as intracellular pathogens through the acquisition of invasion and other genes that mediate invasion of the host epithelial cells. By providing an increased ability to evade the host immune system, the development of diphasic variation in a lineage ancestral to subspecies I, II, IIIb and VI may have been essential to allow the exploitation of a new ecological niche in mammals and birds as a pathogen. Subspecies I then became highly specialized for mammals and birds, with some serotypes adapting to a single host species, e.g. *S. typhi*. However, some serotypes of subspecies I and II have since reverted to monophasic expression of the flagellar genes.

SARA and SARB (*Salmonella* subspecies I)

Of the polyphyletic serotypes described by Boyd *et al.* (1993), three (*S. enteritidis*, *S. Newport* and *S. Typhimurium*) are highly divergent by FAFLP when all three reference collections are brought together. Of the remaining serotypes, five (*S. Choleraesuis*, *S. Muenchen*, *S. Paratyphi B*, *S. Paratyphi C* and *S. Saintpaul*) have some ETs that are found in groups or clusters, while other serotypes have separate, divergent ETs. For example, the two ETs of *Salmonella* Derby (B09 and B10) clustered together by FAFLP with high bootstrap support, but these were identified as being highly divergent by MLEE, belonging to two very distantly related lineages, I and III (Beltran *et al.*, 1988).

S. Paratyphi B ETs were found in FAFLP groups 1, 3 and 4, but the remaining ETs of this serotype were highly divergent and fall outside these groupings. *S. Paratyphi B* strains can be separated according to biotype. Strains that are D-tartrate-positive and slime wall-negative are designated *S. Paratyphi* biotype Java, whereas those that are D-tartrate-negative and slime wall-positive are designated *S. Paratyphi B* biotype Paratyphi B (Duguid *et al.*, 1975; Barker *et al.*, 1988). Strains designated as *S. Paratyphi B* biotype

Java are highly divergent by FAFLP, with two strains being found in cluster D, four in Group 3, two in Group 4 and a further three not falling into any of the groups or clusters. *S. Paratyphi B* biotype Paratyphi B, on the other hand, appears to be a more homogeneous group of strains, the majority of which are identical by FAFLP and are found in Group 1. A further strain of biotype Paratyphi B (ET Pb 5) is found in Group 3. These results correspond well to those of MLEE. Most of the biotype Paratyphi B strains, which cause enteric fever, are members of a globally distributed clone (ET Pb 1). In contrast, the biotype Java strains, which generally cause only gastroenteric disease, are highly heterogeneous (Selander *et al.*, 1990a). This phenomenon is thought to be influenced by host adaptation, with the MLEE results suggesting that clones of human-adapted serotypes, e.g. *S. typhi* and *S. Paratyphi C*, are fewer in number and have less diverse genotypes than those that have a broad host range, e.g. *S. Heidelberg*, *S. typhimurium* and *S. enteritidis* (Selander *et al.*, 1990a). FAFLP shows that the three strains of *S. typhi* cluster together, as do two strains of *S. Paratyphi C*, while the third is found to be more divergent. In contrast, strains of *S. Heidelberg*, *S. typhimurium* and *S. enteritidis*, in particular, are highly divergent by FAFLP.

FAFLP versus MLEE

Changes in the amino acid sequence that do not affect the net electrostatic charge of the protein will not be detected by MLEE. Variation in the nucleotide sequence that does not alter the amino acid sequence will also go undetected by MLEE. It is estimated that 10–20% of sequence variation could therefore go undetected (Selander *et al.*, 1986). In contrast, the migration rate of an FAFLP fragment is determined by its size, which is in turn determined by the position of specific restriction sites and the surrounding sequences. Mutations such as insertions or deletions will be detected by FAFLP. However, substitutions might go undetected unless they occur in the restriction site or in the selective bases surrounding it, or if they have an effect on the secondary structure of the fragment that alters the rate of migration in some way. Therefore, a major advantage of FAFLP above MLEE and current sequence-based approaches is that the genome is sampled throughout its length at both variable and non-variable regions, with little or no bias. By contrast, methods such as MLEE, MLST and other sequence-based approaches only sample small, highly conserved and slowly evolving segments of the genome.

In silico FAFLP modelling on the sequenced *E. coli* K-12 MG 1655 genome revealed that FAFLP, using the selective primer combination *Eco*+0 *Mse*+TA, sampled 0.25% of the genome (Arnold *et al.*, 1999b). We have determined for this study, using the same selective primer combination, that we have the potential to sample approximately 0.85% of a given genome (based on the *S. typhimurium* genome size of 4.5 Mb). How-

ever, each individual strain contains only a proportion of the fragments included in this study. For *S. typhimurium* C01, therefore, 0.19% of the genome is being sampled.

We find that FAFLP is successful in discriminating between the subspecies of *Salmonella* and that it compares well with MLEE in identifying clonal lineages. We propose that FAFLP is a powerful tool for population studies, as it provides an unbiased view of whole-genome variation in natural populations of *Salmonella*.

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