

Prophage Sequences Defining Hot Spots of Genome Variation in *Salmonella enterica* Serovar Typhimurium Can Be Used To Discriminate between Field Isolates[∇]

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Sixty-one *Salmonella enterica* serovar Typhimurium isolates of animal and human origin, matched by phage type, antimicrobial resistance pattern, and place of isolation, were analyzed by microbiological and molecular techniques, including pulsed-field gel electrophoresis (PFGE) and plasmid profiling. PFGE identified 10 profiles that clustered by phage type and antibiotic resistance pattern with human and animal isolates distributed among different PFGE profiles. Genomic DNA was purified from 23 representative strains and hybridized to the composite *Salmonella* DNA microarray, and specific genomic regions that exhibited significant variation between isolates were identified. Bioinformatic analysis showed that variable regions of DNA were associated with prophage-like elements. Subsequently, simple multiplex PCR assays were designed on the basis of these variable regions that could be used to discriminate between *S. enterica* serovar Typhimurium isolates from the same geographical region. These multiplex PCR assays, based on prophage-like elements and *Salmonella* genomic island 1, provide a simple method for identifying new variants of *S. enterica* serovar Typhimurium in the field.

Salmonella enterica is a significant cause of morbidity and mortality for human and veterinary medicine, with multiantibiotic-resistant *S. enterica* serovar Typhimurium being an emerging problem. An important source of *S. enterica* serovar Typhimurium in human infections is contaminated food of animal origin, particularly meat products derived from cattle (9). *S. enterica* serovar Typhimurium can survive in the environment, and once established on a farm, contamination can be difficult to eradicate. *Salmonella* may spread from farm to farm through the exchange of livestock, by wildlife, or in the runoff from fields, and it can disseminate in food chains as a consequence of further cross-contamination at slaughterhouses. Food-borne transmission of common types of *S. enterica* serovar Typhimurium found in cattle, such as definitive phage type 104 (DT104), is well documented for human outbreaks, with sources ranging from roast beef to unpasteurized milk (29). Moreover, animals infected with antibiotic-resistant *Salmonella* are an important source of resistance determinants that can transfer to human-infective *Salmonella* serovars.

Many methods have been developed to phenotypically distinguish between *S. enterica* serovar Typhimurium isolates, including antibiotic susceptibility profiling, phage typing (1), pulsed-field gel electrophoresis (PFGE) (7), and plasmid profiling (26) as well as various PCR-based techniques (8, 15, 19).

However, since the genome sequences of several *S. enterica* strains, including different *S. enterica* serovar Typhimurium strains, are now available, it should be possible to design rational DNA tools based on fully annotated DNA sequences for use in the field to monitor strain diversity. Here, we have used some of the existing classical typing techniques to analyze a matched collection of *S. enterica* serovar Typhimurium strains isolated from animal and human sources and have extended these approaches to include DNA microarray analysis. Using these techniques, we have been able to identify and map regions of variation on the chromosome of *S. enterica* serovar Typhimurium that discriminate between isolates circulating in the same geographical region. Using this information, we have designed multiplex PCR assays that are simple to use and that are able to rapidly distinguish between *S. enterica* serovar Typhimurium isolates in a cost-effective manner. We believe that similar PCR assays, constructed on the basis of regions of variation in other *Salmonella* serovars, have the potential to improve the local epidemiological analysis of *Salmonella* outbreaks.

MATERIALS AND METHODS

Bacterial isolates. Thirty isolates of *S. enterica* serovar Typhimurium of animal origin (prefix A) (26 from cattle feces, 2 from pig feces, and 2 from crow feces) isolated between February 2000 and August 2002 from eight farms were selected from the Wellcome Trust International Partnership Research Award in Veterinary Epidemiology consortium collection. They were chosen to represent a number of phage types and phenotypic antibiotic sensitivity patterns (Table 1). Thirty-one well-characterized human isolates (prefix H) from the Scottish *Salmonella* Reference Laboratory (Glasgow) subsequently were selected to match the animal strains by phage type, antibiotic resistance pattern, and place and time

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TABLE 1. Origin of 30 animal and 31 human *S. enterica* serovar Typhimurium isolates

Isolate	Source	Sample date (day/mo/yr)	Location (animal health district or sending laboratory ^a)	Farm code or location of patient	Note on infection type
Animal					
AWF002005	Bovine	18/02/2000	Central	A03	
AWF002008	Bovine	18/02/2000	Central	A03	
AWF007246	Bovine	15/11/2001	Highlands	S009	
AWF007581	Bovine	06/12/2001	Highlands	S009	
AWF009126	Bovine	16/08/2002	Highlands	S011	
AWF009144	Bovine	16/08/2002	Highlands	S011	
AWF009147	Bovine	16/08/2002	Highlands	S011	
AWX003485	Bovine	30/08/2001	Highlands	S009	
AWX003658	Bovine	30/08/2001	Highlands	S009	
AWX004814	Bovine	13/12/2001	Highlands	S009	
AWX004816	Bovine	13/12/2001	Highlands	S009	
AWX005861	Bovine	16/04/2002	Highlands	S009	
AWX006747	Pig	29/04/2002	Highlands	S010	
AWX006748	Pig	29/04/2002	Highlands	S010	
AWX008764	Crow	16/08/2002	Highlands	S011	
AWX008765	Crow	16/08/2002	Highlands	S011	
AWF004105	Bovine	12/12/2000	North East	S001	
AWF004135	Bovine	12/12/2000	North East	S001	
AWF004526	Bovine	16/01/2001	North East	S001	
AWF004602	Bovine	16/01/2001	North East	S001	
AWX000826	Bovine	10/10/2000	North East	S001	
AWX000836	Bovine	10/10/2000	North East	S001	
AWX000841	Bovine	10/10/2000	North East	S001	
AWX000847	Bovine	10/10/2000	North East	S001	
AWX001996	Bovine	23/01/2001	North East	S006	
AWX001997	Bovine	23/01/2001	North East	S006	
AWX002732	Bovine	19/06/2001	North East	S300	
AWX002743	Bovine	19/06/2001	North East	S300	
AWX002821	Bovine	19/06/2001	North East	S301	
AWX002823	Bovine	19/06/2001	North East	S301	
Human					
H20003447	Human	16/08/2000	Aberdeen	Aberdeen	Sporadic
H20003919	Human	14/09/2000	Aberdeen	Aberdeen	Sporadic
H20021958	Human	29/07/2002	Aberdeen	Aberdeen	Sporadic
H20004019	Human	21/09/2000	Aberdeen	Elgin	Sporadic
H20010345	Human	13/01/2001	Aberdeen	Elgin	Sporadic
H20022475	Human	21/08/2002	Aberdeen	Elgin	Sporadic
H19994475	Human	15/11/1999	Aberdeen	Fraserburgh	Sporadic
H20020688	Human	23/03/2002	Aberdeen	Not available	Sporadic
H19992292	Human	12/08/1999	Arbroath	Arbroath	Sporadic
H19991017	Human	13/05/1999	Ayr	Ayr	Family outbreak
H20014835	Human	13/12/2001	Ayr	Cumnock	Sporadic
H20021651	Human	21/06/2002	Ayr	Irvine	Sporadic
H19990100	Human	26/01/1999	Ayr	Stewarton	Sporadic
H20002800	Human	08/07/2000	Dumfries	Not available	Sporadic
H20004164	Human	05/10/2000	Dumfries	Not available	Sporadic
H19990457	Human	15/03/1999	Dumfries	Thornhill	Sporadic
H20021876	Human	25/07/2002	Dundee	Angus	Sporadic
H20023824	Human	14/11/2002	Dundee	Angus	Sporadic
H19990170	Human	08/02/1999	Dundee	Dundee	Travel history
H20020895	Human	03/04/2002	Dundee	Dundee	Sporadic
H20021856	Human	24/07/2002	Dundee	Dundee	Sporadic
H19963477	Human	09/08/1996	Edinburgh	Not available	Sporadic
H20003668	Human	28/08/2000	Fife	Edinburgh	Sporadic
H20012056	Human	08/06/2001	Fife	Edinburgh	Sporadic
H19990749	Human	20/04/1999	Hawick	Hawick	Sporadic
H19991651	Human	09/07/1999	Monklands	East Kilbride	Sporadic
H20004937	Human	24/11/2000	Monklands	Strathaven	Sporadic
H20023530	Human	25/10/2002	Monklands	Strathaven	Sporadic
H19992329	Human	13/08/1999	Paisley	Paisley	Sporadic
H20004467	Human	20/10/2000	Stirling	Alloa	Sporadic
H20013531	Human	04/09/2001	Stirling	Dunblane	Sporadic

^a Note that the sending laboratory is the local diagnostic microbiology laboratory that submitted the isolate to the reference laboratory. In Scotland, the local diagnostic microbiology laboratory may cover very large geographical areas, particularly if the outlying islands are considered. Thus, the location of the patient was taken from the first three letters/numbers of the regional postal code of the address given. The complete postal code was not available due to patient confidentiality.

of isolation, where possible. The human isolates had been received by the Scottish *Salmonella* Reference Laboratory from 12 regional laboratories between August 1996 and November 2002. Most human isolates (29/31) were from sporadic cases, but one was part of a family outbreak and one patient had a recent travel history. Identification by culture, serology (based on standard laboratory agglutination tests), and phage typing (1) was performed at the aforementioned laboratory. Additional laboratory reference strains included in the analyses were *S. enterica* serovar Typhimurium DT104 (NCTC 13348), *S. enterica* serovar Typhimurium LT2 (ATCC 700220), and *S. enterica* serovar Typhimurium SL1344 (NCTC 13347).

Antibiotic resistance. The phenotypic sensitivity pattern of each isolate was determined by breakpoint antibiotic agar incorporation (10). The antimicrobials and breakpoints (in milligrams per liter) were as follows: ampicillin (A), 128 (high-level breakpoint only); chloramphenicol (C), 8; gentamicin (G), 4; kanamycin (K), 16; streptomycin (S), 16; sulfonamides (sulfathiazole) (Su), 64; spectinomycin (Sp), 64; tetracyclines (T), 128 (high-level breakpoint only); trimethoprim (Tm), 2; furazolidone (Fu), 8; nalidixic acid (Nx), 16; ciprofloxacin, 0.125 and 1.0; cephalixin, 16; cephradine, 16; cefuroxime, 16; ceftriaxone, 1; and cefotaxime, 1. Breakpoint antibiotic agar incorporation was performed by diluting 10 μ l test culture (obtained by overnight culture on Luria-Bertani [LB] agar at 37°C) with 0.5 ml normal saline in the well of a multipoint inoculator plate. Antibiotic-containing Isosensitest agar plates (Oxoid) were inoculated together with a control plate (no antibiotic). An antibiotic-sensitive *S. enterica* serovar Typhimurium strain (42R500) was cultured on all plates as a control. After overnight incubation, growth was recorded on the basis of its variation from the growth on the control plate.

Plasmid DNA extraction. Plasmid DNA was extracted from each isolate using a modified Kado and Liu protocol (14). Each isolate was cultured in nutrient broth at 37°C overnight, and then 1 ml of culture was centrifuged at 13,000 rpm for 5 min and the supernatant was removed. The pellet was resuspended in 20 μ l Tris-EDTA buffer and then mixed with 100 μ l 0.05 M Tris-3% sodium dodecyl sulfate (SDS). After the mixture was heated at 55°C for 30 min, 100 μ l phenol-chloroform was added and emulsified by brief shaking. After being centrifuged at 13,000 rpm for 5 min, 25 μ l of the upper aqueous layer was loaded onto a 0.6% agarose gel containing the *Escherichia coli* transconjugant 39R861 (27), which harbors plasmids of 7.5, 39, 68, and 160 kb, as a size marker. Plasmid molecular masses were calculated by logarithmic comparison to the mass of this marker. The plasmid preparation was repeated for a number of isolates to assess reproducibility.

Genomic DNA extraction. Bacteria were cultured in LB broth or on LB agar overnight at 37°C. Genomic DNA was extracted using either the cetyltrimethylammonium bromide method (Sigma-Aldrich, Ltd., Dorset, United Kingdom) (3) or a QIAGEN tissue DNA extraction kit according to manufacturer's instructions.

Macrorestriction of genomic DNA visualized by PFGE. One milliliter of the overnight culture was resuspended in 1 ml 0.85% NaCl and centrifuged at 13,000 rpm for 5 min. The pellet was resuspended in 0.85% NaCl at a cell density equivalent to a McFarland standard of 3.0. To prepare the plugs, 500 μ l 2% chromosomal agarose (Bio-Rad, Hemel Hempstead, United Kingdom) at 55°C was mixed with an equal volume of cells; 500 μ l of the mixture was dispensed into plug molds and cooled at 4°C for 20 min. The plugs were placed in 2 ml lysis solution (500 mM EDTA, 1% [wt/vol] sarkosyl [aqueous]) overnight at 55°C, with 40 μ l 1-mg/ml proteinase K. The following day, plugs were washed at 55°C three times with water and four times with ET₁₀ (10 mM Tris-Cl, 10 mM EDTA, pH 8); each wash was 15 min long. Plugs were digested with 1 U of XbaI (Roche) overnight at 37°C. The digested plugs were loaded onto a 1% agarose gel and run on a contour-clamped homogeneous electric field DRII system (Bio-Rad) at 14°C with 2 liters of 0.5 \times TBE (50 mM Tris, 50 mM boric acid, 0.5 mM EDTA). The switch times ranged from 6 to 72 s during a 44-h run at 5.4 V/cm. XbaI-digested DNA from *S. enterica* serovar Braenderup H9812 was used as a molecular reference marker every five lanes (22).

Statistical analyses of restriction patterns were performed with BioNumerics software (Applied Maths, St. Martens-Latern, Belgium) using the dice similarity coefficient. A dendrogram was calculated by the unweighted pair group method of averages, with a position tolerance of 1%. Fragments smaller than 30 kb were not included in the final analysis, as recommended by the European guidelines for standardization (20). Different XbaI-PFGE profiles were assigned to different PFGE groups according to a single band difference in restriction pattern (11, 20) rather than according to the criteria of Tenover et al. (24).

Salmonella microarray. *Salmonella* Microarray Generation III is an extension of the previously described *Salmonella* Microarray Generation I array constructed at the Wellcome Trust Sanger Institute (2, 4, 25). Generation III includes additional coding sequences from the *Salmonella* genomes being se-

quenced at The Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/Projects/Salmonella/>). Thus, Microarray Generation III is an essentially nonredundant array that contains features representing the following eight genomes: *S. enterica* serovar Typhi CT18, *S. enterica* serovar Typhi Ty2, *S. enterica* serovar Typhimurium LT2 (ATCC 700220) (17), *S. enterica* serovar Typhimurium DT104 (NCTC 13348), *S. enterica* serovar Typhimurium SL1344 (NCTC 13347), *S. enterica* serovar Enteritidis PT4 (NCTC 13349), *S. enterica* serovar Gallinarum 287/91 (NCTC 13346), and *S. bongori* 12419 (ATCC 43975).

Hybridization procedures. DNA was extracted from 23 isolates that had been selected to represent PFGE profiles 1 to 10, and the different phage types, resistance patterns, and plasmid profiles from animal and human origins, where available. Genomic DNA was competitively hybridized to *Salmonella* Microarray Generation III against *S. enterica* serovar Typhimurium DT104 (NCTC 13348) genomic DNA as the control. Genomic DNA was sonicated (10 s, level 2; Virsonic sonicator) and then fluorescently labeled with Cy5 (test) or Cy3 (control) using the Bioprime kit (Gibco-BRL). Dye-swap labeling experiments also were performed for each test sample. Labeled DNA was purified using an Autoseq G-50 Amersham column, denatured, and precipitated, and the resulting probes were hybridized to the microarray slide for 16 h at 49°C in a hybridization chamber (Genetix X2530). Three slides per isolate were hybridized. Washing procedures were stringent (two washes at 65°C in 2 \times SSC-0.1% SDS for 30 min and two washes at 65°C in 0.1 \times SSC for 30 min [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate]).

Microarray analysis. Hybridization to microarray slides was detected by using a Genepix 4000B scanner (Axon Instruments, Inc.) and quantified by using Genepix Pro software (Axon Instruments, Inc.). Signal intensities were corrected by subtracting the local background values. Normalization was performed across all features on the array before any filtering took place. Data were ordered and labeled according to the gene names of the genomes from which each array feature was derived, and BLAST_N analysis, using the array feature for DNA sequence as the query, was used to identify the gene in the *S. enterica* serovar Typhimurium DT104 (NCTC 13348) genome (loci SDT0001 to SDT4571) that was most similar to the tested gene. Signal ratios were analyzed by three methods to establish the cutoff values for designating genes present, absent, and uncertain: (i) twofold variation, which is the default on GeneSpring microarray analysis software V7.2 (Silicon Genetics); (ii) median value (per strain) \pm 3 standard deviations (28); and (iii) the gene-calling program GACK, which is based on log₂ ratios, with trinary analysis (16). The sensitivity, specificity, and positive and negative predictive values of each method using the E score and bit score were determined for the hybridization results of *S. enterica* serovar Typhimurium DT104 (NCTC 13348) compared to those of *S. enterica* serovar Typhimurium LT2. GACK analysis, using mirroring, produced the highest sensitivity, specificity, and positive and negative predictive values, and it was used to assign the present, absent, or uncertain status to each of the 4,167 loci representing the chromosomal features of *S. enterica* serovar Typhimurium DT104. Information concerning *Salmonella* Microarray Generation III has been deposited in ArrayExpress as A-SGRP-4, and experimental data are available under the accession number E-SGRP-6 (<http://www.ebi.ac.uk/arrayexpress/>).

Multiplex PCR. Oligonucleotides for PCR analyses were designed to explore the main regions of variation highlighted by the microarray data (see Table 3). These regions were *Salmonella* genomic island 1 (SGI1), which is well described for *S. enterica* serovar Typhimurium DT104 (5, 6, 18), and five prophage-like elements. Multiplex PCR was performed on the 23 isolates to confirm the microarray hybridization results. PCRs contained template DNA (1 to 4 μ l), primer pairs (0.5 to 1 μ l), and Invitrogen Platinum PCR Supermix (23 to 46 μ l). The following PCR protocol was used for all oligonucleotide pairs, except for pairs P8, P9, and P10: 94°C for 1 min; 30 cycles of 94°C for 30 s (denaturation step), 65°C for 1 min (annealing step), and 72°C for 5 min; and 72°C for 2 min. For oligonucleotide pairs P8, P9, and P10, the annealing step was 61°C for 1 min.

RESULTS

Origin of *Salmonella* strains and microbiological analysis. Sixty-one *S. enterica* serovar Typhimurium strains collected in Scotland were subjected to typing using established phenotypic techniques. These approaches allowed us to assess the amount of variation present in an *S. enterica* serovar Typhimurium population gathered in a similar time period from the same geographical region. Thirty *S. enterica* serovar Typhimurium strains, representing five different phage types, were obtained

TABLE 2. Results of microbiological analysis of *S. enterica* serovar Typhimurium isolates

Isolate	PFGE profile	Phage type	Antibiotic resistance profile ^a	Plasmid profile (kb)	Hybridized to microarray
Animal					
AWF002005	1	104	ACSSuSpT	96, 3.0	No
AWF002008	1	104	ACSSuSpT	96, 3.0	Yes
AWF004526	1	104	ACSSuSpT	96	No
AWF004602	1	104	ACSSuSpT	99	No
AWX000836	1	104	ACSSuSpT	99	No
AWX000847	1	104	ACSSuSpT	99	No
AWX001996	1	104	ACSSuSpT	97	No
AWX001997	1	104	ACSSuSpT	97	No
AWX002732	1	104	ACSSuSpTNxpCp	97	No
AWX002743	1	104	ACSSuSpTNxpCp	97	No
AWX002821	1	104	ACSSuSpTNxpCp	97	Yes
AWX002823	1	104	ACSSuSpTNxpCp	97	No
AWX000826	1	104b	ACSSuSpT	99	Yes
AWF004105	1	104b	ACSSuSpT	96	No
AWX000841	1	Nontypeable	ACSSuSpT	99	Yes
AWF004135	1	Nontypeable	ACSSuSpT	96	No
AWF007246	2	170	Sensitive ^b	Plasmid free	No
AWF009144	2	170	Sensitive	Plasmid free	No
AWF009147	2	170	Sensitive	Plasmid free	Yes
AWX003485	2	170	Sensitive	Plasmid free	No
AWX005861	2	170	Sensitive	Plasmid free	No
AWX008764	2	170	Sensitive	Plasmid free	No
AWX008765	2	170	Sensitive	Plasmid free	No
AWX003658	2	168a	Sensitive	Plasmid free	Yes
AWF007581	2	Nontypeable	Sensitive	Plasmid free	Yes
AWX004816	2	Nontypeable	Sensitive	70	Yes
AWX004814	4	193	Sensitive	Plasmid free	Yes
AWX006747	7	RDNC	ASSuT	100	Yes
AWX006748	7	RDNC	ASSuT	100	Yes
AWF009126	10	170	SuSpTm	99	Yes
Human					
H19990457	1	104	ACSSuSpT	96	No
H19991017	1	104	ACSSuSpT	93	No
H19992329	1	104	ACSSuSpT	93, 2.5	No
H20002800	1	104	ACSSuSpT	93	Yes
H20003447	1	104	ACSSuSpT	93	No
H20003668	1	104	ACSSuSpT	93	No
H20004164	1	104	ACSSuSpT	104	No
H20010345	1	104	ACSSuSpT	104	No
H20014835	1	104	ACSSuSpT	104	No
H20013531	1	104	ACSSuSpTNx	104, 5.6, 4.0, 3.6, 3.2	No
H19991651	1	104	ACSSuSpTNxpCp	93, 72	No
H20020688	1	104	ACSSuSpTNxpCp	104	No
H19990100	1	104b	ACSSuSpT	96, 72, 5.7, 4.1, 3.1	No
H20021651	1	104b	ACSSuSpT	100, 6.8	Yes
H19990749	1	Nontypeable	ACSSuSpT	93, 7.1	No
H19994475	1	Nontypeable	ACSSuSpT	93, 2.5	No
H20021958	1	Nontypeable	ACSSuSpT	100, 15, 3.2, 2.0	Yes
H19990170	2	170	Sensitive	3.5	No
H20004019	2	170	Sensitive	58	Yes
H20004937	2	170	SSuSp	Plasmid free	No
H20012056	2	170	Sensitive	Plasmid free	No
H20020895	2	170	Sensitive	Plasmid free	No
H20021856	2	170	Sensitive	Plasmid free	Yes
H20021876	2	170	Sensitive	Plasmid free	No
H20022475	2	170	Sensitive	Plasmid free	No
H19992292	3	170	Sensitive	Plasmid free	Yes
H19963477	3	170	SuTm	7.9, 4, 1.9	Yes
H20004467	5	Nontypeable	ACSSuSpTFu	141, 7.6, 3.5, 3.2	Yes
H20023824	6	170	Sensitive	Plasmid free	Yes
H20003919	8	RDNC	ASSuT	123, 49	Yes
H20023530	9	193	Sensitive	100, 6.3	Yes

^a Antibiotic abbreviations: ampicillin, A; chloramphenicol, C; streptomycin, S; sulfonamides, Su; spectinomycin, Sp; tetracyclines, T; trimethoprim, Tm; furazolidone, Fu; nalidixic acid, Nx; low-level (partial) ciprofloxacin resistance, pCp.

^b Sensitive to all antibiotics tested (see Materials and Methods for the agents used).

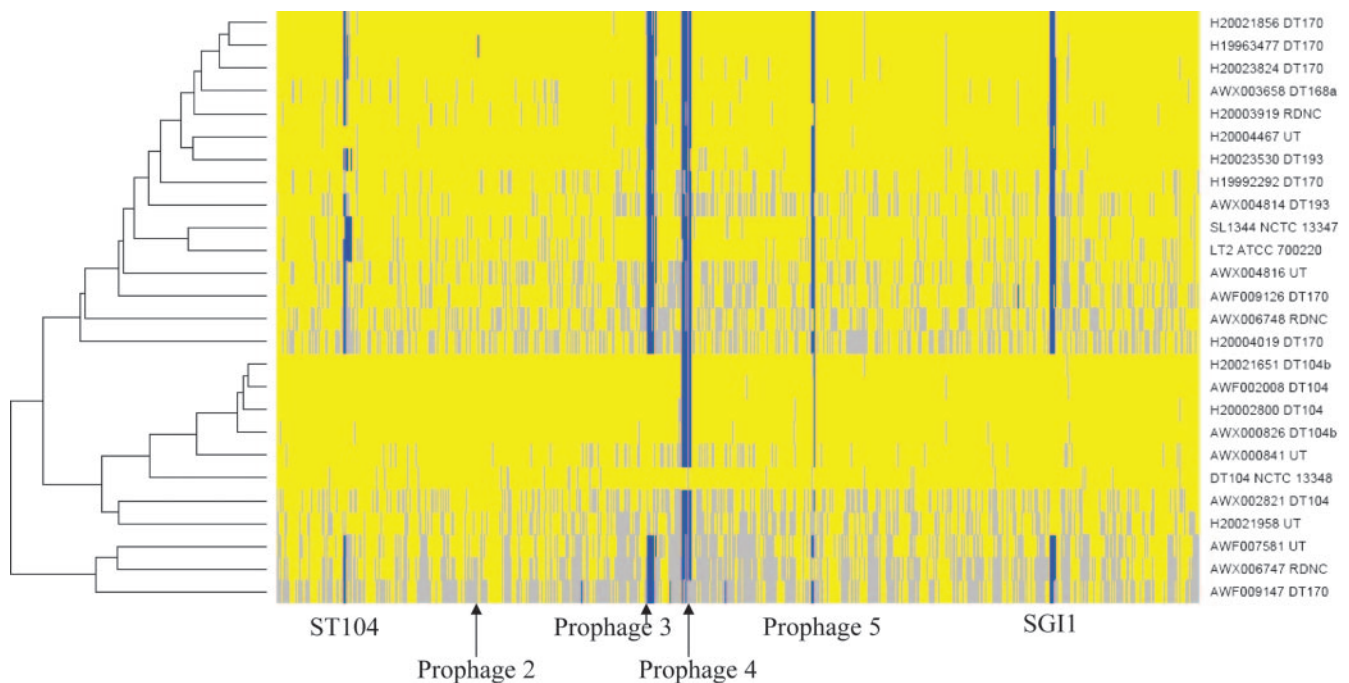


FIG. 1. Analysis of the microarray data by GACK. Test/reference ratios of the 24 *S. enterica* serovar Typhimurium isolates analyzed by microarray were assessed for the presence/absence of genes by using GACK software. The input data set was restricted to the 4,167 chromosomal features (SDT loci) expected to be present in one or more of the isolates. The heat map is plotted in the physical order of the SDT loci according to the order of the loci in the *S. enterica* serovar Typhimurium DT104 (NCTC 13348) genome. The presence of a gene in the test strain that is also present in the control strain (*S. enterica* serovar Typhimurium DT104 [NCTC 13348]) is shown in yellow, the absence or divergence of the gene from its presence in the control strain is shown in blue, and genes for which their presence was uncertain are in gray. The positions of prophage 1/ST104 and prophages 2 to 5 as well as SG11 are indicated.

from animals (26 from cattle, 2 from pigs, and 2 from crows) between 2000 and 2002 (Tables 1 and 2). Four of these isolates were nontypeable. The 31 isolates from humans were obtained from 12 Scottish laboratories between 1996 and 2002 and represented four phage types (Tables 1 and 2), with four isolates being nontypeable. Antimicrobial susceptibility testing revealed eight resistance patterns and included multiresistant isolates, with some exhibiting reduced susceptibility to ciprofloxacin (Table 2).

The isolates were subjected to PFGE analysis using the restriction enzyme XbaI, and 10 PFGE profiles were identified, with DNA fragments ranging from 10 kb to approximately 882 kb. The unweighted-pair-group-method-of-averages dendrogram constructed using BioNumerics software demonstrated clustering of PFGE restriction profiles by antibiotic resistance pattern and phage type. Significantly, there were indistinguishable restriction profiles produced from animal and human isolates of the same phage type and resistance pattern. The most common PFGE profile, PFGE 1, was observed in 33 isolates (16 animal isolates and 17 human isolates), including DT104 (24 isolates), DT104b (4 isolates), and 5 nontypeable isolates. All 33 isolates in PFGE 1 were multiresistant: 26 exhibited the resistance pattern ACSSuSpT (12 animal isolates and 14 human isolates), 6 exhibited the resistance pattern ACSSuSpT-NpxCp (4 animal isolates and 2 human isolates), and 1 exhibited the resistance pattern ACSSuSpTNx (a human isolate). The second most prevalent restriction pattern, PFGE 2, was observed for 18 isolates (10 animal isolates and 8 human iso-

lates) that were all sensitive to antibiotics, with the exception of H20004937, which had the resistance pattern SSpSu. Fifteen PFGE 2 isolates were DT170 (7 animal isolates and 8 human isolates), one was DT168a (an animal isolate), and two were nontypeable (both animal isolates). The eight other PFGE profiles, PFGE 3 to 10, contained three isolates or fewer.

Nine isolates of animal origin and seven isolates of human origin were plasmid free (Table 2), sensitive to the antibiotics, and phage typed as DT170 (14), DT193 (1), or nontypeable (1). The remaining 45 isolates harbored between one and five plasmids, ranging in size from 2 kb to approximately 141 kb. PFGE 1 isolates harbored an approximately 100-kb plasmid of the same size as the serovar-specific plasmid. An approximately 100-kb plasmid was also detected in two RNDC (reacts but does not conform to designated types on phage typing) isolates, one DT170 type and one DT193 type. The plasmid content could be classified into 15 different profiles.

DNA microarray analysis. Although we could detect variations in the *S. enterica* serovar Typhimurium isolates, we were unable to genetically define these regions using the above approaches. Consequently, 23 *S. enterica* serovar Typhimurium isolates, representative of the different PFGE profiles, antibiotic resistance patterns, and phage sensitivity patterns, were subjected to DNA analysis using a composite *Salmonella* DNA microarray (Table 2). Not surprisingly, the microarray analysis revealed high levels of similarity between the genomes of the different *S. enterica* serovar Typhimurium isolates (Fig. 1). However, specific regions were variable, with some differences

TABLE 3. Multiplex PCR for detection of the five prophages harbored by *S. enterica* serovar Typhimurium DT104 (NCTC 13348) and SGI1

Primer	Sequence (5'-3')	Genome coordinates of product (DT104 [NCTC13348])	Product size (bp)	Target
P1For	CGGAAGAGCTGGATAACGAGTTTCTG	365087-367366	2,179	Prophage 1/ST104
P1Rev	GCGAATTATGTTCCGGGAGTATGAC			
P2For	TCACAAGCAATGCGTGGTGTGCAAC	381790-382891	1,101	Prophage 1/ST104
P2Rev	GCAGCACGAACACATGACACTACTAG			
P3For	GGTGA AACCGCGCATTGGCATAGAAG	406501-407203	702	Prophage 1/ST104
P3Rev	ACGCGGGTAGGATCAGAGTACATAG			
P4For	GCAGTACGAACCGTACCCGATACAG	1111820-1112670	850	Prophage 2
P4Rev	CAGTCCGTCACGCCATGCTCAAAC			
P5For	GCTTTCTCGTAACGCCTGCGATTTT	1953476-1954662	1,186	Prophage 3
P5Rev	CGAATTTATTGGGGCAGGTGATGCG			
P6For	TGAAGGCTCTCAGCATATCACCCGTA	1965128-1965939	811	Prophage 3
P6Rev	ATCCACTGCCGAACGTTATCGTGGT			
P7For	AATAGCCCACCGGGAATATTCATC	1995172-1995776	604	Prophage 3
P7Rev	GCTTTGTGATCCATCCAATAGTGAC			
P8For	ATGCTGACGGAAGCGTTCGGGATT	2129791-2128102	1,689	Prophage 4
P8Rev	GATGCGTGGAGATCAGCTTATGCA			
P9For	TAATACCGCGTACGCTCAAGATC	2796632-2797808	1,176	Prophage 5
P9Rev	CTATAACAGTACCGGGAACCTGTTCC			
P10For	CTGGCGCTTAGTCATGTCGGTAATT	2827013-2827557	544	Prophage 5
P10Rev	ACGTTTGGTAAACGCCTTACGCCTT			
P11For	AGTGAAGTAAACGGTCACTCACTGG	4083151-4083812	661	SGI1
P11Rev	ATTGTGCTGACGCTCTGCTTGTGTC			
P12For	CAACTTCCGTAAGTTCAGCTACAGC	4096995-4097976	981	SGI1
P12Rev	TAGCTCTATCCAGCAATGCGGATTG			
P13For	AATTAGTCGGGCTACTTGCATTGCT	4129495-4129852	357	SGI1
P13Rev	ACTTATCTACAGCGTTCTGTCTGCC			

being present in the majority of isolates while others were restricted to particular strains or groups of strains. This limited variation allowed us to perform a simple cluster analysis of the isolates by using a restricted group of 4,167 genes present in *S. enterica* serovar Typhimurium DT104 (NCTC 13348), for which the complete genome sequence was available (Fig. 1).

Seven PFGE 1 *S. enterica* serovar Typhimurium isolates clustered with the reference strain DT104 (NCTC 13348), including three of DT104, two of DT104b, and two that were nontypeable. Interestingly, one region of the reference strain DT104 (NCTC 13348), represented by prophage-like element 4, was absent from all other isolates as revealed by GACK analysis. Also, the SGI1 element of the reference DT104 strain was present only in all PFGE 1 isolates. Visual examination of the microarray profiles highlighted several other regions of the genome that represent significant variation between isolates. Detailed bioinformatic analysis of the microarray data revealed that the majority of these regions of variation corresponded to prophage-like elements within the genome of *S. enterica* serovar Typhimurium DT104 (NCTC 13348). The prophage in DT104 (NCTC 13348) included prophage 1 (also called ST104 [23]; hereafter it is referred to as prophage 1/ST104) (genome coordinates 365588.406646); prophage 2 (genome coordinates 1079201.1124674), which is related to the GIFSY-2 phage (17); prophage 3 (genome coordinates 1954176.1995367); prophage 4 (genome coordinates 2109720.2149064); and prophage 5 (genome coordinates 2797168.2845750) (Fig. 1; Table 3). Analysis of the microarray data corresponding to the prophage elements indicated that prophages 1, 3, and 5 harbored the most variation, while prophages 2 and 4 were mostly conserved (Fig. 2). Thus, although we detected other types of variation, usually limited to single genes, variation was predominantly

associated with some of the prophage-like elements, suggesting that these regions were driving recent evolution in these strains.

Multiplex PCR for phages and SGI1. The microarray analysis identified several significant regions of variation within the genomes of the different *S. enterica* serovar Typhimurium isolates. This information was used to design PCR primers that could form the basis of simple PCR assays to discriminate between these isolates (Table 4). Primers were designed on the basis of different regions of prophages 1 to 5 and, additionally, on the basis of the variant SGI1 antibiotic resistance island known to be present in the antibiotic-resistant DT104 isolates. DNA sequences related to SGI1 were probed by using oligonucleotide pairs P11, P12, and P13. A product was obtained with the oligonucleotide pair P13 from all *S. enterica* serovar Typhimurium isolates, as the amplified region (genome coordinates 4129495.4129852) is located in the core chromosomal sequence downstream of SGI1. Hence, the P13 probes acted as a positive control for the PCR analysis. Products for P11 and P12 were generated from DNA for all seven PFGE 1 isolates and for no others.

The presence of DNA sequences relating to prophage 1/ST104 was monitored using the oligonucleotide pairs P1, P2, and P3. P3 corresponds to DNA sequences encoding structural proteins of prophage 1/ST104, and a product was generated by using DNA from all 23 *S. enterica* serovar Typhimurium isolates. PCR signals for P1 and P2 were detected in the seven PFGE 1 isolates and one other isolate, H20004467, which is a nontypeable strain. Microarray results for the DT170 isolate H19992292 had indicated the presence of sequences relating to prophage 1/ST104 in this genome (Fig. 1), but no PCR product was generated with either the P1 or the P2 oligonucleotide pair, suggesting the presence of additional sequence variation.

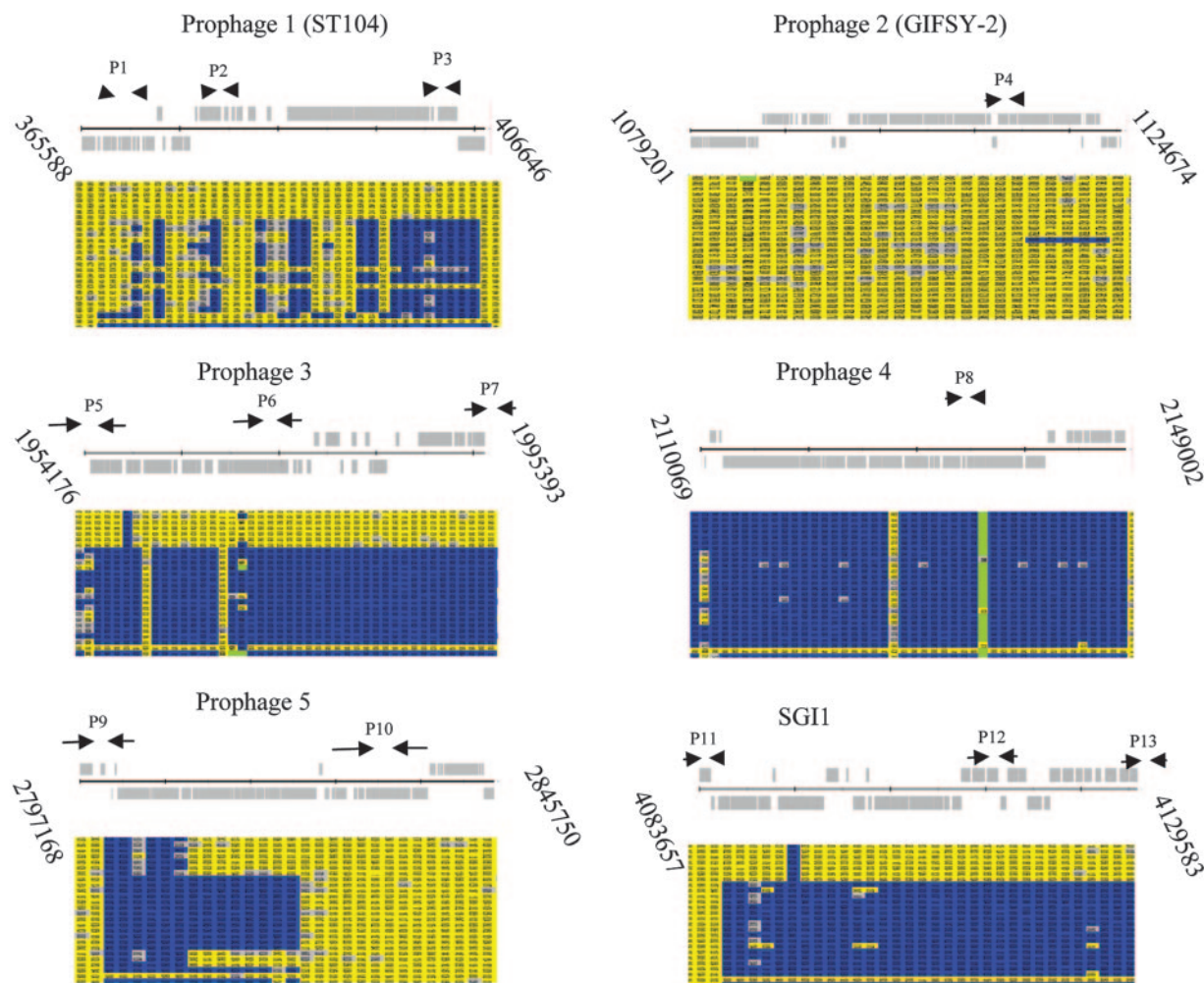


FIG. 2. Schematic diagram of prophages 1 to 5 and SGII, showing oligonucleotide target sites. The gray schematic diagrams represent the predicted open reading frames in the annotated genome of *S. enterica* serovar Typhimurium DT104 (NCTC 13348). The arrows show the location of the primer pairs P1 to P13, as listed in Table 3. The corresponding microarray results, processed by the gene-calling program GACK, are shown underneath each prophage or under SGII. The columns represent the features harbored in each prophage or the genomic island. Yellow represents features that are present in the test strain as well as the control strain (*S. enterica* serovar Typhimurium DT104 [NCTC 13348]), blue represents genes that are in the control strain but are absent in the test strain, and gray indicates that the presence of the gene in the test strain is uncertain. The rows represent each *S. enterica* serovar Typhimurium isolate, in the following order: row 1, AWF002008; 2, AWX002821; 3, H20002800; 4, H20021651; 5, AWX000826; 6, AWX000841; 7, H20021958; 8, H20004019; 9, AWF009126; 10, AWF009147; 11, H20021856; 12, AWX003658; 13, AWX004816; 14, AWF007581; 15, H19963477; 16, H19992292; 17, AWX004814; 18, H20004467; 19, H20023824; 20, AWX006747; 21, AWX006748; 22, H20003919; 23, H20023530; 24, DT104 (NCTC 13348); 25, LT2 (ATCC 700220).

Thus, results from pairs P1 and P2, which are within the regulatory region, indicated divergence between different isolates.

Prophage 2 was found to be present in all 23 isolates probed with oligonucleotide pair P4, and hence GIFSY-2 phage-related DNA is conserved in this population. In contrast, prophage 3, which was probed for by using oligonucleotide pairs P5, P6, and P7, was present only in the seven PFGE 1 isolates.

Prophage 4 is present in the sequenced DT104 isolate, but interestingly it was not detected in any of the 23 other *S. enterica* serovar Typhimurium isolates, including other DT104 isolates, by microarray analysis. The presence of prophage 4-related sequences was probed for by using oligonucleotide pair P8, and interestingly a PCR product of the predicted size was produced by using DNA prepared from all but three isolates (H20004467, AWX006747, and

H20003919), suggesting that these isolates encode sequences relating to prophage 4.

The PCR results obtained with prophage 5-based probe pairs corresponded to the information gleaned from the microarrays. DNA sequences related to prophage 5, probed by P9 and P10, were present in all the PFGE 1 isolates and the three RDNC isolates belonging to other PFGE types. All other *S. enterica* serovar Typhimurium isolates generated a product with P9 but not P10, suggesting that they harbored a variant form of prophage 5.

Thus, these sets of primers confirm aspects of the microarray analysis of variation in specific prophage regions of *S. enterica* serovar Typhimurium and may form the basis of a simple PCR assay for discriminating between different *S. enterica* serovar Typhimurium strains circulating in a geographical area.

TABLE 4. Multiplex PCR results from 23 *S. enterica* serovar Typhimurium isolates selected for microarray analysis

PFGE profile	Isolate	Phage type	Ability of oligonucleotide pair to detect:														
			Prophage 1/ST104			Prophage 2	Prophage 3			Prophage 4	Prophage 5		SGI1				
			P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13		
1	AWF002008	DT104	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1	AWX002821	DT104	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1	H20002800	DT104	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1	H20021651	DT104b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1	AWX000826	DT104b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1	AWX000841	Nontypeable	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
1	H20021958	Nontypeable	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
2	H20004019	DT170	-	-	+	+	-	-	+	+	+	-	-	-	-	+	
2	AWF009147	DT170	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
2	H20021856	DT170	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
2	AWX003658	DT168a	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
2	AWX004816	Nontypeable	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
3	AWF007581	Nontypeable	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
3	H19963477	DT170	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
3	H19992292	DT170	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
4	AWX004814	DT193	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
5	H20004467	Nontypeable	+	+	+	+	-	-	+	-	+	-	-	-	-	+	
6	H20023824	DT170	-	-	+	+	-	-	+	+	+	+	-	-	-	+	
7	AWX006747	RDNC	-	-	+	+	-	-	+	-	+	+	-	-	-	+	
7	AWX006748	RDNC	-	-	+	+	-	-	+	faint	+	+	-	-	-	+	
8	H20003919	RDNC	-	-	+	+	-	-	+	-	+	+	-	-	-	+	
9	H20023530	DT193	-	-	+	+	-	-	+	faint	+	-	-	-	-	+	
10	AWF009126	DT170	-	-	+	+	-	-	+	+	+	-	-	-	-	+	

DISCUSSION

We have used a variety of techniques to examine in detail the relationship between a matched group of *S. enterica* serovar Typhimurium strains isolated from animals and humans in Scotland between 1996 and 2002. One aim of this work was to further define the molecular basis of genome variation within *Salmonella* serovars and to exploit this information to discriminate between isolates in the field by using simple assays. Conventional techniques such as phage and plasmid typing, PFGE, and antibiotic resistance analysis were able to distinguish between different isolates but could not identify the genetic basis of such changes. In contrast, genomic approaches utilizing DNA microarrays were able to highlight hot spots of variation and to link these to annotated and genetically defined regions of DNA. This approach opens up the potential to rationally design specific probes for strain variation and to identify quickly novel genetic variants in the field. Obviously, PCR product-based DNA microarrays are not able to identify variations such as point mutations or DNA insertions not represented on the array. Nevertheless, with this technology we were able to rapidly identify several key areas of genomic variation and to demonstrate that these map to regions encoding prophage-like elements. This observation supports the hypothesis that bacteriophage are a key factor driving the microevolution of *Salmonella* (25), a process that readily can be detected in the sample of strains analyzed in this study.

Using the recently determined genome sequence of the DT104 isolate NCTC 13348, we were able to identify up to five different regions of the genome encoding prophage-like elements, complementing and extending studies carried out by others using non-genome-wide approaches (12, 13). Prophage 1/ST104, also known as PDT17 (21), previously has been de-

scribed as a resident prophage in *S. enterica* serovar Typhimurium DT104 (23). Interestingly, a prophage 1-like element also was detected in the nontypeable *S. enterica* serovar Typhimurium isolates H20004467 and H19992292 (DT170), although only the former was confirmed by multiplex PCR. These observations are of interest, as prophage 1/ST104 previously has not been described outside of phage type DT104. Prophage 2 (GIFSY-2), which is a well-characterized *S. enterica* serovar Typhimurium prophage, was present in all 23 isolates and was thus of little value for discriminating between strains. Prophage 3 was present in the seven PFGE 1 isolates and, together with SGI1, could form the basis of a diagnostic test for DT104.

Significantly, although these techniques could distinguish between isolates of *S. enterica* serovar Typhimurium, organisms derived from humans or animals did not fall into two mutually exclusive groups. This observation serves to illustrate the close genetic relationship between animal and human *Salmonella* strains circulating in the same geographical region and emphasizes the zoonotic nature of these infections. It seems that very similar strains of *S. enterica* serovar Typhimurium were causing disease in animals and humans in the same geographical location and in the same time frame. Antibiotic-resistant strains of *S. enterica* serovar Typhimurium were present in both the human and animal populations. Interestingly, antibiotic-sensitive isolates from animals tended to cluster with sensitive isolates from humans, and antibiotic-resistant isolates also grouped together, irrespective of source.

In conclusion, the examination of the DNA sequences of the different prophages present in *S. enterica* serovar Typhimurium DT104 (NCTC 13348) allowed us to design PCR primer sets that could be used to interrogate the genomes of different field

isolates in multiplex PCR assays. These simple tests were used to rapidly distinguish between different *S. enterica* serovar Typhimurium isolates by using basic equipment and reagents. Thus, we have developed a rational DNA sequence-based assay for discriminating between *S. enterica* serovar Typhimurium isolates that can be applied even in a simple laboratory environment. Importantly, we believe that such assays could be refined to form the basis of a simple rapid test to distinguish between *S. enterica* serovar Typhimurium isolates in a diagnostic or surveillance setting. Further sequence analyses are under way to define the prophage content of multiple *S. enterica* serovar Typhimurium isolates, with a view to improving the discriminatory power of these assays. This approach easily could be expanded to the design of similar PCR-based assays for additional *Salmonella* serovars. The advantage of this approach is that it is based on defined DNA sequences physically mapped in the *Salmonella* genomes, and it targets known hot spots for evolutionary change. Hence, these signatures reasonably might be expected to detect the early emergence of pathogenic variants of *Salmonella* in the field.

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