Genetic Types, Gene Repertoire, and Evolution of Isolates of the *Salmonella enterica* Serovar 4,5,12:i:— Spanish Clone Assigned to Different Phage Types

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*Salmonella enterica* subsp. *enterica* 4,[5],12:i:— is one of the most prevalent serovars associated with human infections worldwide. Two multidrug-resistant clones, designated Spanish and European clones, are recognized as having importance for public health and are subject to control measures in the European Union. In this study, 23 clinical isolates belonging to the Spanish clone were characterized by multilocus sequence typing, multiple-locus variable number tandem repeat analysis (MLVA), PCR amplification and sequencing, and a DNA microarray targeting 263 genes, in order to provide new insights into their origins and further evolution. The derived data were compared with information available from other studies for *S*. 4,[5],12:i:— isolates of both the Spanish and the European clones, to identify differential molecular markers which could be potentially used as surveillance tools in the control of dissemination of this serovar. The isolates analyzed were assigned to sequence type 19 and to 17 MLVA patterns, with 3-13-16-NA-311 being the most prevalent. Highly similar virulence, metabolic, and prophage-associated gene profiles were identified, but DNA mobility markers distinguished five genotypes. Two types of deletions, caused by insertion of IS26, presumably donated by pUO-STmRV1-like plasmids typically found in the Spanish clone, affected the *fljAB* operon and surrounding DNA. The Spanish and European clones differ in sequence type, MLVA patterns, gene repertoire, and *fljAB* deletion type. The observed variability supports an independent evolution of the two successful monophasic clones from different *Salmonella enterica* serovar Typhimurium ancestors and can be taken into consideration for epidemiological surveillance.

Since the mid-1990s, *Salmonella enterica* subsp. *enterica* serovar 4,[5],12:i:— has increasingly been reported to be associated with human clinical samples, a wide range of animal species, and food-related sources in many countries around the world (1). Mainly due to its connection with an increasing number of human cases of salmonellosis, a number of studies have focused on its emergence and evolution (1, 2). It was shown that *S*. 4,[5],12:i:— is closely related to *S*. Typhimurium (3–11). The monophasic serovar does not express the second-phase flagellar antigen, and different mutations and/or deletions affecting the *fljAB* operon (encoding a negative regulator of the *flfC* gene for the first-phase flagellar antigen and the second-phase antigen, respectively) and surrounding genes have been detected (6, 9, 10, 12, 13). Additionally, far from being a unique clonal group, *S*. 4,[5],12:i:— isolates recovered around the world present distinct phage types, resistance types, pulsed-field gel electrophoresis (PFGE) profiles, and multiple-locus variable-number tandem repeat analysis (MLVA) patterns. Thus, its evolution from *S*. Typhimurium through multiple independent emergence events has been suggested (10, 12).

In Europe, *S*. 4,[5],12:i:— is considered a threat to human health, mainly linked to the consumption of contaminated pork (3, 10), although other infection sources, such as poultry, cattle, and companion animals, have been reported (1, 2). Therefore, it has been included in the European Union actions to control *Salmonella* infections (http://eur-lex.europa.eu). Over the last 2 decades, two major resistant clonal lines have emerged in Europe. The European clone, particularly common since 2000, harbors a chromosomal region responsible for resistance to ampicillin, streptomycin/spectinomycin, sulfonamides, and tetracycline (ASSuT type) (10, 14–16). The Spanish clone, first reported in Spain in 1997, displays plasmid-mediated resistance up to seven antimicrobial drugs: ampicillin, chloramphenicol, gentamicin, streptomycin/spectinomycin, sulfonamides, tetracyclines, and trimethoprim (ACGSuTTp type) (3, 11, 17). The genes are carried by a family of IncA/C plasmids which harbor (pUO-STmRV1-like) or do not harbor (pUO-STmR1-like) genes of pSLT, the specific virulence plasmid of serovar Typhimurium (11, 17). Multidrug-resistant *S*. 4,5,12:i:— isolates belonging to the Spanish clone and ascribed to phage type U302 have been shown to differ from *S*. Typhimurium LT2 by five major deletions, designated clusters I to V (6). They include deletions of the allantoic-glyoxylate operon (cluster I, genes STM0517 to STM0529), three regions with the prophage Fels-1-, Gifsy-1-, and Fels-2-related genes (clusters II, III, and IV, respectively), and the *fljAB* region (cluster V, deletion from STM2758 to iroB, due to the insertion of an IS26 element) (6). In a recent study, PFGE and MLVA techniques revealed that *S*. 4,5,12:i:— phage type U302 isolates belonging to the Spanish clone represent a closely related group, al-

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FIG 1 UPGMA dendrogram generated by the MLVA patterns identified in the S. 4,5,12:i:- collection. Phage type and PFGE XbaI profiles as previously described by García et al. (11) are included (9). The variable DNA mobility elements detected by the microarray are shown for each isolate. These include replication proteins of IncN and IncI1-y incompatibility plasmid groups and transposases of the insertion sequences IS1, STY340, IS440, and IS2651, which were taken into account to establish five (I to V) mobility genotypes. A gray box indicates that the element is present and a white box that it is absent.

though they show variation in the deletions affecting the \textit{fljAB} region (13).

In order to provide new insights regarding the emergence and gene repertoire of the S. 4,5,12:i:- Spanish clone, a collection of human isolates classified not only as U302 (which is the predominant phage type of the clone) but also as other phage types (U310, DT193, or not typeable with the phage library used) and previously reported to be carriers of pUO-StrmRV1-like IncA/C plasmids (11) was characterized using a variety of molecular typing methods and a DNA microarray, and the bases of their monophaseic phenotype were investigated. The detected typing profiles, gene repertoires, and deletions in the \textit{fljAB} region were compared with data available from other authors for S. 4,5,12:i:- isolates of both the Spanish and the European clones in order to investigate their origin and diversity and to identify differential markers which could be useful for surveillance purposes.

**MATERIALS AND METHODS**

**Bacterial isolates.** Twenty-one S. 4,5,12:i:- isolates belonging to the Spanish clone, with no known epidemiological relationship, and recovered in four hospitals in Asturias (Spain) were investigated (Fig. 1). They were associated with sporadic cases of enteric salmonellosis and represent all clinical cases reported in Asturias between 2000 and 2003 for this serovar. The isolates were given designation consisting of LSP (for Laboratorio de Salud Pública) followed by a code number/year of isolation. Nineteen isolates had been previously investigated with regard to phage type, PFGE profile, plasmid content, and antimicrobial susceptibility and responsible genes (11). Two additional isolates (LSP 127/00 and LSP 148/00) were similarly characterized for the present study. Both were U302, displayed two novel PFGE XbaI profiles, and were resistant to sulfonamides (encoded by \textit{sul1} and \textit{sul2}) and tetracycline (\textit{tetA}). Two other S. 4,5,12:i:- isolates, recovered in 1997 and 1998 and previously found to carry pUO-StrmRV1 and pUO-StrmR1 (LSP 389/97 and LSP 272/98, respectively), were also included in the study (11, 17), and S. Typhimurium LT2(pSLT) was used as a positive or negative control in PCR amplifications and DNA microarray analysis.

**DNA purification.** S. 4,5,12:i:- isolates were grown in Luria-Bertani broth for 16 to 18 h at 37°C with gentle shaking. Total DNA from individual isolates was extracted and purified using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) following the manufacturer’s standard protocol with minor modifications (1.6-ml initial culture aliquot, addition of 25 μl of proteinase K, and extended lysis time of 3.5 h). The quality and quantity of DNA were spectrophotometrically determined by measuring the optical density at 230, 260, and 280 nm.

**MLVA and MLST analyses.** MLVA was performed following the protocol of Lindstedt et al. (18). Four chromosomal loci and one plasmid locus were analyzed based on the presence of tandem repeats. The size measurements for each locus were estimated using an ABI 310 genetic analyzer (Life Technologies GmbH, Darmstadt, Germany). Variable number of tandem repeats (VNTR) allele numbers were assigned as described by Larsson et al. (19) and combined to give a single MLVA pattern for each isolate (order of loci, STTR9-STTR5-STTR6-STTR10p-STTR3). VNTR allele numbers were imported as character values into the BioNumerics 6.0 software (Applied Maths, NV, Sint-Martens-Latem, Belgium), and cluster analysis of the categorical coefficient was performed using the unweighted pair group method of analysis (UPGMA) algorithm with arithmetic averages. Multilocus sequence typing (MLST) was carried out using previously reported primers specific for seven housekeeping genes (\textit{aroC, dnaA, hsdM, hisD, purE, sucA, and thrA}), and the sequence type (ST) was assigned according to the MLST database (http://mlst.ucc.ie/mlst). For these typing methods and the macrorestriction PFGE XbaI typing published by García et al. (11), Simpson’s index of diversity (SID) and 95% confidence intervals (CI) around the SID were calculated using the Comparing Partition website (http://darwin.phylloviz.net/ComparingPartitions/index.php?link=Tool).

**DNA microarray analysis.** DNA microarray analysis was performed as previously described (20). A total of 263 gene-specific 57- to 60-mer oligonucleotides, previously designed using the Array Designer 4.1 program (Premier Biosoft, Palo Alto, CA) according to Salmonella sequences from GenBank database (http://www.ncbi.nlm.nih.gov/), were included. The genes were categorized into five main groups according to their localization on the \textit{Salmonella} genome and the functionality of the corresponding products: virulence (104 probes), metabolism (19 probes), serotyping (33 probes), DNA mobility (58 probes), and resistance (49 probes). As negative controls, three oligonucleotides derived from different \textit{Arabidopsis thaliana} genes were included. The genomic DNA of each isolate was fluorescently labeled using a genomic labeling kit (Invitrogen, Karlsruhe, Germany) and further purified with PureLink spin columns (Invitrogen) according to the manufacturer’s instructions. The hybridization was performed for 18 h at 42°C in a Slide Booster SB401 (Implen, Munich, Germany), and posthybridization washing steps were carried out using an Advawash AW400 washing station (Implen). Microarray signals were captured with a GenePix 4000B laser scanner (Axon, Foster City, CA), and fluorescent images were analyzed with GenePix Pro 4.1 software (Axon). Signal values were normalized in order to present each data point as presence or absence. “Uncertain” hybridization probe signals (cutoff values between 0.25 and 0.4) were verified by PCR amplification as previously described (20), and individual decisions were made for the presence or absence of each target.

**PCR-based characterization of gene deletions affecting the Fels-2 prophage and the \textit{fljAB} region.** PCR primer sets were designed to test for the presence or absence of Fels-2 prophage genes (cluster IV), which are not part of the microarray, and for a detailed analysis of cluster V (from

García et al.
Chromosomal deletions in cluster V were further delineated by DNA sequencing (Magrogen Europe, The Netherlands) of the generated PCR fragments.

RESULTS

Typing of S. 4,5,12:i:− isolates by MLVA and MLST. Seventeen different MLVA profiles were identified for the 23 S. 4,5,12:i:− isolates analyzed in this study, with a S/D value of 0.953 and 95% CI of 0.895 to 1.000 (Fig. 1). Only three patterns—3-13-16-NA-311 (5 isolates), 3-13-19-NA-311 (2 isolates), and 3-13-17-NA-310 (2 isolates)—were represented by more than one isolate. Variability was found in three of five VNTR loci: STTR5 (5 alleles), STTR6 (9 alleles), and STTR3 (2 alleles). A unique allele was identified for locus STTR9, while none of the isolates was positive for STTR10pl, located on the virulence plasmid of S. Typhimurium (Fig. 2; also, see Table S1 in the supplemental material).

Virulence gene repertoire. All S. 4,5,12:i:− isolates showed highly similar virulence profiles, taking into account 104 probes corresponding to genes located on Salmonella pathogenicity islands (SPI-1 to SPI-5 and SPI-7) (35 probes), pathogenicity islets (32 probes), fimbrial operons (22 probes), prophages (11 probes), and pSLT (4 probes). Probes for SPI-1 to SPI-5 gave positive signals in all isolates, while markers for SPI-7 (present in Salmonella enterica serovar Typhi, S. Paratyphi C, and some strains of S. Dublin) were absent, as were gipA (encoding a Peyer’s patch-specific virulence factor), sopH1 (Salmonella type III effector protein), sodCIII (putative Cu/Zn superoxide dismutase), and sopE1 (Salmonella type III effector protein) genes carried by Gifsy-1, Gifsy-3, Fels-1, and SopEF prophages, respectively. Two additional prophage genes, STY4625 and STY4631, characteristically found on Fels-1, and SopE2, encoding an effector protein which introduces into the host cell through the SPI-2 type III secretion system, were all positive for 13 of 22 probes, in agreement with the redundancy of fimbria operons detected in Salmonella (22). In all, only two isolates displayed different virulence gene patterns. One isolate (LS 303/02) was negative for sopD2, encoding an effector protein which introduces into the host cell through the SPI-2 type III secretion system, while the spvC and spvR genes of pSLT were absent in an isolate (LS 272/98) which harbors the resistance plasmid pUO-STmR1 (11).

DNA mobility genotypes. The array also contains 58 probes targeting transposition genes of different insertion sequences (IS): IS common regions (ISCR), which are found adjacent to antimicrobial resistance genes (23); repA replication genes or associated iterons designed according to Carattoli et al. (24) for plasmids of diverse incompatibility (Inc) groups; and the traT gene, which is involved in pSLT transfer by conjugation (see Fig. S1 in the supplemental material). The isolates shared the same mobility gene repertoire for 51 of the 58 markers tested, yielding positive hybridization for 13 probes and negative hybridization for 38 probes. All were positive for the repA gene of the IncA/C plasmid group, consistent with the presence of pUO-STmR/RV1-like plasmids, and the ISCR2 (linked to sul2 in IncA/C plasmids [11]) and ISCR3 (also carried by the IncA/C plasmids; P. García, unpublished data) elements. The same result was obtained for recC (which encodes a subunit of exonuclease V involved in homologous recombination and DNA repair), for the insertion sequences IS200 (reported as a 1-kb fragment in the flbB-flaA intergenic region of S. Typhimurium [25]), and for IS26. None of the isolates was positive for traT. Seven probes, specific for repA IncN, repC IncI1-γ, insA IS1, insB IS1, tnpA STY343, tnpA IS406 (annotated as IS440 in sul3 integrins), and tnpA IS26 (Fig. 1), gave variations allowing the assignment of the isolates to mobility genotypes I to V (Fig. 1).

Additional gene markers. All investigated isolates were confirmed as S. 4,5,12:i:−, since serotype markers gave positive/negative results consistent with serological results. Thus, they were positive for Salmonella, Salmonella subsp. I, and serogroup B markers and for the O:5 antigen-encoding gene (oafA) (data not shown).
shown). In addition, they all tested positive for the resistance genes previously identified by PCR (11; this study), which explain the resistance phenotypes of the isolates under study, and for the *acrF* (acridine resistance) and *copR* (copper resistance) probes. With regard to metabolic genes, the same profile was shared by all isolates showing positive hybridization for 17 markers. Only two genes involved in metabolic functions were absent: *glox*, located within the region encoding the allantoin-glyoxylate pathway (cluster I), and *STY4221*, which encodes a putative aminotransferase in *S. Typhi* CT18.

**Genetic basis for the lack of the second flagellar antigen.** By microarray analysis, relevant genes for the expression of the second-phase flagellar antigen, *fljA*, *fljB*, and *hin*, were found to be absent in all isolates, which explains the mono/monotypic phenotype. Identification of the start and end points of the deletions through PCR amplification and sequencing (Fig. 2) revealed two different types, which have already been reported for U302 isolates of the Spanish clone (23 isolates analyzed). Several resistance genes carried by IncA/C plasmids in the Spanish clone (*cmlA1*, *aac(3)-IV, ludA2, sul1, sul3, and dfrA12* and the chromosomal resistance region in the European clone [strkr-strB and intB]) are also differential.

**DISCUSSION**

The results reported here demonstrate that a collection of *S. 4,5,12;i:-* isolates of the Spanish clone assigned to several phage types constitute a consistent clonal line related to *S. Typhimurium*. By MLST, all isolates were assigned to ST19, which is the most common ST in the latter serovar (*http://mlst.ucc.ie/mlst/dbs/Senterica*). The MLST scheme, based on housekeeping genes with high stability, is appropriate for estimating long-term evolution and shows a strong correlation with serovar (26). ST19 was likewise found in both *S. Typhimurium* and *S. 4,5,12;i:-* isolates from the United States analyzed by Hoelzer et al. (27) and in *S. 4,5,12;i:-* isolates with the typical resistance genes and IncA/C plasmids of the Spanish clone from manure samples in Portuguese piggeries (28). In contrast to the Spanish clone, the ASSuT monophasic European clonal belonging to ST34, a single-locus variant of ST19 (with *dnaN19* instead of *dnaN7*) also associated with *S. Typhimurium* (28, 29). On the other hand, MLVA analysis resolved 17 patterns with variations detected in the STTR5, STTR6, and STTR3 loci. These patterns differ from profiles found among the European monophasic isolates (10, 29, 30). MLVA has been proposed as an alternative to PFGE due to its higher discriminatory power, which makes it particularly useful for genotyping of highly clonal bacteria and the investigation of outbreaks (19). In this study, MLVA proved to be efficient in distinguishing closely related *S. 4,5,12;i:-* isolates of the Spanish clone, but comparison of the SID values calculated for MLVA (0.953 [this study]) and for PFGE XbaI analysis (0.972 [11]) indicates that both methods are similarly appropriate for the discrimination of these isolates. The high SID values obtained with PFGE could be explained by fragments smaller than 30 kb which derive from *puO-StmR/RV1*-like plasmids (P. García, unpublished data) but were taken into account to define the PFGE patterns, although they actually represent horizontally acquired DNA. In fact, a lower SID (0.948), although still comparable to that estimated for MLVA, was obtained after the plasmid bands were subtracted from the profiles. Of note, isolate clusters determined by these techniques did not exactly correspond, as expected because of the independent evolution of their targets, i.e., VNTR loci and XbaI restriction sites for MLVA and PFGE, respectively. Additionally, no correlation was found between the MLVA or PFGE XbaI patterns with the five mobility genotypes derived from microarray data, consistent with lateral transfer of most of these variable markers, which cannot be taken into account to establish clonal relationships. In fact, IncN, IS440 (carried by a *sul3* integron), and ISEcp1 are known to be located within the IncA/C scaffold (11; P. García, unpublished data), while IncI1-γ and probably IS1 are carried by a coresident plasmid, as both were detected together in a single isolate (LSP 1142/03). In any case, the Spanish *S. 4,5,12;i:-* isolates analyzed in this study proved to be more homogeneous than monophasic groups from other countries (12), even though they belonged to different phage types, were obtained from four hospitals, and, with a possible exception (LSP 37/00 and LSP 40/00, which share

<table>
<thead>
<tr>
<th>Gene (function)</th>
<th>Location</th>
<th>Presence (%) in Spanish clone</th>
<th>Presence (%) in European clone</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>glox</em> (glycerate kinase II)</td>
<td>Cluster I, allantoin-glyoxylate pathway</td>
<td>Absent</td>
<td>Present (98.38)</td>
</tr>
<tr>
<td><em>gipa</em> (Peyer’s patch-specific virulence factor)</td>
<td>Cluster III, Gifsy-1 prophage</td>
<td>Absent</td>
<td>Present (96.77)</td>
</tr>
<tr>
<td>Several ORFs (Table S1)</td>
<td>Cluster IV, Fels-2 prophage</td>
<td>Absent</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>iroB</em> (putative glycosyl transferase)</td>
<td>Cluster V, <em>fljAB</em> operon and flanking genes</td>
<td>Absent</td>
<td>Present (100)</td>
</tr>
<tr>
<td><em>hdld</em> (putative virulence factor, assembly-LSP related protein)</td>
<td>ST104B prophage</td>
<td>Present</td>
<td>Absent (98.38)</td>
</tr>
<tr>
<td><em>irsA</em> (putative transcriptional regulator, macrophage survival)</td>
<td>ST104B prophage</td>
<td>Present</td>
<td>Absent (98.38)</td>
</tr>
<tr>
<td><em>spvC</em> (virulence factor for mouse systemic phase infection)</td>
<td>IncA/C plasmid</td>
<td>Present</td>
<td>Absent (95.16)</td>
</tr>
<tr>
<td><em>orfA</em> of ISCR2, <em>orf2</em> of ISCR3 (DNA mobility)</td>
<td>IncA/C plasmid</td>
<td>Present</td>
<td>Absent (95.16)</td>
</tr>
<tr>
<td><em>tphA</em> of Tn21 (DNA mobility)</td>
<td>IncA/C plasmid</td>
<td>Present</td>
<td>Absent (95.16)</td>
</tr>
<tr>
<td><em>tphA</em> of IS10 (DNA mobility)</td>
<td>Chromosomal resistance region</td>
<td>Absent</td>
<td>Mobility (85.48)</td>
</tr>
</tbody>
</table>

* a Genetic location of gene markers according to Garazia et al. (6) for clusters I to V, Hermans et al. (31) for ST104B prophage, García et al. (11; P. García, unpublished data) for IncA/C plasmids, and Lucarelli et al. (16) for the chromosomal resistance region.

* The genes included here were either absent or present in 100% of the isolates belonging to the Spanish clone (23 isolates analyzed). Several resistance genes carried by IncA/C plasmids in the Spanish clone (*cmlA1*, *aac(3)-IV, ludA2, sul1, sul3, and dfrA12*) and the chromosomal resistance region in the European clone (strkr-strB and intB) are also differential.

* Based on data available for 64 isolates (10; E. Hauser, unpublished data; P. García, unpublished data).
identical properties and came from the same hospital), were epidemiologically unconnected.

Differences between Spanish S. 4,5,12:i− and S. Typhimurium LT2 genomes have been mainly identified on five genomic clusters (see the introduction). All S. 4,5,12:i− isolates analyzed in this study exhibited deletions in these clusters, but variations were identified in comparison with monophasic isolates from other countries. For instance, German and U.S. S. 4,[5],12:i− isolates were reported as being positive for Gifsy-1 (cluster III) markers (10, 12), and monophasic isolates of the European clone carry the glxK (STM0525) gene of the allantoin-glyoxylate operon (cluster I) (P. García, unpublished data; E. Hauser, unpublished data), both absent in the Spanish clone. Interestingly, the hldD and irsA genes, carried by a DT104-specific prophage (ST104B) and suggested to be involved in virulence (30), were both found in our isolates of the Spanish clone but not in most isolates of the European clone analyzed so far (10). These and other noticeable differences between the Spanish and European clones are compiled in Table 1. In conjunction with the resistance profiles, these differential markers could be used for a tentative ascription of new S. 4,[5],12:i− isolates to one of the two predominant monophasic clonal lines in Europe.

Previous studies have identified various deletion patterns in Spanish, European, and U.S. S. 4,[5],12:i− isolates, affecting the fliAB operon and flanking genes (6, 10, 12, 13). Thus, U.S. S. 4,5,12:i− isolates exhibit a major deletion spanning 76 genes but still conserve the hin gene (12), while European monophasic isolates commonly show a 16-gene deletion, including fliA, fliB, and hin (10). Other genetic types displaying partial deletions and point mutations were also detected in isolates from America and Europe (9, 10, 29). The S. 4,5,12:i− isolates analyzed in this study showed two deletion types spanning STM2757 to iredB, which were apparently caused by insertion of IS26. This insertion sequence has previously been associated with the same deletions in U302 isolates of the Spanish clone, and the IncF plasmid pU302L, found in S. Typhimurium U302 and carrying five copies of IS56, has been proposed as its donor (13). However, pUO-STmRV1 could be a more likely candidate, since more than 10 copies of IS26 have been detected in this plasmid (P. García, unpublished data), and large (170 kb), nonconjugative IncA/C plasmids have been found in DT104 and U302 biphasic S. Typhimurium isolates from Portugal sharing the predominant AGCSSuTTP type of the S. 4,5,12:i− Spanish clone (28).

In all, multidrug-resistant S. 4,5,12:i− isolates of the Spanish clone, assigned to U302, U310, or DT193 or not typeable with the phage library used, were confirmed to be a monophasic variant of S. Typhimurium, with limited variability. Results indicated that transposition of IS26, presumably donated by pUO-STmRV1-like plasmids originally acquired by biphasic S. Typhimurium, were involved in the deletion of the fliAB operon and surrounding genes and hence are responsible for the monophasic phenotype displayed by the isolates. The S. 4,5,12:i− Spanish clone differs from the European clone in a number of genetic characteristics which could be used as convenient markers for the epidemiological surveillance of both clonal lines. The observed variation also supports the independent evolution of the two successful monophasic clones from different S. Typhimurium ancestors.

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