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Salmonella enterica Serotype 4,5,12:i:−, an Emerging *Salmonella* Serotype That Represents Multiple Distinct Clones▽†

Y. Soyer,[1] A. Moreno Switt,[1] M. A. Davis,[2] J. Maurer,[3] P. L. McDonough,[4] D. J. Schoonmaker-Bopp,[5] N. B. Dumas,[5] T. Root,[5] L. D. Warnick,[4] Y. T. Gröhn,[4] and M. Wiedmann[1]*

Department of Food Science[1] and Department of Population Medicine and Diagnostic Sciences,[4] Cornell University, Ithaca, New York; Washington State University, Pullman, Washington[2]; University of Georgia, Athens, Georgia[3]; and Wadsworth Center, New York State Department of Health, Albany, New York[5]

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The prevalence, among human clinical cases, of *Salmonella enterica* serotype 4,5,12:i:−, a serotype antigenically similar to *Salmonella enterica* serotype Typhimurium but lacking second-phase flagellar antigens, has increased considerably over the last 10 years. To probe the evolution and ecology of this emerging serotype, we characterized 190 *Salmonella* isolates initially classified as *Salmonella* serotypes 4,5,12:i:− ($n = 90$) and Typhimurium ($n = 100$) and obtained from various sources in the United States and Spain. These isolates were characterized into six sequence types (determined by multilocus sequence typing [MLST]) and 79 pulsed-field gel electrophoresis types. The majority of *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates (85 and 84 isolates, respectively) represented a single MLST type. Existing genome information revealed different genome deletions (which included genes responsible for phase 2 flagellum expression) in four Spanish *Salmonella* serotype 4,5,12:i:− isolates and one U.S. *Salmonella* serotype 4,5,12:i:− isolate. Fifty-nine isolates of both serotypes, representing different sources and geographical locations as well as different molecular subtypes, were thus screened for the presence of six genes and one specific region, all of which were previously found to show variable presence among *Salmonella* serotype 4,5,12:i:− and Typhimurium strains. All *Salmonella* serotype 4,5,12:i:− isolates lacked the phase 2 flagella genes *fljA* and *fljB*, which were present in all *Salmonella* serotype Typhimurium isolates. While all Spanish *Salmonella* serotype 4,5,12:i:− isolates carried the same deletion surrounding *fljAB*, all but two U.S. isolates showed a different genomic deletion; the two atypical U.S. isolates represented the "Spanish" deletion genotype and a unique deletion genotype. *Salmonella* serotype 4,5,12:i:− thus appears to represent at least two common clones, which cannot easily be differentiated with standard diagnostic procedures.

Salmonella spp. are one of the most common causes of bacterial food-borne diseases worldwide (34). In the United States nontyphoidal *Salmonella* serotypes cause an estimated 1.4 million human salmonellosis cases, including approximately 550 deaths annually (27). Serotyping with the Kaufmann-White scheme is used commonly as a first step to differentiate *Salmonella* isolates. Serotyping of *Salmonella* isolates is based on lipopolysaccharide moieties on the cell surface (O antigens) and the flagellar proteins (H antigens), as well as capsular protein antigens (Vi antigen), which are only found in a few *Salmonella* serotypes (e.g., *Salmonella enterica* serotype Typhi). According to the Kaufmann-White scheme, *Salmonella* includes over 2,500 recognized serotypes (20). Many *Salmonella* bacteria are motile due to peritrichous flagella (28), which include a basal body, a propeller, and a hook. The motility of *Salmonella* depends on the rotation of the flagellar propeller (i.e., the filament), which includes either FliC (phase 1 antigen) or FljB (phase 2 antigen) flagellin (11). Most *Salmonella* serotypes, including *Salmonella enterica* serotype Typhimurium, are biphasic, meaning that they can express two distinct flagellar antigens (i.e., phase 1 and phase 2 antigens). Regulation of phase 1 and 2 antigen expression is under the control of the recombinase Hin. This recombinase facilitates inversion of a promoter element so that it either (i) transcribes *fljB* (which encodes the phase 2 antigen FljB) and *fljA* (which encodes a repressor of *fliC*, the gene encoding the phase 1 antigen FliC) (4, 37) or (ii) does not transcribe either of these genes. If this promoter is located in an orientation that does not allow for transcription of *fljB* and *fljA*, the lack of a repression of *fliC* transcription leads to expression of phase 1 flagellar antigens.

Salmonella enterica serotype 4,5,12:i:− is a serotype that appears to be antigenically similar and genetically closely related to *Salmonella* serotype Typhimurium (which has the antigenic formula 4,5,12:i:1,2) but lacks expression of the second-phase flagellar antigen, which is 1,2 in *Salmonella* serotype Typhimurium (28). *Salmonella* serotype 4,5,12:i:− was the sixth most common *Salmonella* serotype among cases of human disease in the United States in 2006 (10) and the fourth most common serotype among human isolates in Spain in 1998 (18). Overall, the prevalence of *Salmonella* serotype 4,5,12:i:− among human cases has increased considerably in many countries in the world over the last 10 years (9, 10, 18, 29, 36). This *Salmonella* serotype has also been responsible for a number of human salmonellosis outbreaks over the last decades, including in Spain (1998), the United States (2004 and 2007), and Luxemburg (2006). *Salmonella* serotype 4,5,12:i:− has been isolated, particularly over the last decade, from a number of different foods and animals (1, 6, 13, 29, 38). While a number

* Corresponding author. Mailing address: Department of Food Science, 412 Stocking Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 254-2838. Fax: (607) 254-4868. E-mail: mw16@cornell.edu.

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TABLE 1. *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates used in this study

Serotype and state or country	No. of isolates from[a]						
	Bovine	Human	Poultry	Food	Others	Nondomestic birds	Total
Salmonella serotype 4,5,12:i:−							
Georgia	2 (2)	0	10 (7)	0	0	1 (1)	13 (10)
New York	7 (5)	9 (4)	0	2 (1)	0	0	18 (10)
Washington	0	40 (10)	0	0	2 (1)	0	42 (11)
Spain	0	11	0	2	0	0	13 (10)
Total							86 (41)
Salmonella serotype Typhimurium							
Georgia	6	0	5 (2)	0	0	1 (1)	12 (3)
New York	22	29 (4)	0	0	0	0	51 (4)
Washington	1	6 (2)	0	0	0	0	7 (2)
Spain[b]	0	5	0	8	17	0	30 (5)
Total							100 (14)
Inconsistent (*Salmonella* serotype Typhimurium or 4,5,12:i:−)[c]							
Georgia	0	0	0	0	0	1	1 (1)
Washington	3 (1)	0	0	0	0	0	3 (3)
Total							4 (4)
Total							190 (59)

[a] Numbers in parentheses represent numbers of isolates that were used for further PCR screens to determine the presence/absence of selected genes and one specific region.

[b] Spanish *Salmonella* serotype Typhimurium isolates in the category "Other" were obtained from water and other environments; detailed information for Spanish isolates is in Table S1 in the supplemental material.

[c] These isolates were serotyped as *Salmonella* serotype 4,5,12:i:− in one replicate and *Salmonella* serotype Typhimurium in another replicate (including one isolate that was classified as *Salmonella* serotype 4,5,12:i:− in two replicates and *Salmonella* serotype Typhimurium in one replicate) and were thus designated "inconsistent."

of separate studies, using molecular subtyping and characterization tools (e.g., genomic microarrays and PCR assays to test for gene presence/absence), have shown that *Salmonella* serotype 4,5,12:i:− isolates from Spain (15, 18) and the United States (1, 2, 38) are genetically closely related to *Salmonella* serotype Typhimurium, we are not aware of any comparative studies of *Salmonella* serotype 4,5,12:i:− isolates from Europe and the United States that have been published to date. In order to provide a better understanding of the transmission, ecology, and evolution of *Salmonella* serotype 4,5,12:i:−, we have assembled a collection of 190 *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates from various sources and from two countries, the United States and Spain. These isolates were characterized by different molecular subtyping methods (i.e., multilocus sequence typing [MLST] and pulsed-field gel electrophoresis [PFGE]), followed by characterization of selected isolates for genomic deletions that may be responsible for the lack of phase 2 flagellum expression.

MATERIALS AND METHODS

***Salmonella* isolates.** A total of 190 *Salmonella* isolates initially identified as *Salmonella* serotypes Typhimurium ($n = 100$) and 4,5,12:i:− ($n = 90$) were used in this study (Table 1). These isolates were obtained from different states in the United States, including New York (69 isolates), Washington (52 isolates), and Georgia (26 isolates), and in Spain (43 isolates), as well as from different sources, including human clinical cases, foods, and cattle, poultry, and other warm-blooded animals (Table 1). Human isolates from New York State and Washington State were obtained from the New York State Department of Health and the Washington State Department of Health, respectively. Bovine isolates from New York State and Washington State were obtained from the Animal Health Diagnostic Center at Cornell University and the Washington Animal Disease Diagnostic Laboratory, Pullman, respectively; isolates from foods collected in New York State were obtained from the Food and Drug Administration (FDA) (see Table S1 in the supplemental material). *Salmonella* serotype Typhimurium and 4,5,12:i:− isolates from Georgia have previously been described (38). *Salmonella* serotype Typhimurium and 4,5,12:i:− isolates from Spain have also been described previously (5, 18) and were provided by J. Garaizar, University of the Basque Country, Vitoria-Gasteiz, Spain. Detailed information for all isolates (see Table S1 in the supplemental material), including serotype, source, gene sequence data, allelic types (ATs), and PFGE patterns are available via the Pathogen Tracker website (http://www.pathogentracker.net).

While serotype data were provided for all isolates, isolates that were initially classified as *Salmonella* serotype 4,5,12:i:− but contained an intact copy of the phase 2 flagellum gene *fljB* were resubmitted for serotyping at the National Veterinary Service Laboratories (USDA APHIS VS, Ames, IA). Isolates that were classified as *Salmonella* serotype 4,5,12:i:− in one replicate and *Salmonella* serotype Typhimurium in another replicate (including one isolate that was classified as *Salmonella* serotype 4,5,12:i:− in two replicates and *Salmonella* serotype Typhimurium in one replicate) were designated "inconsistent serotype" isolates.

MLST. While traditional MLST schemes target seven housekeeping genes (24), we initially used a previously reported MLST scheme targeting three genes (i.e., *manB*, *mdh*, and *fimA*) (2, 33) to characterize all isolates used in this study. In addition to these three genes, we also sequenced an 826-nucleotide fragment of a fourth gene (*aroC*) in all isolates to determine whether the use of additional genes would increase discriminatory power. *aroC* was chosen as an additional gene because it was found to represent the greatest number of different ATs among all isolates in the seven-gene MLST database for *Salmonella* in July 2007 (http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica). ATs for *fimA*, *mdh*, and *manB* and three-gene sequence types (STs) were assigned to be consistent with previous studies published by our group (2, 3, 33). ATs for *aroC* were also assigned to be consistent with the seven-gene MLST Max Planck Institute database (http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica). For example, three-gene ST6 includes the same AT combination for three genes as reported in two studies by Alcaine et al. (2, 3), while *aroC* AT 18 is identical to AT AROC18 in the seven-gene MLST database (http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica). STs were also determined based on ATs for all four genes; these STs do not correspond to any previously reported STs.

Salmonella DNA used as a template for PCRs performed for MLST was purified using the QIAmp DNA mini kit (Qiagen Inc., Chatsworth, CA). PCR primers for *manB*, *mdh*, and *fimA* have been previously reported (2, 33); PCR

TABLE 2. PCR conditions and primers for six genes and one region that show variable presence among *Salmonella* serotype 4,5,12:i:− isolates from the United States and Spain

Gene	Function[a]	Amplicon size (bp)	Primers (5′ to 3′)[b]	Temp, °C (time) for:		
				Denaturation	Annealing	Extension
fljA	Repressor of phase 1 flagellin gene	642	F, TTC ATT AGG TCC CCT CCG G; R, ATT CAG CCC CGT GAA TTC GGG	95 (10 min)	55 (45 s)	72 (1 min)
fljB	Phase 2 flagellin structural protein	561	F, TTTACCGTCTACGCCACCC; R, GGTACTACACTGGATGTAT CGGG	95 (10 min)	52 (45 s)	72 (1 min)
hin	H inversion: regulation of flagellar gene expression	570	F, TGG CTA CTA TTG GGT ATA TTC GGG; R, AAT TCA TTC GTT TTT TTA TGC GGC	95 (10 min)	52 (45 s)	72 (1 min)
STM1053-1997		614	F, CCA TTT TTA TAC TGC CAG TCG CC; R, CAG CGA AAT ACT GAT GGC GG	95 (10 min)	55 (45 s)	72 (1 min)
STM2740	Integrase, phage family	980	F, AAT GTG GAG ATC GCT GGC GCG; R, AGT TCG CCG CCG AAC CCC	95 (2 min)	55 (45 s)	72 (1.5 min)
STM2757	Putative cytoplasmic protein	717	F, ATG ATG ATG GCG TAA TGG CGC; R, AAA ACG TTC CGG TGC GGC G	95 (10 min)	55 (45 s)	72 (1 min)
iroB	Glucosyltransferase homolog	858	F, TTC GAT TCG GAA GCG GGT TAT CGC CG; R, CTC GCG AAG CGC GCG	95 (2 min)	65–55 TD[c] (45 s)	72 (1.5 min)

[a] Gene functions for *Salmonella* serotype Typhimurium LT2 were obtained from the J. Craig Venter Institute Comprehensive Microbial Resource website.
[b] R, reverse primer; F, forward: primer.
[c] TD, touchdown PCR; annealing temperatures decreased 0.5°C/cycle during the first 20 cycles, followed by 20 cycles at 55°C.

primers for *aroC* amplification were obtained from the *Salmonella enterica* MLST database at Max Plank Institute (http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica). All primers used are summarized in Table S2 in the supplemental material. PCR products were purified using exonuclease I (USB, Cleveland, OH) and shrimp alkaline phosphatase (USB, Cleveland, OH). Purified PCR products were sequenced using Big Dye Terminator chemistry and AmpliTaq-FS DNA polymerase, and sequencing reactions were analyzed using the Applied Biosystems automated 3730 DNA analyzer at the Cornell University Life Sciences Core Laboratories Center. Sequences were assembled and proofread using SeqMan and aligned using the Clustal W algorithm implanted in MegAlign (DNAStar Inc., Madison, WI).

Phylogenetic analysis. Phylogenetic analysis was performed using all three-gene STs found among *Salmonella* serotype Typhimurium and 4,5,12:i:− isolates as well as three-gene STs available for other serotypes (e.g., STs reported by Alcaine et al. ([2]). As *manB* is duplicated in some isolates, thus yielding sequence data not suitable for phylogenetic analyses (2), STs representing sequences with *manB* duplications were not included in the phylogenetic analyses. For each unique ST, the sequences of the three genes were concatenated. Concatenated sequences were aligned using MegAlign (DNAStar Inc., Madison, WI) and MACCLADE version 4.08 (Sinauer Associates Inc., Sunderland, MA). MODELTEST (30) was used to determine the best-fitting model of evolution (i.e., TrN+I+G), which was used for construction of a maximum-likelihood (ML) tree. The ML tree was constructed, using the concatenated three-gene MLST sequences, using PAUP* Portable version 4.0b10 for Unix (35). No phylogenetic analyses were performed on the four-gene MLST data, as insufficient ST data are available for serotypes other than *Salmonella* serotypes Typhimurium and 4,5,12:i:−.

PFGE. XbaI PFGE was performed according to the Centers for Disease Control and Prevention PulseNet protocol (31). Analysis of PFGE types was performed using the BioNumerics Software package (Applied Maths, Austin, TX). Similarity analysis was performed by using the Dice coefficient, and clustering was performed using the unweighted pair group method with arithmetic mean.

SID. Simpson's index of diversity (SID) was calculated as previously described (23).

Analysis of microarray and genome sequence data to identify gene deletions in *Salmonella* serotype 4,5,12:i:−. In order to identify gene deletions and other genomic differences between *Salmonella* serotypes 4,5,12:i:− and Typhimurium, we used (i) comparative genomic microarray data on gene presence/absence patterns in four Spanish *Salmonella* serotype 4,5,12:i:− isolates (reported by Garaizar et al. [18]) and (ii) the full genome sequence data for the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM23701 (GenBank accession no. NZ_ABAO00000000; http://msc.jcvi.org/salmonella/salmonella_enterica_subsp__enterica_serovar_4__5__12_i___str__cvm23701/index.shtml) (32) and *Salmonella* serotype Typhimurium LT2 (AE006468). Genomic microarray data reported by Garaizar et al. (18) revealed one genomic deletion (termed cluster V) in *Salmonella* serotype 4,5,12,:i:−, which included deletion of *fljB* (encoding phase 2 flagella, thus providing a functional explanation for the absence of phase 2 flagellar expression observed in *Salmonella* serotype 4,5,12:i:−) as well as a second deletion (termed cluster IV) located approximately 16 kb 5′ of cluster V. BLAST searches were used to determine whether genes in cluster IV (genes STM2694 to STM2740) and cluster V (genes STM2758 to STM2773) as well as genes in the intervening regions and upstream and downstream were present in the *Salmonella* serotype 4,5,12:i:− isolate CVM23701 genome. Specifically, BLAST searches were used to determine whether *Salmonella* serotype Typhimurium LT2 genes STM2691 through STM2775 were present in the CVM23701 genome. BLAST searches were performed using the National Center for Biotechnology Information (NCBI) BLAST tools and gene sequences downloaded from the J. Craig Venter Institute Comprehensive Microbial Resource. BLAST searches were also used to determine whether genes in three other clusters (I, II, and III), which were previously reported to be present in *Salmonella* serotype Typhimurium LT2, but absent in Spanish *Salmonella* serotype 4,5,12:i:− isolates, were present in the genome sequence for the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM23701.

PCR-based characterization of gene deletion patterns in representative *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates. Based on our analyses of (i) the genomic microarray data reported by Garaizar et al. (18) and (ii) the *Salmonella* serotype 4,5,12:i:− isolate CVM23701 genome, we designed PCR primers to test for the presence of selected genes in clusters IV and V and adjoining regions (Table 2). We initially designed eight primer sets for genes that are at the junctions of clusters IV and V (as reported by Garaizar et al. [18]); these primers target STM2692, STM2694, STM2740, STM2741, STM2757, STM2758, STM2773 (*iroB*), and STM2774 (Fig. 1 shows primer locations). In addition, we designed primer sets for (i) two genes (*fljA* and *fljB*) absent from both the *Salmonella* serotype 4,5,12:i:− isolates from Spain (based on the genomic microarray data reported by Garaizar et al. [18]) and the CVM23701 genome and for (ii) one gene (*hin*) present in CVM23701 and absent in the *Salmonella* serotype 4,5,12:i:− isolates from Spain. We also designed one set of primers targeting a region found upstream of *hin* in only the CVM23701 genome; this region was designated the STM1053-1997 region, as the primers designed

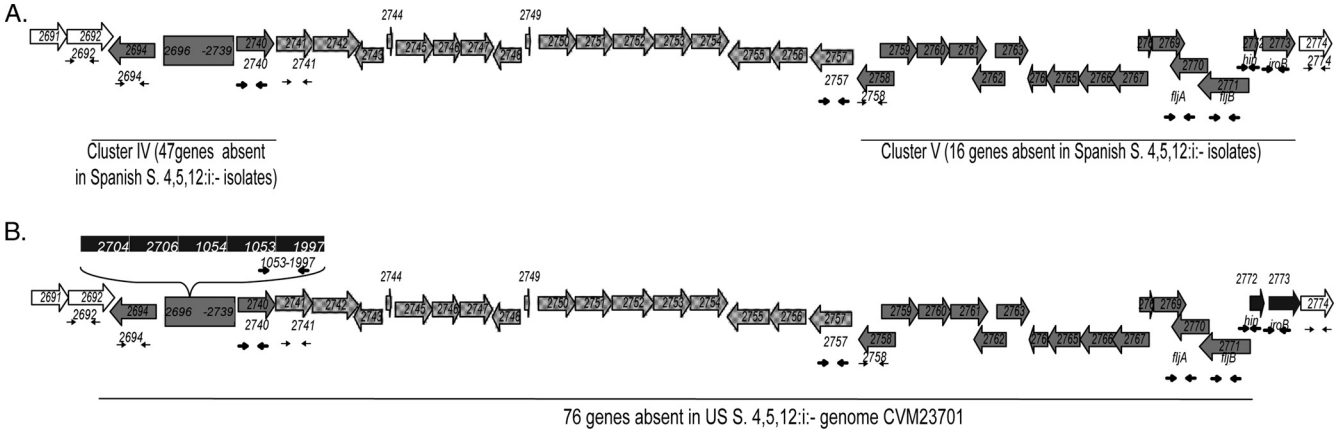


FIG. 1. Deduced genome structure for the genomic region between STM2691 and STM2774 for four *Salmonella* serotype 4,5,12:i:− isolates from Spain (based on genomic microarray data reported by Garaizar et al. [18]) (A) and for the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM 23701 (based on an unfinished genome sequence reported by Rosovitz et al. [http://msc.jcvi.org/salmonella/salmonella_enterica_subsp__enterica_serovar_4__5__12_i___str__cvm23701/index.shtml]) (B). Genes are represented as open arrows or boxes; gene numbers (e.g., 2691) represent locus numbers based on primary annotation of *Salmonella* serotype Typhimurium LT2 (with the prefix "STM"). White represents the genes present in *Salmonella* serotype Typhimurium LT2 and all *Salmonella* serotype 4,5,12:i:− isolates, gray represents genes present in *Salmonella* serotype Typhimurium LT2 and absent from both U.S. and Spanish *Salmonella* serotype 4,5,12:i:− isolates, and a halftone pattern represents genes present in *Salmonella* serotype Typhimurium LT2 and Spanish *Salmonella* serotype 4,5,12:i:− isolates but absent from the U.S. *Salmonella* serotype 4,5,12:i:− isolate. Black represents a unique insertion in the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM 23701, which includes genes with full or partial homology with the *Salmonella* serotype Typhimurium LT2 genes STM1054 (94% homology with LT2 over 79% gene length), STM1053 (93% homology with LT2 over 85% gene length), STM1997 (92% homology with LT2 over 42% gene length), STM2704 (87% homology with LT2 over 100% gene length), and STM2706 (87% homology with LT2 over 18% gene length). *hin* and *iroB*, which are present in LT2 and the U.S. *Salmonella* serotype 4,5,12:i:− isolate, are also shown in black. Small arrows represent PCR primers, including five primer sets (see Table S2 in the supplemental material) used only for an initial screen of six isolates (two *Salmonella* serotype 4,5,12:i:− isolates each from Spain and the United States and one *Salmonella* serotype Typhimurium isolate each from Spain and the United States) (shown as thin black arrows) and seven primer sets (Table 2) used to screen a total of 59 isolates (shown as thick arrows).

are located in genes with homology to STM1053 (forward primer) and STM1997 (reverse primer) (Fig. 1). PCR was performed on DNA purified using the QIAmp DNA mini kit (Qiagen Inc., Chartsworth) as detailed below, using either Ampli *Taq* Gold (Applied Biosystems, Foster City, CA) or Go *Taq* (Promega, Madison, WI).

All PCR primers were used initially to screen for gene presence/absence among four *Salmonella* serotype 4,5,12:i:− isolates (two each from Spain and the United States) as well as two *Salmonella* serotype Typhimurium isolates (one each from Spain and the United States). Subsequently, primers targeting six genes (i.e., STM2740, STM2757, *fljA*, *fljB*, *hin*, and *iroB*) and the STM1053-1997 region (Table 2 lists all primers) were used to screen for presence/absence of the selected genes among 59 representative isolates, representing *Salmonella* serotypes 4,5,12:i:− (41 isolates) and Typhimurium (14 isolates) as well as all four isolates with inconsistent serotype data (i.e., serotyped as *Salmonella* serotypes 4,5,12:i:− and Typhimurium). These isolates were selected to represent all PFGE types and STs found among the Spanish isolates. Isolates obtained in the United States were selected to represent the most common PFGE types found among different isolate sources (e.g., human, food, cattle, poultry, and nondomestic birds); for *Salmonella* serotype 4,5,12:i:−, isolates from the United States were selected to ensure inclusion of at least one representative of each ST and PFGE.

Nucleotide sequence accession number. The representative sequence for *Salmonella* isolate FSL S9-102 has been deposited in GenBank with accession no. FJ763347.

RESULTS AND DISCUSSION

In order to better understand the evolution, ecology, and genetic characteristics of *Salmonella* serotype 4,5,12:i:−, we characterized 190 *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates from the United States and Spain using a variety of molecular methods. Overall, our data indicate that (i) *Salmonella* serotypes 4,5,12:i:− and Typhimurium represent a highly clonal group, which can be differentiated by PFGE; (ii) U.S. and Spanish *Salmonella* serotype 4,5,12:i:− isolates show different patterns of gene deletion in the regions encoding phase 2 flagella and represent distinct PFGE patterns; and (iii) in addition to two common *Salmonella* serotype 4,5,12:i:− genotypes (designated here the "Spanish" and the "U.S." *Salmonella* serotype 4,5,12:i:− clones), other 4,5,12:i:− genotypes exist. We thus conclude that *Salmonella* serotype 4,5,12:i:− most likely represents multiple clones that emerged through independent deletion events.

***Salmonella* serotypes 4,5,12:i:− and Typhimurium represent a highly clonal group, which can be differentiated by PFGE.** Among the 190 *Salmonella* isolates initially characterized as *Salmonella* serotypes Typhimurium (100 isolates) and 4,5,12:i:−, we identified six distinct STs based on a four-gene MLST scheme (Table 3). While the three-gene MLST scheme initially used had previously been shown to provide discriminatory power similar to that for a seven-gene MLST scheme (33), sequencing of a fourth gene (*aroC*) was included because the three-gene MLST allowed for only limited discrimination among the isolates used. Even with a four-gene MLST, a single ST (ST1) represented the vast majority of *Salmonella* serotype Typhimurium and 4,5,12:i:− isolates; 84 out of 100 *Salmonella* serotype Typhimurium and 85 out of 86 *Salmonella* serotype 4,5,12:i:− isolates were classified as ST1. Analyses of the relevant genes in the genomes of *Salmonella* strain LT2 and the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM23701 showed that these two strains also represent ST1. One *Salmonella* serotype 4,5,12:i:− isolate from Spain represented ST3; ST3 also represented seven U.S. *Salmonella* serotype Typhimurium

TABLE 3. Distribution of four-gene STs among *Salmonella* isolates

Four-gene ST[a]	No. of isolates			
	Salmonella serotype 4,5,12:i:−	*Salmonella* serotype Typhimurium	Inconsistent *Salmonella* serotype[b]	Total
1	85	84	3	172
2	0	1	0	1
3	1	7	1	9
7	0	5	0	5
8	0	1	0	1
9	0	2	0	2

[a] STs were based on ATs for partial *fimA*, *mdh*, *manB*, and *aroC* sequences.
[b] These isolates were serotyped as *Salmonella* serotype 4,5,12:i:− in one replicate and *Salmonella* serotype Typhimurium in another replicate (including one isolate that was classified as *Salmonella* serotype 4,5,12:i:− in two replicates and *Salmonella* serotype Typhimurium in one replicate) and were thus designated "inconsistent."

isolates and one U.S. isolate with inconsistent serotype data (i.e., serotyped as *Salmonella* serotypes 4,5,12:i:− and Typhimurium in replicate experiments). ST3 differs from ST1 by only one nucleotide difference in *manB*. While *Salmonella* serotype 4,5,12:i:− represented only two STs (SID = 0.02), *Salmonella* serotype Typhimurium isolates represented six STs (SID = 0.29), indicating considerably higher ST diversity among the *Salmonella* serotype Typhimurium isolates characterized. Guerra et al. (21) previously also proposed that *Salmonella* serotype 4,5,12:i:− represents a lower diversity than *Salmonella* serotype Typhimurium, even though their molecular subtype study only used 16 *Salmonella* serotype 4,5,12:i:− and 2 *Salmonella* serotype Typhimurium isolates from Spain.

Phylogenetic analyses of three-gene MLST data (Fig. 2) also supported that *Salmonella* serotypes 4,5,12:i:− and Typhimurium are genetically closely related and highly clonal, as shown by the fact that all *Salmonella* serotype 4,5,12:i:− and Typhimurium STs form a single branch with strong bootstrap support. This observation is consistent with a number of studies (2, 14) that have shown that *Salmonella* serotype Typhimurium is highly clonal. The observation that all *Salmonella* serotype 4,5,12:i:− isolates characterized here share identical STs with Typhimurium isolates is consistent with a number of studies (see, e.g., references 1, 6, 13, 14, 16, and 38) that have shown, using different molecular subtyping methods (e.g., PFGE and MLST), that *Salmonella* serotype 4,5,12:i:− isolates are genetically and phenotypically closely related to *Salmonella* serotype Typhimurium. While according to serological characterization, *Salmonella* serotype 4,5,12:i:− is also closely related to *Salmonella* serotypes Lagos (4,5,12:i:1,5), Agama (4,12:i:1,6), Farsta (4,12:i:e,n,x), Tsevie (4,12:i:e,n,z_{15}), Cloucester (1,5,12,27:i:l,w), and Tumodi (1,4,12:i:z_6) and to an unnamed subspecies II serotype (4,5,27:i:z_{35}) (28), we are not aware of any data indicating that any of these serotypes might be an ancestor of a *Salmonella* serotype 4,5,12:i:− strain. Echeita et al. (16) specifically reported that two genomic regions, i.e., a 1,000-bp *fliB-fliA* intergenic region and a 162-bp region specific for DT104 and DT U302 phage types, were absent in *Salmonella* serotype Lagos but present in *Salmonella* serotype Typhimurium phage types DT104 and DT U302, as well as in Spanish *Salmonella* serotype 4,5,12:i:− isolates; these data suggest that Spanish *Salmonella* serotype 4,5,12:i:− isolates are genetically closely related to *Salmonella* serotype Typhimurium DT104 and DT U302 and are unlikely to have originated from a *Salmonella* serotype Lagos ancestor. In our analysis of *aroC* ATs (including *aroC* ATs obtained from the Max Planck Institute MLST website at http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica), we also found that the *aroC* AT (i.e., AROC146) for the only *Salmonella* serotype Lagos isolate in this database was distinct from the *aroC* AT found among all *Salmonella* serotype 4,5,12:i:− isolates and all but one *Salmonella* serotype Typhimurium isolate characterized here (i.e., AT 10, which differs by four nucleotides from AT AROC146), further supporting that *Salmonella* serotype Lagos is unlikely to be the ancestor of *Salmonella* serotype 4,5,12:i:−. Similarly, the *aroC* AT for the one *Salmonella* serotype Agama isolate represented in the Max Planck Institute MLST database represents an *aroC* AT (AROC136), which is distinct from AT 10 (four nucleotide differences between AT 10 and AT AROC136). These data indicate that *Salmonella* serotype Agama is unlikely to be the ancestor of *Salmonella* serotype 4,5,12:i:−. While, overall, these data suggest that *Salmonella* serotype 4,5,12:i:− is a monophasic variant of *Salmonella* serotype Typhimurium, the rare *Salmonella* serotypes Farsta, Tsevie, Cloucester, Tumodi, and subspecies II serotype 4,5,27:i:z_{35} cannot be definitively excluded as ancestors of *Salmonella* serotype 4,5,12:i:− until isolates representing these serotypes have been characterized by molecular methods and compared to *Salmonella* serotype Typhimurium and 4,5,12:i:− isolates.

As PFGE has been shown to be a highly discriminatory subtyping method for a number of *Salmonella* serotypes (1, 14, 38), we further characterized all 190 *Salmonella* isolates using PFGE with the enzyme XbaI. Overall, we identified 79 PFGE patterns (SID = 0.96) among all 190 isolates. A total of 29 and 50 PFGE types were differentiated among the 86 and 100 *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates (SID = 0.92 and 0.93, respectively); the four isolates with inconsistent serotypes represented three different PFGE patterns. Overall, these data support previous studies which have shown that *Salmonella* serotype 4,5,12:i:− isolates represent considerable PFGE diversity (1, 21, 38) and that PFGE, in general, allows for more sensitive subtype discrimination among *Salmonella* isolates than MLST (12, 17, 22).

Interestingly, two PFGE patterns (P1 and P71) (Fig. 3) were shared by *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates. PFGE type P1 was found in three *Salmonella* serotype 4,5,12:i:− and four *Salmonella* serotype Typhimurium isolates from the United States, while P71 represented four *Salmonella* serotype 4,5,12:i:− isolates and one *Salmonella* serotype Typhimurium isolate as well as one isolate with an inconsistent serotype, all isolated in the United States. These observations extend previous observations by de la Torre et al. (13) and Zamperini et al. (38). de la Torre et al. (13) showed that at least one XbaI type and one BlnI PFGE type were shared between Spanish *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates, even though these two serotypes never shared the same combined XbaI/BlnI PFGE type (13). However, Zamperini et al. found one combined XbaI/BlnI PFGE type shared by one *Salmonella* serotype 4,5,12:i:− and one *Salmonella* serotype Typhimurium isolate, both isolated from poultry in the United States (38). In general, these observations further support that *Salmonella* serotype 4,5,12:i:− has evolved from a *Salmonella* serotype Typhimurium ancestor.

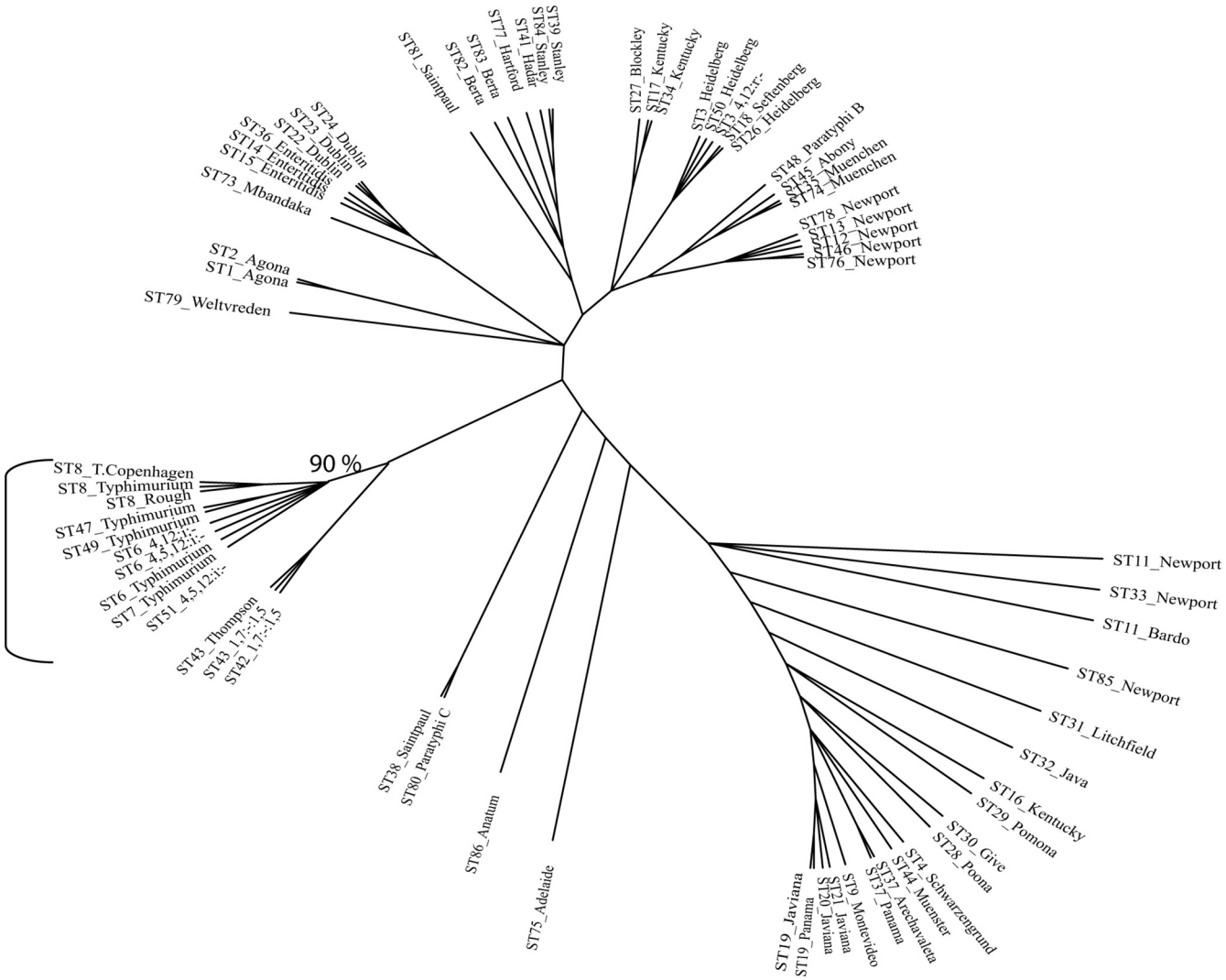
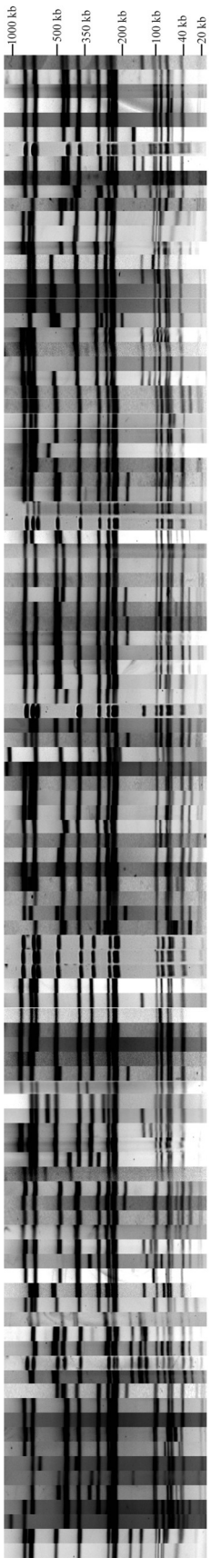


FIG. 2. Phylogenetic tree for all three-gene (*fimA*, *manB*, and *mdh*) STs identified among *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates as well as selected isolates representing other *Salmonella* serotypes (these STs were taken from reference 2). For each unique ST, *fimA*, *manB*, and *mdh* sequences were concatenated and aligned, followed by construction of an ML tree (100 bootstrap replicates), using the TrN+I+G model of evolution (identified by MODELTEST as the appropriate model for the data set used). While a number of nodes in this tree were supported by high bootstrap values, bootstrap support is shown only for the clade containing *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates. Branch lengths do not represent phylogenetic distances.

A total of four PFGE patterns (P12, P19, P35, and P45) were found among both Spanish and U.S. *Salmonella* serotype Typhimurium isolates. P12 represented 14 and 10 *Salmonella* serotype Typhimurium isolates from Spain and the United States, respectively. P35 represented two *Salmonella* serotype Typhimurium isolates from the United States and one *Salmonella* serotype Typhimurium isolate from Spain. PFGE patterns P19 and P45 each represented one *Salmonella* serotype Typhimurium isolate from the United States and one isolate from Spain. Identification of identical XbaI PFGE patterns among *Salmonella* serotype Typhimurium isolates from different continents is consistent with previous studies which have shown that some genetically closely related *Salmonella* strains are distributed worldwide (7, 19), including some other studies that have found *Salmonella* serotype Typhimurium isolates with identical PFGE types in different countries and continents (7). While PFGE patterns for most Spanish *Salmonella* serotype 4,5,12:i:− isolates were similar to each other and different from the patterns of U.S. *Salmonella* serotype 4,5,12:i:− isolates, one PFGE pattern (PFGE type P28) was shared among five Spanish *Salmonella* serotype 4,5,12:i:− isolates and one *Salmonella* serotype 4,5,12:i:− isolate from the United States. This U.S. isolate was obtained from a free-ranging owl in Georgia (38). As most owls are nonmigratory, there is no apparent hypothesis as to the source of an infection with a "Spanish clone" *Salmonella* serotype 4,5,12:i:− isolate in this animal.

U.S. and Spanish *Salmonella* serotype 4,5,12:i:− isolates show different patterns of gene deletion in the regions encoding phase 2 flagella and have different PFGE patterns. A previous study of four multidrug-resistant Spanish *Salmonella* serotype 4,5,12:i:− isolates and *Salmonella* serotype Typhimurium LT2 identified five genomic regions (clusters) that



Isolate No.	Country Isolated from	State Isolated from	Source	Serotype	PFGE Pattern (No. of isolates)	4-gene MLST
FSL S5-534	US	NY	Human	Typhimurium	P15 (1)	1
FSL S5-607	US	NY	Bovine	Typhimurium	P16 (1)	1
FSL R6-144	US	WA	Human	Typhimurium	P12 (24)	1
FSL S9-221	Spain		Enviromental	Typhimurium	P12 (24)	1
FSL S5-845	US	NY	Bovine	Typhimurium	P12 (24)	1
FSL S9-217	Spain		Enviromental	Typhimurium	P14 (1)	1
FSL S9-165	US	WA	Bovine	Inconsistent	P23 (2)	1
FSL S9-214	Spain		Food	Typhimurium	P13 (1)	1
FSL S5-492	US	NY	Human	Typhimurium	P17 (1)	1
FSL R6-131	US	WA	Human	Typhimurium	P24 (1)	2
FSL S5-516	US	NY	Human	Typhimurium	P74 (1)	1
FSL S9-233	Spain		Enviromental	Typhimurium	P84 (1)	1
FSL S9-235	Spain		Food	Typhimurium	P88 (1)	1
FSL S9-112	US	GA	Poultry	Typhimurium	P9 (1)	1
FSL S9-126	US	GA	Bovine	4,5,12:i:-	P49 (1)	1
FSL S9-167	US	WA	Human	4,5,12:i:-	P48 (2)	1
FSL S9-184	US	WA	Human	4,5,12:i:-	P50 (3)	1
FSL S9-121	US	GA	Poultry	4,5,12:i:-	P50 (3)	1
FSL S9-179	US	WA	Human	4,5,12:i:-	P55 (1)	1
FSL S9-128	US	GA	Bovine	Typhimurium	P85 (1)	1
FSL S9-188	US	WA	Human	4,5,12:i:-	P46 (8)	1
FSL S9-109	US	GA	Poultry	4,5,12:i:-	P46 (8)	1
FSL S9-130	US	GA	Bovine	Typhimurium	P41 (1)	1
FSL S5-452	US	NY	Human	Typhimurium	P1 (7)	1
FSL S9-193	US	WA	Human	4,5,12:i:-	P1 (7)	1
FSL S9-114	US	GA	Poultry	4,5,12:i:-	P40 (10)	1
FSL R6-150	US	WA	Human	4,5,12:i:-	P40 (10)	1
FSL S9-129	US	GA	Bovine	Typhimurium	P47 (1)	1
FSL S5-392	US	NY	Human	Typhimurium	P44 (1)	1
FSL S5-527	US	NY	Human	4,5,12:i:-	P42 (3)	1
FSL S9-031	US	NY	Food	4,5,12:i:-	P42 (3)	1
FSL S9-172	US	WA	Avian	4,5,12:i:-	P69 (5)	1
FSL R6-152	US	WA	Human	4,5,12:i:-	P69 (5)	1
FSL S9-212	Spain		Human	Typhimurium	P37 (1)	1
FSL S5-788	US	NY	Bovine	Typhimurium	P45 (2)	9
FSL S9-210	Spain		Enviromental	Typhimurium	P45 (2)	7
FSL S5-536	US	NY	Human	Typhimurium	P18 (1)	9
FSL S9-207	Spain		Food	Typhimurium	P10 (3)	1
FSL S5-462	US	NY	Human	Typhimurium	P38 (1)	1
FSL S5-507	US	NY	Human	Typhimurium	P36 (4)	1
FSL S9-125	US	GA	Poultry	Typhimurium	P36 (4)	1
FSL R6-154	US	WA	Human	Typhimurium	P35 (3)	1
FSL S9-110	US	GA	Poultry	Typhimurium	P35 (3)	1
FSL S9-231	Spain		Enviromental	Typhimurium	P35 (3)	7
FSL S9-228	Spain		Enviromental	Typhimurium	P52 (2)	1
FSL R6-119	US	WA	Human	4,5,12:i:-	P67 (1)	1
FSL S5-936	US	NY	Bovine	Typhimurium	P53 (1)	1
FSL S5-501	US	NY	Human	Typhimurium	P39 (1)	1
FSL S5-896	US	NY	Bovine	Typhimurium	P75 (1)	1
FSL S5-729	US	NY	Bovine	Typhimurium	P8 (1)	1
FSL S5-520	US	NY	Human	Typhimurium	P4 (7)	1
FSL S5-831	US	NY	Bovine	Typhimurium	P4 (7)	1
FSL S9-103	US		Non-dom. bird	Typhimurium	P4 (7)	1
FSL S9-247	Spain		Enviromental	Typhimurium	P19 (2)	1
FSL S5-494	US	NY	Human	Typhimurium	P19 (2)	1
FSL S5-805	US	NY	Bovine	Typhimurium	P7 (2)	1
FSL S5-381	US	NY	Human	Typhimurium	P7 (2)	1
FSL S5-397	US	NY	Human	Typhimurium	P6 (1)	1
FSL S5-653	US	NY	Human	Typhimurium	P20 (1)	1
FSL S5-799	US	NY	Bovine	Typhimurium	P11 (1)	1
FSL S5-394	US	NY	Human	Typhimurium	P25 (1)	1
FSL R6-028	US	WA	Bovine	Inconsistent	P71 (6)	3
FSL R6-124	US	WA	Human	4,5,12:i:-	P71 (6)	1
FSL S9 - 171	US	WA	Canine	4,5,12:i:-	P71 (6)	1
FSL S5-433	US	NY	Bovine	Typhimurium	P71 (6)	1
FSL S5-596	US	NY	Bovine	4,5,12:i:-	P56 (2)	1
FSL S9-115	US	GA	Poultry	4,5,12:i:-	P59 (20)	1
FSL S5-398	US	NY	Human	4,5,12:i:-	P59 (20)	1
FSL S5-737	US	NY	Bovine	4,5,12:i:-	P59 (20)	1
FSL S9-192	US	WA	Human	4,5,12:i:-	P60 (1)	1
FSL S5-526	US	NY	Human	4,5,12:i:-	P61 (1)	1
FSL S5-618	US	NY	Bovine	4,5,12:i:-	P62 (1)	1
FSL S5-759	US	NY	Bovine	4,5,12:i:-	P65 (1)	1
FSL S5-816	US	NY	Bovine	4,5,12:i:-	P64 (2)	1
FSL S9-123	US	GA	Poultry	Typhimurium	P57 (1)	1
FSL S9-124	US	GA	Poultry	Typhimurium	P63 (1)	1
FSL S9-131	US	GA	Bovine	Typhimurium	P51 (1)	1
FSL S9-189	US	WA	Human	4,5,12:i:-	P73 (1)	1
FSL S5-555	US	NY	Bovine	Typhimurium	P76 (3)	1
FSL S5-505	US	NY	Human	Typhimurium	P76 (3)	1
FSL S5-531	US	NY	Human	Typhimurium	P77 (2)	8
FSL S5-564	US	NY	Bovine	Typhimurium	P77 (2)	1
FSL S9-132	US	GA	Bovine	Typhimurium	P78 (1)	1
FSL S9-102	US		Non-dom. bird	Inconsistent	P21 (1)	1
FSL S9-211	Spain		Human	Typhimurium	P79 (1)	1
FSL S9-119	US	GA	Poultry	4,5,12:i:-	P66 (1)	1
FSL S9-133	US	GA	Bovine	Typhimurium	P72 (1)	1
FSL S5-430	US	WA	Bovine	Typhimurium	P2 (1)	3
FSL S5-554	US	NY	Bovine	Typhimurium	P81 (5)	3
FSL R6-161	US	WA	Human	Typhimurium	P81 (5)	1
FSL R6-002	US	WA	Bovine	Typhimurium	P86 (1)	3
FSL S5-796	US	NY	Bovine	Typhimurium	P82 (1)	3
FSL S5-635	US	NY	Human	4,5,12:i:-	P80 (1)	1
FSL S9-101	US		Non-dom. bird	4,5,12:i:-	P28 (6)	1
FSL S9-206	Spain		Human	4,5,12:i:-	P28 (6)	1
FSL S9-245	Spain		Human	4,5,12:i:-	P30 (1)	1
FSL S9-241	Spain		Human	4,5,12:i:-	P29 (1)	3
FSL S9-238	Spain		Human	4,5,12:i:-	P31 (1)	1
FSL S9-239	Spain		Human	4,5,12:i:-	P26 (1)	1
FSL S9-244	Spain		Human	4,5,12:i:-	P27 (1)	1
FSL S9-204	Spain		Human	4,5,12:i:-	P32 (2)	1
FSL S9-237	Spain		Human	4,5,12:i:-	P34 (1)	1
FSL S9-232	Spain		Enviromental	Typhimurium	P87 (1)	7
FSL S9-234	Spain		Enviromental	Typhimurium	P83 (1)	7

were absent in all four Spanish *Salmonella* serotype 4,5,12:i:− isolates, but present in *Salmonella* serotype Typhimurium LT2. As our initial PFGE data indicated that Spanish *Salmonella* serotype 4,5,12:i:− isolates are genetically distinct from most U.S. *Salmonella* serotype 4,5,12:i:− isolates, we analyzed an available genome sequence for a U.S. *Salmonella* serotype 4,5,12:i:− isolate (strain CVM23701 [32]) for the presence of these five clusters (i.e., clusters I to V). BLAST searches against the CVM23701 genome sequences showed that cluster I (STM0517 to STM0529), which includes 13 genes, most of which are involved in allantoin-glyoxylate pathway-related functions, is present in the genome of this U.S. *Salmonella* serotype 4,5,12:i:− isolate, even though this cluster appears to be absent from the four Spanish *Salmonella* serotype 4,5,12:i:− isolates previously characterized by genomic microarrays (18). Cluster II (STM0893 to STM0929), which includes 35 Fels-1 prophage genes and two adjacent genes, was reported to be absent from the four Spanish *Salmonella* serotype 4,5,12:i:− isolates and was not identified in the available unfinished genome of the U.S. *Salmonella* serotype 4,5,12:i:− isolate. As the genes upstream and downstream of cluster II were located on a single contig, we conclude that this cluster is likely absent from the CVM23701 genome. These findings are consistent with the observation that the Fels-1 prophage is present in LT2 but typically is absent in other *Salmonella* serotype Typhimurium isolates (25). Cluster III (STM2616 to STM2617) encodes the Gifsy-1 prophage and was found in the genome of the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM23701, but it was reported to be absent from the four Spanish *Salmonella* serotype 4,5,12:i:− isolates previously characterized (18). The findings that clusters I to III were all absent from these four Spanish *Salmonella* serotype 4,5,12:i:− isolates while only cluster II was absent from the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM23701 provide initial evidence that U.S. and Spanish *Salmonella* serotype 4,5,12:i:− isolates may represent distinct genotypes.

Clusters IV (STM2694 to STM2740) and V (STM2758 to STM2773), which both were reported to be absent in the four Spanish *Salmonella* serotype 4,5,12:i:− isolates (Fig. 1A), are located in close proximity to each other. While cluster IV contains 47 Fels-2 prophage genes, cluster V contains a number of genes associated with different functions, including the *fljAB* operon (18). Notably, deletion of *fljAB* provides a functional explanation for the absence of phase 2 flagellar expression observed in *Salmonella* serotype 4,5,12:i:−, as *fljB* encodes the phase 2 flagellar protein and *fljA* encodes a repressor of *fliC* transcription (which encodes the phase 1 flagellar protein). Initial BLAST searches against the genome sequence for the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM23701 showed that both clusters IV and V were absent from the CVM23701 genome (cluster IV and V as well as intervening genes were located on a single CVM23701 contig, NZ_ABA001000014.1). Two genes located in the 3′ end of cluster V, including STM2772 (*hin*, encodes a recombinase that regulates the regulation of flagellar gene expression) and STM2773 (*iroB*, encodes glucosyltransferase homolog protein), were present in the CVM23701 genome, even though they were reported to be absent from the four Spanish *Salmonella* serotype 4,5,12:i:− isolates, based on genomic microarray data (18). Further analysis of the CVM23701 genome sequence indicated that the region between clusters IV and V (STM2739 to STM2757) was also absent from the CVM23701 genome, indicating that this strain contains a larger deletion than the four Spanish *Salmonella* serotype 4,5,12:i:− isolates; this deletion spans cluster IV and most of cluster V (except for two genes at the 3′ end) as well as the region between these two clusters. Interestingly, in the genome sequence of the *Salmonella* serotype 4,5,12:i:− isolate CVM23701, an approximately 7-kb region is inserted into this deleted section of the genome. This insertion includes two partial Fels-2 genes (STM2704 and STM2706) and three genes homologous to STM1054, STM1053, and STM1997 (*umuC*), which encode two Gifsy-2 prophage genes and a component of DNA polymerase V (*umuC*) (Fig. 1B). We will refer to this insertion as the "STM1053-1997" region; this region is not found in LT2. The presence of this region in CVM23701 suggests the intriguing hypothesis that deletion, in the U.S. *Salmonella* serotype 4,5,12:i:− clone, of clusters IV and V and the intervening region may have been caused by abortive, imprecise excision of a prophage.

As our analyses detailed above indicated that the U.S. *Salmonella* serotype 4,5,12:i:− isolate CVM23701 shows distinct genomic gene presence/absence patterns compared to four Spanish *Salmonella* serotype 4,5,12:i:− isolates previously characterized (18), we designed PCR primers to determine the absence/presence of eight genes that are at the junctions of clusters IV and V (i.e., STM2692, STM2694, STM2740, STM2741, STM2757, STM2758, STM2773 [*iroB*], and STM2774; Fig. 1 shows primer locations) and three genes (i.e., *fljA*, *fljB*, and *hin*) that are responsible for expression of phase 2 flagellar antigen. In addition, a set of primers was designed to allow for the detection of the "STM1053-1997" region, which was found in the CVM23701 genome sequence. While negative PCR results may indicate the absence of a gene or the presence of a distinct allelic variant of a gene, which does not allow for PCR amplification, we surmised that in this study, negative PCR results for *Salmonella* serotype 4,5,12:i:− due to gene diversification (rather than gene absence) are extremely unlikely due to the high genetic similarity between *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates (e.g., as indicated by identical or highly similar MLST types for these two serotypes).

FIG. 3. Representative XbaI PFGE patterns for *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates as well as four isolates with inconsistent serotype data (i.e., isolates that were initially identified as *Salmonella* serotype 4,5,12:i:− but were classified as *Salmonella* serotype Typhimurium when they were resubmitted for serotyping). The PFGE types shown represent all 79 unique types found among the 190 isolates characterized. If identical PFGE types were found among isolates representing two serotypes, different sources (e.g., human and bovine), or different countries, one representative from each group was included in this figure; solid vertical lines indicate multiple isolates with identical PFGE patterns. For example PFGE pattern 28 (P28) was identified in five *Salmonella* serotype 4,5,12:i:− isolates from Spain and one *Salmonella* serotype 4,5,12:i:− isolate from a nondomestic bird in the United States. The number of isolates with a given PFGE type is indicated in parentheses after the PFGE type designation.

Characterization of an initial six isolates (two *Salmonella* serotype 4,5,12:i:− isolates each from Spain and the United States and one *Salmonella* serotype Typhimurium isolate each from Spain and the United States) showed that the Spanish *Salmonella* serotype 4,5,12:i:− isolates had STM2692, STM2740, STM2741, STM2757, and STM2774 but lacked STM2694, STM2758, STM2773 (*iroB*), *fljA*, *fljB*, *hin*, and the STM1053-1997 region. These results confirmed the gene presence/absence patterns previously reported for four Spanish *Salmonella* serotype 4,5,12:i:− isolates (18), except for the fact that the PCR primers for STM2740, which was previously reported as absent in Spanish *Salmonella* serotype 4,5,12:i:−, yielded positive results, indicating the presence of at least part of this gene. The PCR results for the two U.S. *Salmonella* serotype 4,5,12:i:− isolates were consistent with the observations based on our analysis of the CVM23701 genome (representing a U.S. *Salmonella* serotype 4,5,12:i:− isolate). Specifically, the PCR data indicated that the two U.S. *Salmonella* serotype 4,5,12:i:− isolates (i) lack clusters IV and V as well as the intervening region (as supported by negative PCR results for STM2694, STM2740, STM2741, STM2757, STM2758, *fljA*, and *fljB*), (ii) contain *hin* and *iroB* (which are located in the 3′ end of cluster V and absent in the Spanish isolates), and (iii) contain an insertion upstream of the *hin* gene (i.e., the STM1053-1997 region), which is absent in the Spanish *Salmonella* serotype 4,5,12:i:− isolates. These data provided further support that Spanish *Salmonella* serotype 4,5,12:i:− isolates may be distinct from U.S. *Salmonella* serotype 4,5,12:i:− isolates.

To further test the hypothesis that Spanish and U.S. *Salmonella* serotype 4,5,12:i:− isolates represent different clonal groups with distinct genome deletion patterns, we screened 59 *Salmonella* serotype 4,5,12:i:− and Typhimurium isolates from these two countries (representing all PFGE patterns represented among 4,5,12:i:− isolates) for the presence/absence of six genes (i.e., STM2740, STM2757, *fljA*, *fljB*, *hin*, and *iroB*) and the STM1053-1997 region (Table 2). These PCR targets were selected because they (i) allow for clear differentiation of *Salmonella* serotype Typhimurium and 4,5,12:i:− genotypes and (ii) allow for differentiation of the "Spanish" and "U.S." genomic deletion patterns in the cluster IV and V region of *Salmonella* serotype 4,5,12:i:− isolates. The PCR data generated clearly indicated that (i) all Spanish *Salmonella* serotype 4,5,12:i:− isolates show a deletion of clusters IV and V but presence of the intervening region (STM2740 to STM2757) and (ii) all but two U.S. *Salmonella* serotype 4,5,12:i:− isolates show a deletion of clusters IV and V, including a deletion of the intervening region between clusters IV and V, as well as presence of *hin* and *iroB* (which are absent in the Spanish *Salmonella* serotype 4,5,12:i:− isolates) and presence of the STM1053-1997 region. We thus propose that *Salmonella* serotype 4,5,12:i:− isolates from the United States and Spain represent two distinct clones (i.e., the "Spanish" and the "U.S." clones). These findings are consistent with our observations that XbaI PFGE types of Spanish *Salmonella* serotype 4,5,12:i:− isolates generally are clearly distinct from the PFGE patterns for U.S. *Salmonella* serotype 4,5,12:i:− isolates. Interestingly, one *Salmonella* serotype 4,5,12:i:− isolate from the United States (isolated from a free-ranging owl in Georgia) had the same deletion pattern as Spanish *Salmonella* serotype 4,5,12:i:− isolates. As this isolate also shared an identical PFGE pattern (P28 [Fig. 3]) with five Spanish *Salmonella* serotype 4,5,12:i:− isolates, we also provide initial evidence for intercontinental spread of the "Spanish" *Salmonella* serotype 4,5,12:i:− clone.

TABLE 4. Presence/absence of selected genes in isolates representing the Spanish and U.S. *Salmonella* serotype 4,5,12:i:− clones as well as other *Salmonella* isolates

Gene	Presence of genes in[a]:				
	U.S. *Salmonella* serotype 4,5,12:i:− clone (n = 30)	Spanish *Salmonella* serotype 4,5,12:i:− clone (n = 10)	FSL S5-635 (*Salmonella* serotype 4,5,12:i:−; n = 1)	Isolates with inconsistent serotype results (n = 4)[b]	*Salmonella* serotype Typhimurium (n = 14)
STM2740	−	+	+	+	+
STM2757	−	+	+	+	+
STM1053-1997	+	−	−	−	−
fljA	−	−	−	+	+
fljB	−	−	−	+	+
hin	+	−	−	+	+
iroB	+	−	+	+	+

[a] + and − signs designate positive and negative PCR results, indicating the presence or absence of a gene, respectively.

[b] These isolates were serotyped as *Salmonella* serotype 4,5,12:i:− in one replicate and *Salmonella* serotype Typhimurium in another replicate (including one isolate that was classified as *Salmonella* serotype 4,5,12:i:− in two replicates and *Salmonella* serotype Typhimurium in one replicate) and were thus designated "inconsistent."

Interestingly, Matiasovicova et al. (25) suggested that multidrug-resistant *Salmonella* serotype Typhimurium might have evolved from a *Salmonella* serotype Typhimurium ancestor that first lost the region including STM0517-0529 (designated cluster I by Garaizar et al. [18]), allowing the utilization of allantoin as a sole nitrogen source, followed by acquisition of the *Salmonella* genomic island 1, which includes genes responsible for multidrug resistance. Since multidrug-resistant Spanish *Salmonella* serotype 4,5,12:i:− isolates lack cluster I (18), while the U.S. *Salmonella* serotype 4,5,12:i:− (CVM23701) contains this cluster, one might hypothesize that Spanish *Salmonella* serotype 4,5,12:i:− strains might have emerged from multidrug-resistant *Salmonella* serotype Typhimurium, while U.S. *Salmonella* serotype 4,5,12:i:− might have emerged from non-drug-resistant *Salmonella* serotype Typhimurium through independent events. Future studies of larger sets of multidrug-resistant and pansusceptible *Salmonella* serotype Typhimurium and 4,5,12:i:− isolates from different countries will be needed, though, to test this hypothesis.

In addition to two common *Salmonella* serotype 4,5,12:i:− clones (i.e., the "Spanish" and the "U.S." clones), we identified one rare *Salmonella* serotype 4,5,12:i:− genotype in North America. In addition to the common "Spanish" and "U.S." *Salmonella* serotype 4,5,12:i:− clones described above, we also identified one rare *Salmonella* serotype 4,5,12:i:− genotype in North America. Specifically, a human *Salmonella* serotype 4,5,12:i:− isolate from New York State (isolate FSL S5-635) (Table 4) was found to lack *hin* and the STM1053-1997 region, which are both present in the typical U.S. *Salmonella* serotype 4,5,12:i:− isolates, but contained *iroB*, which is typically absent in the Spanish clone. This isolate also was positive in the PCR

assays targeting STM2741 and 2757, suggesting that this isolate did maintain the genomic region between clusters IV and V, which is present in the Spanish but absent in the U.S. *Salmonella* serotype 4,5,12:i:− clone. This isolate thus seems to be similar to the Spanish clone but shows a deletion pattern different from that of Spanish clone isolates at the 3′ end of cluster V (Table 4). Further characterization of this isolate will be needed to determine whether it represents a third emergence event, independent of both the emergence of the "Spanish" and the "U.S." clones of *Salmonella* serotype 4,5,12:i:−, or whether it represents an evolutionary intermediate related to the Spanish *Salmonella* serotype 4,5,12:i:− clone. While this isolate represents a unique PFGE pattern not found among any other *Salmonella* serotype 4,5,12:i:− or Typhimurium isolates, it was classified as ST1, the same ST that represented the majority of *Salmonella* serotype Typhimurium isolates (84/100) as well as the majority of Spanish and U.S. clone *Salmonella* serotype 4,5,12:i:− isolates (12/13 and 73/73, respectively). This indicates that this strain most likely also emerged from a *Salmonella* serotype Typhimurium ancestor. Overall, our findings suggest that *Salmonella* serotype 4,5,12:i:− represents multiple genotypes, possibly indicating a strong selective pressure for loss of phase 2 flagellum expression.

We also identified four isolates from the United States (FSL S9-102, FSL S9-165, FSL S9-166, and FSL R6-084) that were initially determined to be *Salmonella* serotype 4,5,12:i:− but were found in the PCR screens to contain *fljA*, *fljB*, and *hin*, three genes critical for phase 2 flagellar expression (Table 4). PCR screens for other genes in clusters IV and V indicated that both of these clusters were present in these four isolates. As Zamperini et al. (38) suggested that mutations in *fljB* (the gene encoding phase 2 flagella) may also cause a *Salmonella* serotype 4,5,12:i:− phenotype, we sequenced the 1,521-nucleotide *fljB* open reading frame in three of these isolates (isolates FSL S9-165 and FSL S9-166 showed the same PFGE type [P23], and thus *fljB* was sequenced for only one of these isolates). All of these isolates had an identical *fljB* sequence, which showed one synonymous single-nucleotide polymorphism compared to *Salmonella* serotype Typhimurium LT2; these isolates did not show any nonsynonymous changes or other mutations that would explain a lack of phase 2 flagellum expression. These four isolates were thus submitted to National Veterinary Service Laboratories for serotype confirmation. While isolates FSL S9-166 and FSL R6-084 were reserotyped as *Salmonella* serotype Typhimurium, FSL S9-102 was reserotyped twice, once as *Salmonella* serotype 4,5,12:i:− and once as *Salmonella* serotype Typhimurium, and FSL S9-165 was reserotyped as *Salmonella* serotype Typhimurium twice. These results are consistent with previous reports (26) that serotyping of *Salmonella* may sometimes be difficult to reproduce and suggest that *Salmonella* serotype Typhimurium isolates may sometimes be misclassified as *Salmonella* serotype 4,5,12:i:− (and vice versa). While the four specific isolates with inconsistent serotype results characterized here appear to represent *Salmonella* serotype Typhimurium (based on genetic evidence for the presence of intact phase 2 genes and at least one serotype result characterizing them as *Salmonella* serotype Typhimurium), it is tempting to speculate that these isolates may show reduced phase 2 flagellum expression, which could be responsible for the inconsistent serotype data. This hypothesis would need to be tested further by expression analyses (e.g., quantitative reverse transcription-PCR analysis).

Conclusions. Overall, our observations suggest that *Salmonella* serotype 4,5,12:i:− evolved through multiple independent emergence events, most likely from *Salmonella* serotype Typhimurium ancestors. *Salmonella* serotype 4,5,12:i:− isolates from Spain and the United States appear to represent two different clones with distinct geographical distributions. This hypothesis is supported by multiple independent pieces of evidence. First, different genome-wide deletion patterns were found in four Spanish *Salmonella* serotype 4,5,12:i:− isolates (as previously determined by genomic microarrays [18]) and one U.S. *Salmonella* serotype 4,5,12:i:− isolate (based on an available whole genome sequence [32]). In particular, clusters I and III were present in the U.S. *Salmonella* serotype 4,5,12:i:− isolate (CVM23701), even though these clusters were reported to be absent in Spanish *Salmonella* serotype 4,5,12:i:− isolates (18). Second, genome analyses and PCR-based mapping showed clearly distinct deletion patterns in the genome region up- and downstream of the genes encoding proteins critical for phase 2 flagella and phase variation (i.e., *fljA*, *fljB*, and *hin*) in all Spanish and all but two U.S. *Salmonella* serotype 4,5,12:i:− isolates. Specifically, the Spanish isolates showed two deletions (of clusters IV and V), while the majority of U.S. isolates showed a single larger deletion (encompassing both clusters IV and V as well as the intervening region) with a 3′ junction different from that observed in the Spanish isolates. These findings provide another example of a *Salmonella* serotype of considerable public health relevance that represents at least two independent genetic lineages. For example, *Salmonella* serotype Newport has previously been shown to represent two distinct genetic lineages, including one lineage that contains predominantly pansusceptible isolates and one that contains predominantly multidrug-resistant isolates (2, 8, 22). In addition, the multiple independent emergences of *Salmonella* serotype 4,5,12:i:− and subsequent ecological success of multiple lineages (as evidenced by common isolation from human clinical cases in both Spain and the United States) suggest a strong selective pressure for loss of phase 2 flagella or a closely linked genotype. Future efforts to define the possible selection for loss of phase 2 flagella and to understand the specific *Salmonella* serotype 4,5,12:i:− genotypes circulating in countries other than the United States and Spain will be critical for understanding of the ecology and evolution of human disease-associated nontyphoidal *Salmonella*.

Findings of particular clinical relevance include the following: (i) PFGE allows for sensitive subtype discrimination of the emerging *Salmonella* serotype 4,5,12:i:−; (ii) *Salmonella* serotype 4,5,12:i:− appears to represent at least two common clones, which cannot easily be differentiated with standard diagnostic procedures (but can easily be discriminated with the PCR primers described here); and (iii) serological misclassification of *Salmonella* isolates as *Salmonella* serotype 4,5,12:i:− or Typhimurium may occasionally occur.

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