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Multilocus Sequence Typing Supports the Hypothesis that Cow- and Human-Associated *Salmonella* Isolates Represent Distinct and Overlapping Populations▽†

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A collection of 179 human and 156 bovine clinical *Salmonella* isolates obtained from across New York state over the course of 1 year was characterized using serotyping and a multilocus sequence typing (MLST) scheme based on the sequencing of three genes (*fimA*, *manB*, and *mdh*). The 335 isolates were differentiated into 52 serotypes and 72 sequence types (STs). Analyses of bovine isolates collected on different farms over time indicated that specific subtypes can persist over time on a given farm; in particular, a number of farms showed evidence for the persistence of a specific *Salmonella enterica* serotype Newport sequence type. Serotypes and STs were not randomly distributed among human and bovine isolates, and selected serotypes and STs were associated exclusively with either human or bovine sources. A number of common STs were geographically widespread. For example, ST6, which includes isolates representing serotype Typhimurium as well as the emerging serotype 4,5,12:i:–, was found among human and bovine isolates in a number of counties in New York state. Phylogenetic analyses supported the possibility that serotype 4,5,12:i:– is closely related to *Salmonella* serotype Typhimurium. *Salmonella* serotype Newport was found to represent two distinct evolutionary lineages that differ in their frequencies among human and bovine isolates. A number of *Salmonella* isolates carried two copies of *manB* (33 isolates) or showed small deletion events in *fimA* (nine isolates); these duplication and deletion events may provide mechanisms for the rapid diversification of *Salmonella* surface molecules. We conclude that the combined use of an economical three-gene MLST scheme and serotyping can provide considerable new insights into the evolution and transmission of *Salmonella*.

Salmonella is a gram-negative pathogen found in a wide range of animal species including birds, mammals, and reptiles. In the United States, *Salmonella* causes an estimated 1.4 million food-borne illnesses a year (39), and salmonellosis accounts for over half of all food-borne disease outbreaks linked to bacterial pathogens (41). Whereas *Salmonella* infections in developed countries are typically acquired through the consumption of contaminated food and water (12, 13, 16), direct contact with infected animals is also an important source of human infections (14, 17, 33). Human nontyphoidal *Salmonella* infections generally manifest as gastroenteritis (40) and, less commonly, as systemic infections, which may require antibiotic treatment (52).

Characterization of *Salmonella* isolates and human and animal salmonellosis surveillance have traditionally used serotyping for subtyping and strain differentiation (11). This technique relies upon the immunoreactivity of *Salmonella* O and H antigens. The O antigen is found in the lipopolysaccharide layer of the bacterial cell wall, whereas the H antigen is the filamentous portion of the flagella (11). Even though over 2,500 different *Salmonella* serotypes can be differentiated (11), some serotypes are commonly associated with human salmonellosis infections (e.g., *Salmonella enterica* serotype Typhimurium, *S. enterica* serotype Enteritidis, *S. enterica* serotype Newport, and *S. enterica* serotype Heidelberg) (11), limiting the value of serotyping for human disease surveillance. More discriminatory subtyping methods, such as phage typing (3, 32) and pulsed-field gel electrophoresis (PFGE), are thus commonly used to subtype *Salmonella*, particularly as part of national and international salmonellosis surveillance systems (8, 9, 24, 25, 50). These methods are generally used in conjunction with serotyping, since serotyping information can still provide valuable information about host-associated subtypes, the emergence of new subtypes, and historical trends in the association of specific *Salmonella* subtypes with different hosts species. Although serotyping, PFGE, and phage typing, particularly if used in combination, can provide a high level of subtype discrimination, none of these methods provides appropriate information to infer phylogenetic relationships among *Salmonella* isolates and subtypes. Multilocus sequence typing (MLST) is a subtyping method that determines the nucleotide sequences of full or partial housekeeping genes. Advantages of MLST include not only that the resulting DNA sequences data are nonambiguous and easily compared between laboratories, e.g., through large World Wide Web-based databases (24, 25, 50), but also that the DNA sequence data generated can be used to infer phylogenetic relationships among isolates, providing improved insight into the evolution and ecology of *Salmonella* subtypes. Even though MLST originally was defined as

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a sequencing-based subtyping approach that includes the sequencing of 450 to 600 nucleotide (nt) fragments for six to seven housekeeping genes (38, 51), *Salmonella* MLST schemes described in the literature have used sequencing of three to four genes (37, 49); some of these MLST schemes also included the sequencing of virulence or virulence-associated genes, e.g., *spaM* and *fimA* (23, 49). Although a seven-gene *Salmonella* MLST scheme is available through a WWW page maintained by the Max Plank Institute for Infection Biology in Berlin, Germany (6), we chose a previously described three-gene MLST, which was shown to provide discriminatory power similar to that of a seven-gene MLST (49), for the study reported here, because the sequencing of fewer genes provides a more economical subtyping approach.

The transmission of *Salmonella* from animal populations to humans represents a continuing concern (5), particularly because the incidence of human salmonellosis cases in the United States does not appear to have decreased substantially despite considerable efforts to reduce food-borne diseases (44). Whereas a variety of animal reservoirs (e.g., poultry, pigs, cattle, and reptiles) can serve as direct or indirect, food-borne sources for human *Salmonella* infections, our study reported here focused on probing the subtype diversity and epidemiology of human- and cow-associated *Salmonella* populations. Even though source attribution of sporadic salmonellosis cases is difficult, a number of human salmonellosis outbreaks have been linked to contaminated foods of bovine origin (milk or beef) (41). In addition, it has been suggested that antibiotic use in dairy cattle is selecting for multidrug-resistant (MDR) *Salmonella* strains, which may be transmitted to the human population via food (4, 13), as supported by the isolation of multidrug-resistant *Salmonella* strains from cattle (2, 36, 53) and human salmonellosis outbreaks linked to beef products contaminated with multidrug-resistant *Salmonella* strains (13, 15). Furthermore, direct contact between cattle and humans may also be involved in the transmission of *Salmonella* between these populations (33, 48). We assembled a set of 335 *Salmonella* strains isolated from humans and cattle in the same general region (New York state and Vermont) over the same time (2004) for characterization using serotyping and a three-gene MLST scheme in order to allow population-based comparisons of human- and cow-associated *Salmonella* subtypes and to evaluate the potential for cow-associated subtypes to be transmitted to humans. By comparison, a previous study by our group (49), which used isolates predominantly from domestic animals, reported the development of a three-gene MLST scheme but did not use this subtyping method to characterize the transmission of *Salmonella* between different host species.

MATERIALS AND METHODS

***Salmonella* isolates.** A total of 335 *Salmonella* isolates, including 179 human and 156 bovine isolates, were included in the study reported here (see Table S1 in the supplemental material). All isolates were obtained from clinical salmonellosis cases that occurred between January and December 2004. All human *Salmonella* isolates were received from the New York State Department of Health (NYSDOH). Specifically, for each month in 2004, 10 to 20 human clinical *Salmonella* isolates were randomly selected from all human clinical isolates received by the NYSDOH during that month for inclusion in our study. The majority of human isolates ($n = 165$) were obtained from patients residing in the counties outside the five New York City boroughs, a total of 11 isolates represented residents of New York City boroughs, and patient residence was unknown for 3 isolates. The 156 bovine clinical *Salmonella* isolates were obtained from the Animal Health Diagnostic Center at Cornell University; isolates included in our study were obtained from specimens from cattle with signs of clinical salmonellosis, which represented either routine veterinary submissions ($n = 14$) or submissions that were part of a prospective study on the frequency of clinical bovine salmonellosis ($n = 142$). The prospective study enrolled 831 dairy herds in the Northeast United States. Participating farms and veterinary practices were asked to submit fecal samples from dairy cattle with clinical signs consistent with salmonellosis to the Animal Health Diagnostic Center at Cornell University for diagnostic testing. For enrolled herds, *Salmonella* culture and antimicrobial susceptibility testing along with diagnostic testing for other diseases with similar signs were paid for by the study. The majority of bovine isolates ($n = 140$) were obtained from farms located in New York; 16 isolates were obtained from farms in the neighboring state of Vermont. Bovine *Salmonella* isolates were selected so that only one isolate for each serotype isolated on a given farm from specimens collected on the same date would be included. For a number of farms, isolates from specimens collected on different dates during the study period were included in the study. In addition, some farms had multiple isolates with different serotypes obtained from samples collected on the same day. If multiple isolates from a given herd were characterized, they were obtained from different animals. Overall, animal isolates were obtained from a total of 64 different farms, including 56 and 8 farms located in New York and Vermont, respectively.

Human and bovine *Salmonella* isolates were serotyped at the NYSDOH and the National Veterinary Services Laboratory (USDA-APHIS-VS, Ames, IA) using standard procedures (22).

PCR, DNA sequencing, and MLST. The MLST scheme used here was based on PCR amplification and sequencing of three genes (*manB*, *fimA*, and *mdh*) as previously reported (49). As also previously described (49), the full *fimA* open reading frame (558 nt) and partial *manB* and *mdh* open reading frames (640 nt and 520 nt, respectively) were sequenced and used for allele assignments. While *Salmonella* lysates for PCR were initially prepared as previously described (49), preparation of purified *Salmonella* DNA using the QIAamp DNA Mini kit (QIAGEN Inc., Chatsworth, CA) replaced this lysate protocol to provide more consistent PCR results.

PCR amplification of *manB*, *fimA*, and *mdh* was performed essentially as previously described (2). All PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc.) and quantified using either a fluorescent DNA quantitation kit (Bio-Rad, Hercules, CA) or a Nanodrop spectrophotometer (NanoDrop Technologies Inc., Willmington, DE). Purified PCR products were sequenced by the Biotechnology Resource Center at Cornell University or by Macrogen Inc. (Geumcheon-gu, Seoul, Korea) as previously described (2). All sequences were assembled and proofread using SeqMan and aligned using the Clustal W algorithm in MegAlign (DNAStar, Madison, WI).

Allele assignments for individual genes were performed using DnaSP 4.0 (45); two sequences were assigned different allelic types if they differed by at least one nucleotide. Sequence types (STs) were assigned so that isolates that have identical allelic types for all three genes have the same ST. Allelic types and STs were assigned to be consistent with previous studies that used the same MLST scheme (2, 49), i.e., ST6 in the study reported here is identical to ST6 reported previously by Alcaine et al. (2).

Phylogenetic analyses. Phylogenetic analyses were performed essentially as previously described (2). Briefly, neighbor-joining (NJ) and maximum likelihood (ML) trees were constructed for each gene using one representative sequence for each allelic type for a given gene. PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) was used to construct NJ and ML trees. MODELTEST (43) was used to find the most likely model of DNA substitution for a given gene for ML trees (i.e., TrNef+G for *fimA* and *mdh* and HKY+I+G for *manB*); NJ trees were constructed using the HKY85 nucleotide substitution model. Each tree was rooted with the *manB*, *fimA*, or *mdh* sequence for *Escherichia coli* O157:H7 (31), which served as the outgroup. Phylogenetic trees were constructed based on individual sequences only and were not based on concatenated sequences, since a number of *manB* sequences showed mixed bases due to the presence of two gene copies (49), preventing their inclusion in the phylogenetic analyses.

Statistical analyses. The frequency distributions of serotypes and STs between human and bovine isolates were compared using the chi-square test of independence. For comparisons where one or more of the expected values were <5, Fisher's exact test was performed. P values of <0.05 were considered statistically significant. Chi-square and Fisher's exact tests were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). P values for exact tests for large contingency tables (e.g., 2 by 10) were determined by a Monte Carlo simulation using SAS version 9.1.

Simpson's index of discrimination (SID) was calculated as described previously by Hunter and Gaston (34); this index provides an indication of the discrimina-

TABLE 1. Farms with multiple sample submission dates resulting in the isolation of *Salmonella* from clinically infected cattle

Farm	No. of farm sample dates with positive *Salmonella* samples	Subtype(s) isolated[a] (no. of isolates)
510	20	ST11/serotype Newport (20), ST6/serotype 4,5,12:i:– (1)
261	22	ST6/serotype 4,5,12:i:– (18), ST17/serotype Kentucky (5), ST6/serotype Typhimurium (1)
223	15	ST60/serotype Infantis (15)
329	5	ST9/serotype Montevideo (1), ST44/serotype Muenster (3), ST62/serotype Thompson (1)
186	4	ST75/serotype Adelaide (1), ST8/serotype Typhimurium (2), ST8/serotype Typhimurium var. Copenhagen (1)
524	5	ST6/serotype 4,5,12:i:– (1), ST11/serotype Newport (4)
152	4	ST11/serotype Newport (4)
490	4	ST11/serotype Newport (4)
163	3	ST60/serotype Infantis (1), ST11/serotype Newport (2)
259	3	ST44/serotype Muenster (3)
488	3	ST11/serotype Bardo (1), ST11/serotype Newport (3)
584	3	ST2/serotype Agona (2), ST6/serotype Typhimurium (1)
97	2	ST8/serotype Typhimurium var. Copenhagen (2)
105	2	ST11/serotype Newport (1), ST8/serotype Typhimurium (1)
125	2	ST6/serotype Typhimurium (2)
208	2	ST6/serotype Typhimurium (2)
303	2	ST11/serotype Newport (2)
320	2	ST11/serotype Newport (2)
415	2	ST9/serotype Montevideo (1); ST6/serotype Typhimurium (1)
764	2	ST6/serotype Typhimurium (2)

[a] Subtype is represented as ST/serotype.

tory power of a given subtyping method (34) as well as an estimate of the subtype diversity within a given population (26). The 95% confidence intervals for SID were calculated as described previously by Grundmann et al. (26), and SIDs were considered significantly different if 95% confidence intervals did not overlap. All calculations were performed using Microsoft (Seattle, WA) Excel.

Among the bovine *Salmonella* isolates included in this study, multiple isolates obtained from the same farm (but different animals) at different sampling times showed the same serotypes and STs, indicating the reisolation of a persistent subtype on a given farm. Therefore, only one isolate representing each unique serotype/ST combination for a given farm was included in the summary statistics as well as in the chi-square test and SID calculations to avoid an overrepresentation of a subtype due to resampling. For example, while 20 *Salmonella* Newport isolates with ST11 were obtained from farm 510 (Table 1), only one of the isolates with this subtype combination from farm 510 was included in isolate numbers used for statistical analyses. When this approach was used to include only one isolate representing each unique serotype/ST combination found on a given farm, a total of 78 bovine isolates were obtained for inclusion in the statistical analyses reported here (i.e., see Table 2 through 5).

Access to detailed isolate information. All isolate information for this study, including isolate source, gene sequence data, and allele assignments, can be accessed via the PathogenTracker website at http://www.pathogentracker.net; isolates specifically included in the study reported here are linked to the reference for the manuscript.

RESULTS AND DISCUSSION

A total of 335 *Salmonella* strains isolated from human and bovine clinical cases that occurred in New York and a neighboring state (Vermont) were characterized by serotyping and MLST targeting three selected genes in order to better understand the ecology and epidemiology of human- and bovine-associated *Salmonella* strains. Analyses of serotypes and MLST data indicate that (i) MLST provides subtype discrimination of *Salmonella* that is only slightly more sensitive than serotyping; (ii) *Salmonella* subtypes isolated from cattle and humans represent distinct and overlapping populations; (iii) a number of *Salmonella* STs, such as ST6, which includes the emerging serotype 4,5,12:i:–, are geographically widespread among human and/or bovine populations; (iv) *Salmonella* serotype Newport represents two distinct phylogenetic lineages that appear to be host specific; and (v) duplication and deletion events in *manB* and *fimA* may provide a mechanism for the rapid diversification of *Salmonella* surface molecules.

MLST provides subtype discrimination of *Salmonella* that is only slightly more sensitive than serotyping. The 335 *Salmonella* isolates included in this study could be differentiated into a total of 52 serotypes and 72 STs. *fimA* and *mdh* sequence data allowed the differentiation of 40 and 28 allelic types, respectively. Analysis of *manB* sequence data revealed a total of 33 isolates that showed reproducible double peaks at 1 to 15 nt positions, consistent with the presence of two copies of *manB* in these isolates, as previously reported for a smaller number of isolates (2). For each *manB* nucleotide position with a double peak, an International Union of Pure and Applied Chemistry (IUPAC) ambiguity nucleotide code indicating the presence of the two bases found (e.g., Y indicates the presence of C or T) was used to designate the final sequences for a given isolate. Isolates were assigned different *manB* allelic types if two isolates differed in their nucleotide sequences, including the ambiguous nucleotides (mixed bases), consistent with similar approaches that have been used to assign subtypes based on 16S rRNA gene sequence data that indicated the presence of multiple distinct 16S rRNA genes in a given organism (46). Using this approach, a total of 55 *manB* allelic types were differentiated. The numbers of polymorphic sites in *manB*, *fimA*, and *mdh* were 76, 260, and 85, respectively (calculated based on the allelic types reported here and in reference 49).

When serotype and MLST typing data were combined to assign overall subtypes (i.e., only isolates with the same serotype and ST were considered the same subtype), a total of 81

TABLE 2. Distribution of *Salmonella* serotypes among STs and human and bovine isolates

Serotype[a]	ST(s)	No. of isolates from:	
		Humans	Dairy cattle[b]
Serotype 4,5,12:i:–	6, 40	12	3
Serotype Agona	1, 2	3	4
Serotype Enteritidis***	14, 36	26	0
Serotype Heidelberg*	3, 26, 50	10	0
Serotype Mbandaka	64, 65, 73	4	1
Serotype Montevideo	9, 56, 57, 67	4	3
Serotype Muenster*	44	1	5
Serotype Newport***	11, 13, 33, 46, 76, 78	18	25
Serotype Saint Paul	38, 81	5	0
Serotype Thompson	43, 62	5	3
Serotype Typhimurium	6, 7, 8, 47, 49	30	19
Serotype Urbana	52	5	0

[a] Only serotypes that occurred more than five times are listed separately (for animal isolates, only one isolate with a given serotype and ST per farm was counted). Additional serotypes that occurred less than five times are as follows (numbers of human and unique bovine isolates per farm are included in parentheses): serotype 4,12:i:– (one bovine isolate), serotype Abony (two human isolates), serotype Adelaide (one human isolate and one bovine isolate), serotype Agbeni (one human isolate), serotype Anatum (three human isolates), serotype Arechavaleta (one human isolate), serotype 4,12:r:– (two human isolates), serotype Bardo (two bovine isolates), serotype Berta (two human isolates), serotype Blockley (one human isolate), serotype Braenderup (one human isolate), serotype 1,7:–:1,5 (three human isolates), serotype Cubana (one human isolate), serotype Dublin (two human isolates), serotype Give (one human isolate), serotype Hadar (two human isolates), serotype Hartford (one human isolate), serotype Havana (one bovine isolate), serotype Infantis (two human and two bovine isolates), serotype Javiana (four human isolates), serotype Kentucky (two bovine isolates), serotype Kintambo (one human isolate), serotype Litchfield (one human isolate), serotype Muenchen (three human isolates), serotype Nyanza (one human isolate), serotype Oranienburg (one human isolate and one bovine isolate), serotype Panama (three human isolates), serotype Paratyphi B (one human isolate), serotype Paratyphi B var. Java (two human isolates), serotype Paratyphi C (one human isolate), serotype Pomona (one human isolate), serotype Poona (two human isolates), serotype Rough o:i:1,2 (one bovine isolate), serotype Rublislaw (one human isolate), serotype Schwarzengrund (three human isolates), serotype Senftenberg (one human isolate), serotype Stanley (two human isolates), serotype Typhimurium var. Copenhagen (four bovine isolates), serotype Weltvreden (one human isolate), and serotype Worthington (one human isolate). Serotypes that differ significantly in frequency among human and animal isolates, as determined by chi-square test or Fisher's exact test, are marked with * ($P < 0.05$) or *** ($P < 0.001$).

[b] Only one isolate representing each unique serotype/ST combination found on a given farm was counted; these numbers were used to avoid the overrepresentation of a subtype due to resampling on a given farm (e.g., when isolates with the same serotype/ST combination were isolated on different dates on the same farm) (Table 1).

subtypes were differentiated. Analysis of combined serotype/ST-based subtypes allowed us to analyze the *Salmonella* diversity on the 20 farms from which *Salmonella* isolates were collected over multiple visits in 2004 (Table 1). On some farms, a given *Salmonella* subtype persisted over time; e.g., on one farm, an ST11 *Salmonella* serotype Newport strain was isolated from samples collected on 20 separate dates from 20 different animals (Table 1). These data showed the persistence of *Salmonella* subtypes over time in cattle on 18 farms, including the persistence of *Salmonella* serotype Newport ST11 on a total of 9 farms. We thus conclude that *Salmonella* serotype Newport ST11 may be characterized by its particular ability for persistence, consistent with a recent report that also showed the persistence of multidrug-resistant *Salmonella* serotype Newport strains on two farms in Washington state in the United States (18).

Among human isolates, a total of nine instances where two or three isolates with the same subtype (i.e., the same serotype and ST) were obtained from humans in the same county within <2 months of each other were observed. Whereas five of these instances are likely to represent small clusters, in the other four instances, a retrospective analysis showed that two or three isolates with the same ST had been obtained from the same patient. In one case, both isolates were obtained from the same patient on the same day; the time spans between obtaining the first and the last isolates for the other three patients were 2, 27, and 54 days, respectively (isolates were serotype Typhimurium, serotype 4,5,12:i:–, and serotype Abony, respectively).

A total of 17 serotypes included two or more STs, including the differentiation of six and five STs within isolates of serotype Newport and serotype Typhimurium. Among the 72 STs differentiated in this study, 7 STs included isolates representing multiple serotypes. These findings are consistent with previous studies, which also found the differentiation of multiple serotypes within a given ST (2, 49). The overall discriminatory abilities, as determined by SID, for serotyping and MLST were 0.917 and 0.920, respectively. The SID for combined serotype and MLST data was 0.943. Even though other subtyping methods, particularly PFGE, appear to provide even more sensitive subtype discrimination (23), we conclude that serotyping and MLST provide the appropriate subtype discrimination to evaluate the ecology and epidemiology of human and bovine *Salmonella* isolates, particularly since MLST data allow the phylogenetic analysis and definition of *Salmonella* clonal groups (49), which are defined here as groups of isolates that have closely related STs as determined by phylogenetic analyses and thus are presumed to have derived from a recent common ancestor.

***Salmonella* subtypes isolated from cattle and humans represent distinct and overlapping populations.** The *Salmonella* isolates characterized in our study represented 35 and 6 serotypes unique to human and bovine isolates, respectively, as well as 11 serotypes found among both host species. The most common human-associated serotypes were serotype Typhimurium, serotype Enteritidis, serotype Newport, serotype 4,5,12:i:–, and serotype Heidelberg, largely consistent with CDC data for 2003, which listed serotype Typhimurium, serotype Enteritidis, serotype Newport, serotype Heidelberg, and serotype Javiana as the five most commonly reported human *Salmonella* serotypes in the United States (11). This indicates that the human isolates included in our study are similar in their serotype composition to isolates associated with human clinical disease previously collected throughout the United States. Comparison of the frequency distributions of serotypes among human and bovine isolates using an overall chi-square analysis (with all serotypes that occurred less than five times grouped into a single category, termed "rare serotypes") showed that serotypes were not randomly distributed among human and bovine isolates ($P < 0.001$; Monte Carlo estimation of exact test). Pairwise chi-square tests (comparing the frequency of a given serotype to the frequency of all other serotypes) showed that serotype Enteritidis and serotype Heidelberg were overrepresented among human isolates, whereas serotype Newport and serotype Muenster were overrepresented among bovine isolates. Serotype Enteritidis and serotype Heidelberg were exclusive to human isolates, consistent with their well-recognized association with poultry and partic-

TABLE 3. Distribution of *Salmonella* STs among human and bovine isolates and among different counties in New York state

ST[a]	Serotype(s)	No. of isolates from:		County origin of isolates (no. of isolates) from[c]:	
		Humans	Cattle[b]	Humans	Dairy cattle[b]
3*	Serotype Heidelberg, serotype 4,12:r:–	10	0	Dut (1), Eri (1), Fra (1), Mon (1), Nas (2), New (1), One (1), Suf (2)	None
6	Serotype Typhimurium, serotype 4,12:i:–, serotype 4,5,12:i:–	38	20	Alb (1), Brx (1), Cha (1), Cmg (1), Cor (1), Dut (1), Eri (3), Fra (1), Kin (1), Mon (1), Nas (4), Ono (2), Ora (1), Ots (1), Stl (1), Ste (2), Suf (7), Tom (1), War (1), Was (2), Wes (4)	Cat (1), Cli (3), Cor (1), Gen (1), Nia (1), Ont (1), Orl (1), Ren (3), Stl (1), Tom (1), Was (1), Wyo (5)
8***	Serotype Typhimurium, serotype Rough o:i:1,2, serotype Typhimurium var. Copenhagen	0	7	None	Cay (1), Cno (1), Osw (2), Was (3)
11***	Serotype Newport, serotype Bardo	9	27	Cno (1), Nia (1), Put (1), Sar (1), Suf (1), Uls (1), Unk (1), Way (1), Wes (1)	Cay (1), Cno (1), Cvt (2), Cli (2), Cor (1), Eri (1), Fvt (6), Gen (1), Lvt (1), Lew (1), Nia (3), One (2), Ono (1), Sen (1), Stl (1), Wyo (2)
14**	Serotype Enteritidis	19	0	Brm (1), Dut (1), Eri (3), Kin (1), Mon (2), Nas (3), Ono (2), Ora (1), Suf (2), Unk (1), Wes (2)	None
36	Serotype Enteritidis	7	0	Alb (1), Del (1), Eri (1), Mon (1), Nas (1), Tom (1), Wes (1)	None
44**	Serotype Muenster	1	5	Cmg (1)	Fra (1), Liv (1), Orl (1), Wyo (2)
52	Serotype Urbana	5	0	Cli (2), Sch (3)	None
62	Serotype Thompson	4	2	Nas (1), Sar (1), Uls (1), Way (1)	Liv (1), Nia (1)
Rare**	Various serotypes	86	17	NS	NS

[a] Only STs that occurred five or more times are listed separately; the STs with fewer than five isolates were grouped into a category termed "rare"; STs that differ significantly in frequency among human and bovine isolates, as determined by chi-square test or Fisher's exact test, are marked with * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

[b] Only one isolate representing each unique serotype/ST combination found on a given farm was counted; these numbers were used to avoid the overrepresentation of a subtype due to resampling on a given farm (e.g., when isolates with the same serotype/ST combination were isolated on different dates on the same farm) (Table 1).

[c] Counties are depicted in Fig. 1. County codes are as follows: Alb, Albany; Brx, Bronx; Brm, Broome; Cat, Cattaraugus; Cay, Cayuga; Cha, Chautauqua; Cmg, Chemung; Cno, Chenango; Cvt, Chittenden, VT; Cli, Clinton; Cor, Cortland; Del, Delaware; Dut, Dutchess; Eri, Erie; Fra, Franklin; Fvt, Franklin, VT; Gen, Genesee; Kin, Kings; Lvt, Lamoille, VT; Lew, Lewis; Liv, Livingston; Mon, Monroe; Nas, Nassau; New, New York; Nia, Niagara; One, Oneida; Ono, Onondaga; Ont, Ontario; Ora, Orange; Orl, Orleans; Osw, Oswego; Ots, Otsego; Put, Putnam; Ren, Rensselaer, Sar, Saratoga; Sch, Schenectady; Sen, Seneca; Stl, St. Lawrence; Ste, Steuben; Suf, Suffolk; Tom, Tompkins; Uls, Ulster; Unk, Unkown; War, Warren; Was, Washington; Way, Wayne; Wes, Westchester; Wyo, Wyoming. NS, not shown.

ularly chickens (11, 27, 47) and their rare presence in cattle (11, 27). In addition to being common among human isolates, serotype Typhimurium and serotype Newport were also the two most common bovine-associated serotypes, consistent with both U.S.-wide CDC data (11) and the fact that human salmonellosis outbreaks caused by these two serotypes have been linked to the consumption of contaminated beef and dairy products (12, 13, 42). While this finding provided evidence that cattle may be an important reservoir for these two serotypes, the sources of human *Salmonella* serotype Newport infections probably depend on the specific *Salmonella* serotype Newport clonal group, as discussed below. In addition to the chi-square test data, which showed that the individual serotype frequency differed among bovine and human isolates, SIDs also showed that human serotype diversity (SID = 0.931) was significantly higher than bovine serotype diversity (SID = 0.832), further supporting the hypothesis that distinct *Salmonella* populations are associated with these two host species.

MLST identified 56 and 6 STs that were unique to human and bovine isolates, respectively, as well as 10 STs that were found in both host species. A comparison of the frequency distributions of STs among human and bovine isolates using an overall chi-square analysis (with all STs that occurred less than five times being grouped into a single category, termed "rare STs") showed that STs were not randomly distributed among human and bovine isolates ($P < 0.001$; Monte Carlo estimation of exact test). Pairwise chi-square tests (comparing the frequency of a given ST to the frequency of all other STs) showed that two and three STs were overrepresented among isolates from humans (ST3 and ST14) and cattle (ST8, ST11, and ST44), respectively (Table 3), further supporting the finding that certain subtypes were not distributed evenly among human and cattle isolates. ST3 and ST14, which were overrepresented among human isolates, represent serotype Heidelberg and serotype 4,12:r:– (ST3) as well as serotype Enteritidis (ST14), consistent with the overrepresentation of these serotypes among human isolates as discussed above. Interestingly, ST8, which includes isolates classified as serotype Typhimurium and related serotypes (Table 3), was overrepresented among bovine isolates and was never isolated from human clinical cases, potentially indicating that this specific ST shows bovine host specificity. Alternatively, this ST may not yet have been transmitted to humans in significant numbers. ST11 and ST44, which were also more common among bovine isolates than human isolates, represented serotype Newport and serotype Bardo (ST11) as well as serotype Muenster (ST44), consistent with an overrepresentation of these serotypes among bovine isolates (Table 2). Thus, MLST data not only confirmed

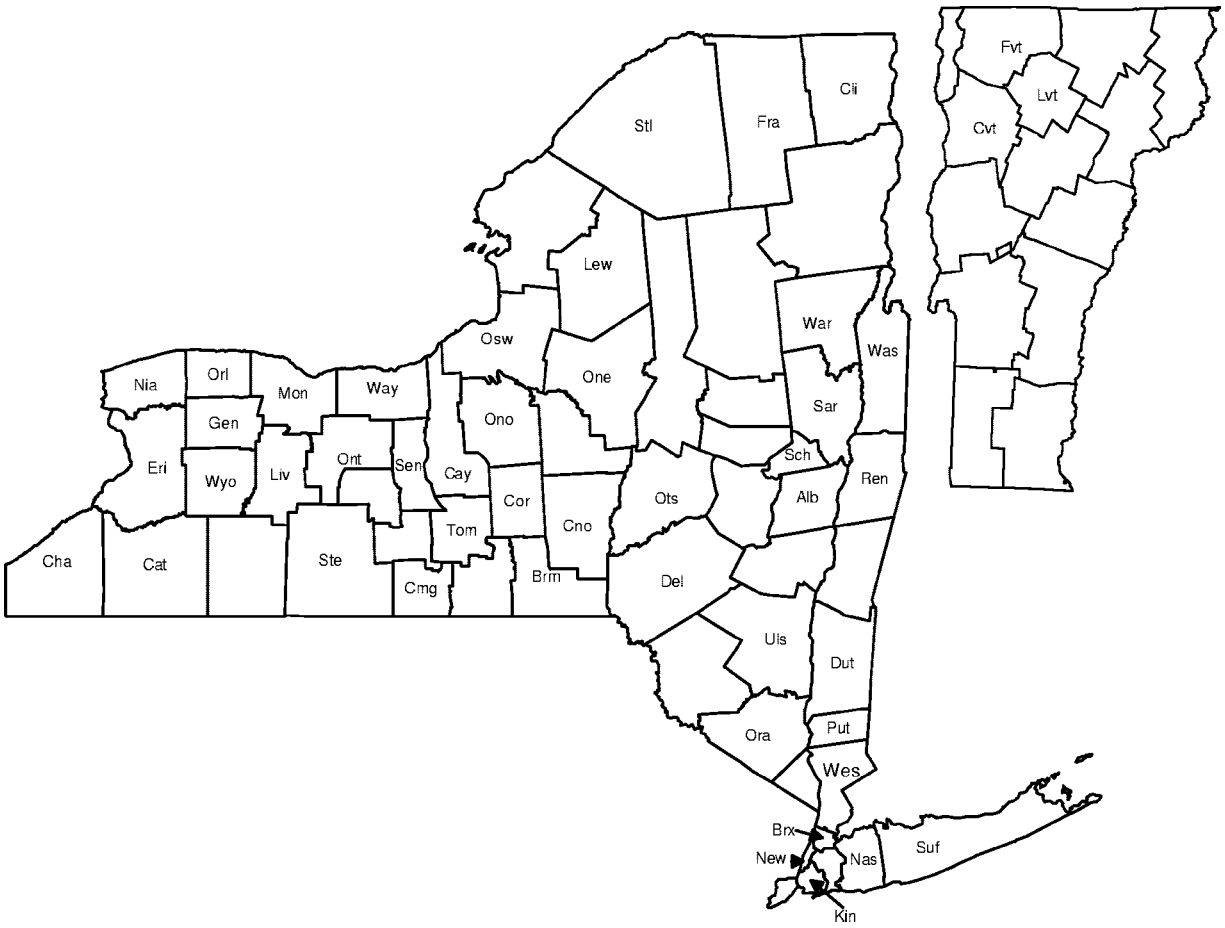

FIG. 1. Map of New York and Vermont counties where *Salmonella* isolates were obtained. The map was drawn using MapViewer 4.01 (Golden Sofware, Inc., Golden, CO). County codes are as follows: Alb, Albany; Brx, Bronx; Brm, Broome; Cat, Cattaraugus; Cay, Cayuga; Cha, Chautauqua; Cmg, Chemung; Cno, Chenango; Cvt, Chittenden, VT; Cli, Clinton; Cor, Cortland; Del, Delaware; Dut, Dutchess; Eri, Erie; Fra, Franklin; Fvt, Franklin, VT; Gen, Genesee; Kin, Kings; Lvt, Lamoille, VT; Lew, Lewis; Liv, Livingston; Mon, Monroe; Nas, Nassau; New, New York; Nia, Niagara; One, Oneida; Ono, Onondaga; Ont, Ontario; Ora, Orange; Orl, Orleans; Osw, Oswego; Ots, Otsego; Put, Putnam; Ren, Rensselaer; Sar, Saratoga; Sch, Schenectady; Sen, Seneca; Stl, St. Lawrence; Ste, Steuben; Suf, Suffolk; Tom, Tompkins; Uls, Ulster; Unk, Unkown; War, Warren; Was, Washington; Way, Wayne; Wes, Westchester; and Wyo, Wyoming.

the host association of specific serotypes but also identified a bovine-associated ST within serotype Typhimurium, which could not have been identified based on serotype data alone.

SID also showed that human ST diversity (SID = 0.935) was significantly higher than bovine ST diversity (SID = 0.806), providing additional support for the hypothesis that human- and cow-associated *Salmonella* isolates represent distinct populations. The higher ST and serotype diversity found among human *Salmonella* isolates likely reflects the fact that human *Salmonella* infections can originate from a number of distinct source populations and reservoirs (e.g., birds, reptiles, cattle, and pigs) and indicates that a large number of subtypes found in these different hosts have the ability to cause human disease.

A number of *Salmonella* STs, such as ST6, which includes emerging serotype 4,5,12:i:–, are geographically widespread among human and/or bovine populations. Analysis of geographic source data for human and bovine isolates (Table 3) showed that a number of STs are distributed widely across New York state (Fig. 1), including ST3, ST14, and ST36, which were found only among human isolates, as well as ST8, which was found only among bovine isolates. ST6 and ST11, the two most common STs found in this study, were widely distributed among both human and bovine populations in New York state. Interestingly, ST6 not only contains *Salmonella* serotype Typhimurium isolates but also includes serotype 4,5,12:i:– and serotype 4,12:i:– (Table 3), indicating that these serotypes share a common ancestor, consistent with data from phylogenetic trees (Fig. 2), which grouped ST6 with other *Salmonella* serotype Typhimurium STs (ST7, ST8, ST47, and ST49). PFGE, random amplified polymorphic DNA, plasmid profile, and ribotyping-based studies on small isolate sets (<50 isolates) also previously concluded that serotype 4,5,12:i:– originated from a *Salmonella* serotype Typhimurium ancestor (19, 28). The ecology and epidemiology of *Salmonella* serotype 4,5,12:i:– are of particular interest, since it appears to represent an emerging human disease-associated *Salmonella* serotype (11). In 2003, serotype 4,5,12:i:– was identified as the 14th most common human *Salmonella* serotype in the United States (11), but due to difficulties in the classification of this serotype, many serotype 4,5,12:i:– isolates may have simply been reported as

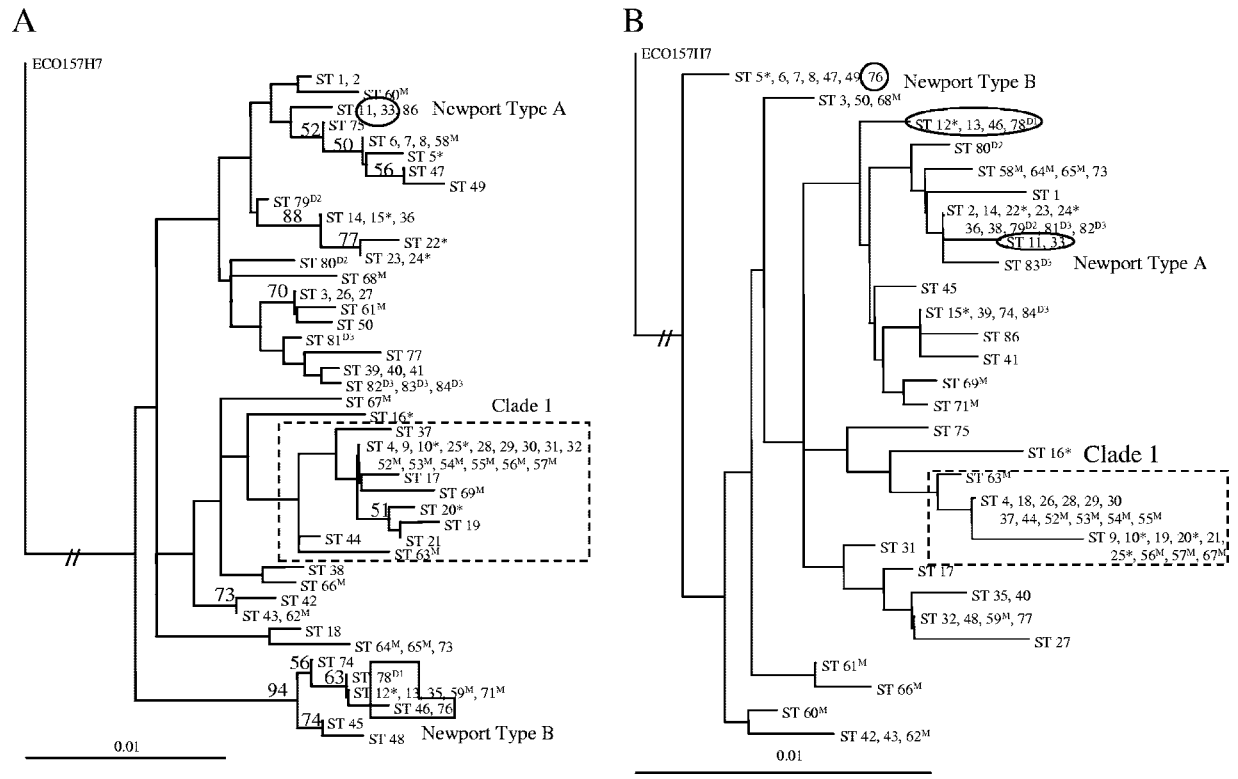

FIG. 2. Phylogenetic trees based on *Salmonella fimA* (A) and *mdh* (B) gene sequences. Neighbor-joining trees were built in PAUP* using one representative isolate for each sequence type, including STs previously described by Sukhnanand et al. (49), which were not represented among the isolates found here (these STs are indicated by a *). ML trees (not shown) displayed similar clusterings and topologies. Bootstrap analyses were performed using 5,000 replications, and bootstrap values >50 are indicated. M indicates STs representing isolates that contain a *manB* gene duplication; the branch labeled as "clade 1" represents a number of related STs, including STs (ST52 to ST57, ST63, and ST67 in the *mdh* tree) that correspond to isolates with two *manB* copies, indicating a common ancestral event that was responsible for the two *manB* copies found in these isolates. D1, D2, and D3 indicate STs representing isolates that contain *fimA* deletion types D1 through D3 (Table 6). STs representing the two distinct *Salmonella* serotype Newport groups (designated types A and B) are marked by circles or boxes and the respective type.

subspecies I, group B, and its prevalence may thus have been greater than previously reported (11). Serotype 4,5,12:i:– was also implicated in a food-borne salmonellosis outbreak in New York City in 2002 (1). The observation that this serotype was the fourth most common human-associated serotype in our study and that ST6, which includes serotype 4,5,12:i:– as well as other related serotypes (Table 3), is the most common human disease-associated and second most common cattle-associated ST (Table 3) indicates the public health importance of this specific ST, including the potential importance of bovine hosts as a reservoir for this ST.

***Salmonella* serotype Newport represents two distinct phylogenetic lineages that appear to be host specific.** MLST data showed that *Salmonella* serotype Newport isolates represented six STs (Table 2). Phylogenetic trees based on the sequenced regions of *fimA* (Fig. 2A) and *mdh* (Fig. 2B) revealed that the serotype Newport STs represented two genetically distinct clonal groups, consistent with preliminary data for three bovine and two avian serotype Newport isolates (49) as well as a recent MLST study on 81 *Salmonella* serotype Newport isolates (30). Based on the larger data set (43 serotype Newport isolates, including only one isolate representing each unique serotype/ST combination found on a given farm) reported here, we have designated these two lineages *Salmonella* serotype Newport types A and B. The observation that a single serotype Newport type B ST (ST76) clusters separately from other type B isolates in the *mdh* tree (Fig. 2B) likely represents horizontal gene transfer events of an *mdh* allele and does not affect this conclusion, since this ST groups with isolates of type B in the other trees. Overall, serotype Newport type A represented two STs (ST11 and ST33) and includes 11 human and 25 bovine isolates (counting only one bovine isolate representing each unique serotype/ST combination for a given farm); ST11 represents 9 human and all 25 bovine isolates. This is consistent with previous data showing that ST11 was associated with bovine sources (49). *Salmonella* serotype Newport type B represented four STs (ST13, ST46, ST76, and ST78), and all isolates of this type ($n = 7$) were obtained from human clinical cases; one additional *Salmonella* serotype Newport type B ST (ST12) was found in a previous study (49) but was not among the isolates characterized here. Statistical analysis (chi-square test) showed that the frequency distributions of human and bovine isolates among these two *Salmonella* serotype Newport types differ significantly ($P < 0.001$), indicating that these types differ in their host associations. Since a previous study has shown that *Salmonella* serotype Newport isolates of avian origin are grouped into type B (49), we hypothesize that *Salmonella* serotype Newport types A and B represent a cow- and a

TABLE 4. Serotypes that include at least one isolate that carries two *manB* copies[a]

Serotype	ST(s) of human isolates (no of isolates) within a given serotype that carry:		ST(s) of cattle isolates (no. of isolates) within a given serotype that carry[b]:	
	Two *manB* copies	One *manB* copy	Two *manB* copies	One *manB* copy
Serotype Agbeni	63 (1)	None	None	None
Serotype Braenderup	61 (1)	None	None	None
Serotype 1,7:–:1,5	58 (1)	42 (1), 43 (1)	None	None
Serotype Cubana	71 (1)	None	None	None
Serotype Havana	None	None	69 (1)	None
Serotype Infantis	60 (2)	None	60 (1)	None
Serotype Kintambo	59 (1)	None	None	None
Serotype Mbandaka	64 (2)	73 (2)	65 (1)	None
Serotype Montevideo	56 (2), 57 (1), 67 (1)	None	None	9 (3)
Serotype Nyanza	66 (1)	None	None	None
Serotype Oranienburg	53 (1)	None	53 (1)	None
Serotype Poona	55 (1)	28 (1)	None	None
Serotype Rublislaw	54 (1)	None	None	None
Serotype Thompson	62 (4)	43 (1)	62 (2)	43 (1)
Serotype Urbana	52 (5)	None	None	None
Serotype Worthington	68 (1)	None	None	None

[a] Serotypes for which all isolates carry only a single *manB* copy are not included in this table.
[b] Only one isolate representing each unique serotype/ST combination found on a given farm was counted; these numbers were used to avoid the overrepresentation of a subtype due to resampling on a given farm (e.g., when isolates with the same serotype/ST combination were isolated on different dates on the same farm) (Table 1).

bird-associated lineage, respectively, and that both can be transmitted to humans. Antimicrobial susceptibility testing showed that all 7 serotype Newport type B isolates were sensitive to 15 commonly used antibiotics, whereas 8 of the 14 tested type A isolates showed an MDR phenotype, with resistance to at least ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (J. Richards, S. D. Alcaine, P. McDonough, and M. Wiedmann, unpublished data). These results indicate that MDR *Salmonella* serotype Newport, an emerging pathogen of public health relevance (11, 20, 29), represents a bovine-associated lineage, at least in our study population. We thus conclude that cattle may represent a reservoir for MDR *Salmonella* serotype Newport (particularly ST11), since this strain has been found to commonly persist in cattle herds (our data reported here; see also reference 18). We further conclude that MLST of human serotype Newport isolates may provide a means for linking human salmonellosis cases or outbreaks to likely food sources, particularly since human outbreaks of *Salmonella* serotype Newport infections have been linked to both beef (13) and poultry (7) sources, and also provides a useful method for monitoring the sources and spread of MDR *Salmonella* serotype Newport.

Duplication and deletion events in *manB* and *fimA* may provide a mechanism for rapid diversification of *Salmonella* surface molecules. DNA sequence data for *manB* revealed the presence of two copies of *manB* in a total of 27 human and 6 bovine isolates (Table 4), as demonstrated by the presence of mixed bases at a number of nucleotide positions after PCR products were sequenced. This was also observed in a previous study where we reported and confirmed, using cloning and sequencing of *manB* PCR products, the presence of two *manB* alleles in three *Salmonella* serotype Montevideo isolates (49). Phylogenetic analyses of the duplicate *manB* genes found in these serotype Montevideo isolates showed that an additional *manB* copy had likely been introduced into an ancestral strain by horizontal gene transfer from a different *Salmonella* serotype similar to serotype Javiana (49). In the study reported here, we found evidence for the presence of two *manB* genes in 16 *Salmonella* serotypes (including serotype Montevideo) and in 19 STs (Table 4). *fimA*- and *mdh*-based phylogenetic

TABLE 5. Serotypes that include at least one isolate that carries a deletion in the 3′ end of *fimA*[a]

Serotype	*fimA* deletion type	ST(s) of human isolates (no. of isolates) within a given serotype that carry:		ST of cattle isolates (no. of isolates[b]) within a given serotype that carry:	
		fimA deletion	No *fimA* deletion	*fimA* deletion	No *fimA* deletion
Serotype Newport	D1	78 (3)	11 (9), 13 (1), 33 (2), 46 (2), 76 (1)	None	11 (25)
Serotype Weltvreden	D2	79 (1)	None	None	None
Serotype Paratyphi C	D2	80 (1)	None	None	None
Serotype Saint Paul	D3	81 (1)	38 (4)	None	None
Serotype Berta	D3	82 (1), 83 (1)	None	None	None
Serotype Stanley	D3	84 (1)	39 (1)	None	None

[a] Serotypes for which all isolates carry no deletion in the 3′ end of *fimA* are not included in this table.
[b] Only one isolate representing each unique serotype/ST combination found on a given farm was counted; these numbers were used to avoid the overrepresentation of a subtype due to resampling on a given farm (e.g., when isolates with the same serotype/ST combination were isolated on different dates on the same farm) (Table 1).

TABLE 6. DNA and amino acid sequences of *fimA* deletion types D1, D2, and D3[a]

Deletion type	DNA sequence	Amino acid sequence
Wild type	GCACGCTATAAGGCAACCGCCGCCGCCACGACGCCAGGCCAGGCTAAT	ARYKATAAATTPGQANADATFIMKYE
D1	GCACGCTATAAGGCAACCGCCGCCGCCGCGACGC**C**AGGC-----TAAT	ARYKATAAAATPG
D2	GCACGCTATAAGGCAACCGCCGCC---ACGACGCCAGGCCAGGCTAAT	ARYKATAA-TTPGQANADATFIMKYE
D3	GCACGCTATAAGGCAACCGCCGCCGCG---ACGCCAGGCCAGGCTAAT	ARYKATAAA-TPGQANADATFIMKYE

[a] Nucleotide sequences surrounding the deletions in the 3′ region of *fimA* and amino acid sequences surrounding the deletions in the 3′ region of *fimA* are shown. The first amino acid corresponds to the first codon shown in the DNA sequence. Whereas the amino sequence includes the stop codon, the nucleotide sequence shown is missing the nucleotides encoding the last 10 amino acids.

trees (Fig. 2) showed that STs representing isolates with two *manB* copies represent a number of distinct subtypes, indicating that multiple independent horizontal gene transfer events (or duplication events) contributed to the presence of two *manB* copies in different *Salmonella* subtypes. While positive selection for the presence of multiple *manB* copies could account for this observation, expression of both *manB* copies would be necessary and needs to be established in future experiments. Several STs (ST52 to ST57, ST63, and ST67) that include isolates with two *manB* copies formed a similar cluster in *fimA* and *mdh* trees (designated clade 1 in Fig. 2). This may be the result of a common ancestral event (e.g., horizontal gene transfer or *manB* duplication) that was responsible for the two *manB* genes found in these isolates. Although the benefit of two distinct copies of *manB* in a single isolate remains to be determined, the fact that *manB* encodes phosphomannomutase, an enzyme that is part of the chemical pathway necessary to produce GDP-D-mannose, an important sugar subunit of the *Salmonella* O antigen (35), may suggest that the presence of two *manB* copies provides a possible mechanism to rapidly generate serotype diversity. This is consistent with the observation that isolates in clade 1 that carry two *manB* copies represented a total of six serotypes, including serotype Agbeni, serotype Montevideo, serotype Poona, serotype Oranienburg, serotype Rublislaw, and serotype Urbana. Further research, including the generation and characterization of *manB* deletion mutants, is clearly needed to better understand the potential contributions of multiple *manB* copies to generating *Salmonella* serotype diversity.

Sequence analysis of *fimA* revealed three different deletions in the 3′ coding region of *fimA* (Tables 5 and 6), including a 5-bp deletion (deletion type D1) that leads to a premature stop codon and that is found only in the three ST78 isolates. Deletion types D2 (found in two STs) and D3 (found in four STs), on the other hand, are both similar to each other and represent in-frame 3-bp deletions that result in the loss of an alanine or a threonine (Table 6). A deletion similar to D2 and D3 was also previously reported for *Salmonella* serotype Typhi (10). The two STs carrying deletion type D2 clustered separately from each other in the *fimA* and *mdh* tree (Fig. 2), and the four STs carrying *fimA* deletion type D3 represented two and three branches in the *fimA* and *mdh* trees, respectively. One ST with deletion type D2 clustered with two STs with D3 in the *mdh* tree. A *manB* tree (not shown) also further supported the finding that some STs with the same *fimA* deletion represent distinct branches. Overall, these data indicate that identical *fimA* deletion types may represent either (i) two or more separate deletion events or (ii) horizontal transfer of small *fimA* fragments carrying a given deletion among distinct *Salmonella* subtypes. Whereas *fimA* encodes FimA, the major shaft subunit of type 1 fimbriae in *Salmonella enterica*, the second subunit of type 1 fimbriae, FimH, is responsible for the binding of the fimbriae to specific sugars and cell types (21). The binding specificity of type 1 fimbriae has been proposed to be due largely to the interaction between the FimA and FimH subunits rather than due to the primary structure of the FimH subunit alone (21). This suggests that variations in the shaft subunits (e.g., FimA) could lead to changes in sugar specificity and thus changes in binding. Although functional studies are needed to characterize the phenotypic importance of the *fimA* deletion described here, the occurrence of multiple independent deletion events and their apparent fixation in different lineages after horizontal gene transfer indicate potential selective advantages associated with these deletions, such as changes in the specificity or avidity of fimbrial binding to host cell sugars. In this context, it is interesting that the *fimA* deletions were found only in *Salmonella* isolates from human sources (Table 5), possibly because selection for these deletions is limited to specific hosts.

Conclusions. The data reported here show that the combined use of serotyping with MLST and phylogenetic analyses can provide important information on the evolution, ecology, and epidemiology of *Salmonella* subtypes associated with different host species. This approach provides insights that cannot be achieved with traditional subtyping methods that do not allow phylogenetic analyses. Our results specifically support the hypothesis that human and bovine *Salmonella* isolates represent separate, but overlapping, populations that include widely distributed subtypes found in both host populations (e.g., ST6, which includes the emerging serotype 4,5,12:i:–) as well as host-specific subtypes, including host-specific clonal groups and host-specific STs within a given serotype (e.g., ST8, a bovine-associated ST, which includes serotype Typhimurium isolates). A definition of host-specific and host-associated subtypes not only will improve our understanding of the biology of this important mammalian pathogen but will also improve our ability to recognize sources of infection in human salmonellosis cases and outbreaks. Importantly, our study also indicates that cattle may be a reservoir for many of the common human disease-associated serotypes and STs that are not associated with avian hosts.

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