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Contribution of Horizontal Gene Transfer and Deletion Events to Development of Distinctive Patterns of Fimbrial Operons during Evolution of *Salmonella* Serotypes

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Only certain serotypes of *Salmonella* represent 99% of all human clinical isolates. We determined whether the phylogenetic distribution of fimbrial operons would account for the host adaptations observed for *Salmonella* serotypes. We found that three fimbrial operons, *fim*, *lpf*, and *agf*, were present in a lineage ancestral to *Salmonella*. While the *fim* and *agf* fimbrial operons were highly conserved among all *Salmonella* serotypes, sequence analysis suggested that the *lpf* operon was lost from many distantly related lineages. As a consequence, the distribution of the *lpf* operon cannot be explained easily and may be a consequence of positive and negative selection in different hosts for the presence of these genes. Two other fimbrial operons, *sef* and *pef*, each entered two distantly related *Salmonella* lineages and each is present only in a small number of serotypes. These results show that horizontal gene transfer and deletion events have created unique combinations of fimbrial operons among *Salmonella* serotypes. The presence of *sef* and *pef* correlated with serotypes frequently isolated from common domesticated animals.

The genus *Salmonella* and the species *Escherichia coli* are phylogenetically closely related, diverging from a common ancestor between 100 and 160 million years ago (21, 40). The gene order on the chromosome has been highly conserved between these two organisms (45), and corresponding genes have an average of 84% sequence identity (50). These properties make *E. coli* and the genus *Salmonella* a unique pair with which to identify evolutionary changes in genomes. A comparison of the genetic map of *E. coli* K-12 with that of serotype *Salmonella typhimurium*, which identified some 30 large DNA segments present only in one of these organisms, provided the first evidence that horizontal gene transfer mediated by plasmids or phage was a major driving force during the evolution of these bacteria (45). The genetic material received by the genus *Salmonella* by way of horizontal gene transfer includes several segments encoding virulence factors. The transferred DNA may vary in size from large genetic elements, such as the pathogenicity islands SPI 1 (34) and SPI 2 (51), phage (38), and virulence plasmids (27), to fimbrial operons located on the chromosome (5), or even single genes inserted in a housekeeping operon (52). Although acquisition of these virulence genes has significantly influenced the evolution of *Salmonella* serotypes, little is known about their ancestry. The first step in understanding how these virulence factors contributed to shape this successful pathogen is to determine the point in evolution when they were acquired.

The classification of the genus *Salmonella* into serotypes according to the Kauffmann and White scheme is not particularly useful for evolutionary studies since it is based on three surface markers and therefore does not provide a good basis for estimating genetic relatedness among strains (43). The inherent weakness of this system was pointed out by Selander and coworkers, who showed by multilocus enzyme electrophoresis (MLEE) that many *Salmonella* serotypes are not of monophyletic origin (8, 49). An analysis of DNA relatedness (20, 30), MLEE (10, 44), and sequence analysis (9, 32, 36, 37) established taxonomic schemes which reflect the actual phylogenetic relationships among *Salmonella* serotypes. According to this current nomenclature the genus *Salmonella* contains only two species, *Salmonella bongori* (44) and *Salmonella enterica* (29), the latter of which is further subdivided into subspecies designated by roman numerals. Thus, the antigen formulas of the Kauffmann and White scheme describe serotypes rather than species (e.g., *S. enterica* serotype Typhimurium, although Latin binomials for serotypes are still in use for practical reasons, e.g., *S. typhimurium*). This differentiation into species and subspecies involved changes in the host range of *Salmonella* serotypes. For example, *S. enterica* subspecies I is predominantly isolated from warm-blooded animals, whereas *S. bongori* and the other subspecies of *S. enterica* are most frequently isolated from reptiles (12, 43, 56). The importance of these changes in host range is highlighted by the fact that 99% of clinical isolates belong to *S. enterica* subspecies I (43). What are the genetic alterations that allowed *S. enterica* subspecies I to change its host range to include warm-blooded animals as hosts? While fimbriae have been shown to determine the host range of enteropathogenic *E. coli* (25, 28, 42), little is known about the virulence factors involved in determining host specificity of *Salmonella* serotypes. Several fimbrial operons, including *fim* (14), *lpf* (5), *pef* (26), *sef* (16), and *agf* (22), have been identified in *Salmonella* serotypes. Did horizontal transfer of these fimbrial operons play a role during the evolution of *S. enterica* subspecies I? To answer these questions, we determined the distribution of fimbrial operons among two collections of *Salmonella* serotypes with known phylogenetic relationships (10, 44).

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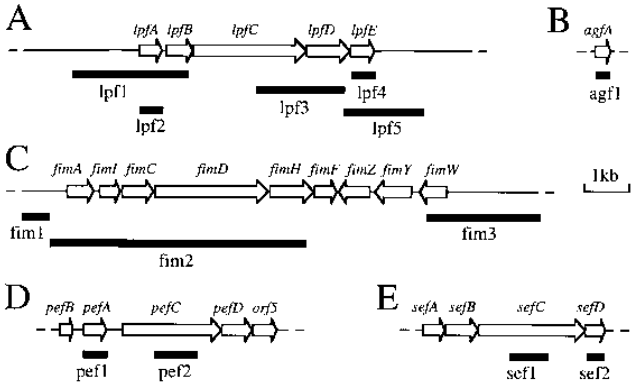

FIG. 1. Positions of DNA fragments (black bars) used as probes for detection of fimbrial operons. The positions of genes within the *lpf* (A), *agf* (B), *fim* (C), *pef* (D), and *sef* (E) fimbrial operons are indicated by arrows.

MATERIALS AND METHODS

Bacterial strains. *Salmonella* reference collection B (SARB) has been reported on recently (10). A collection of *Salmonella* serotypes representing *S. bongori* and six subspecies of *S. enterica* has been described by Reeves et al. (44). *E. coli* strains TA One Shot and DH5α were purchased from Invitrogen and Gibco BRL, respectively.

PCR and generation of nucleotide probes. The primers for PCR amplification of a region between open reading frame 103 (orf103) and orf101 in *E. coli* and the corresponding regions on the chromosomes of *Salmonella* serotypes were 5′-CAATGACAATAATATCGCCGAATTCGGCTC-3′ and 5′-CGGCCAAAC AGTGAAAGAAGACG-3′. Amplification was achieved by 25 cycles of amplification with *Taq* polymerase, the conditions for each cycle being 95°C for 1 min, 50°C for 1 min, and 72°C for 6 min. PCR products were cloned into the vector pCRII and transformed into the *E. coli* strain TA One Shot (TA cloning kit; Invitrogen). To generate nucleotide probes, DNA fragments were labeled with the DNA labeling and detection kit (nonradioactive) from Boehringer Mannheim. Plasmid pISF101 (13) was digested with *Hpa*I-*Sph*I, and probes fim1, fim2, and fim3 were generated by labeling the restriction fragments depicted in Fig. 1. The inserts of plasmids pMS1067, pMS1039, and pMS1048 (5) were labeled to produce probes lpf1, lpf3, and lpf5, respectively. Primers 5′-TTGCTCTGTCTG CTCTCGCTGTAG-3′ and 5′-CATGATTCTCTTCCTGAGCCTCC-3′ were used for PCR amplification of the *lpfA* gene from serotype *S. typhimurium* (Fig. 1). Primers for amplification of *lpfE* were 5′-TTTGATGCCAGCGTGTTTACT G-3′ and 5′-AGTAGACCACCAGCAGAGGGAAAG-3′ (Fig. 1). The *pefA*-specific nucleotide probe pef1 has been described previously (6). The insert of plasmid pPE1 (6) was labeled to generate probe pef2. A 1,103-bp internal fragment of *sefC* was amplified by PCR from *Salmonella enteritidis* En1 with primers 5′-GCGAAAACCAATGCGACTGTAG-3′ and 5′-CCCACCAGAAA CATTCATCCC-3′ (Fig. 1). Primers 5′-TCAACTATTAAAGCACAAGAAC-3′ and 5′-TTATAATTCAATTTCTGTCGC-3′ were used to amplify a 374-bp fragment of *sefD* from serotype *S. enteritidis* En1 (Fig. 1). A 151-bp fragment of *agfA* from serotype *S. typhimurium* was amplified with primers 5′-TGCAAAGCGAT GCCCGTAAATC-3′ and 5′-TTAGCGTTCCACTGGTCGATGGTG-3′ (Fig. 1).

Southern hybridization. Isolation of chromosomal DNA was performed as recently described (3). Chromosomal DNA was restricted with *Eco*RI, and the fragments were separated on a 0.5% agarose gel. Southern transfer of DNA onto a nylon membrane was performed as previously described (3). Hybridization was performed at 65°C in solutions without formamide. Two 15-min washes were performed under nonstringent conditions at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate. Hybrids were detected with the labeling and detection kit (nonradioactive) from Boehringer Mannheim.

Nucleotide sequencing. To compensate for PCR errors, products from two independent reactions were cloned in each case, and sequencing was performed by the dideoxy chain termination method (47) with an AutoRead sequencing kit (Pharmacia) and an ALF automatic sequenator. The nucleotide sequences were analyzed with programs blastX and blastN (1).

RESULTS

Distribution of fimbrial operons among strains of *S. enterica* subspecies I. The frequencies of homologs of the *lpf* and *pef* operons have not been established within *Salmonella* serotypes (5, 6). The distributions of the *agf* (22), *sef* (15, 54), and *fim* operons (53) have produced some very useful epidemiological data. However, strains of the same serotype might be only distantly related in genotype (8, 49), and hence these earlier reports provided little information about the evolutionary origins of fimbrial operons. To obtain clues as to when during evolution fimbrial operons entered the gene pool of the genus *Salmonella*, we determined their distribution by using the SARB collection, which includes 72 strains representing 37 serotypes of *S. enterica* subspecies I (10). Hybridization with probes fim1, fim2, fim3, and agf1 (Fig. 1) revealed that the *fim* and *agf* fimbrial operons were acquired in a lineage ancestral to *S. enterica* subspecies I. DNA probes lpf2, lpf3, and lpf4 (Fig. 1) each produced identical hybridization patterns (Fig. 2). These hybridization patterns may best be explained by repeated loss of the *lpf* operon, although their generation by repeated acquisition of *lpf* genes cannot be ruled out. Probes lpf1 and lpf5 contain sequences upstream and downstream of the *lpf* operon; these sequences are also present at the corresponding location in the *E. coli* chromosome (5). These probes detected homologous sequences in all strains of the SARB collection, indicating that a putative repeated loss of the *lpf* operon never involved large deletions of upstream or downstream sequences (Fig. 2). Probes sef1 and sef2 (Fig. 1) both hybridized with chromosomal DNA of two phylogenetic lineages (Fig. 2), suggesting recent acquisition by two lateral transfer events. Further support for this idea comes from the atypical base composition of *sef*. The G+C content, which in the case of *S. enterica* averages 52%, is considered to be a phylogenetic characteristic of a species (2). However, the genes *sefABCD* have a G+C content of only 35.2% (15, 16), which suggests that this operon was obtained horizontally from an organism with low G+C content. Probes pef1 and pef2 (Fig. 1) detected signals in two distantly related lineages within *S. enterica* subspecies I (Fig. 2). The restricted phylogenetic distribution of *pef* is evidence for its recent acquisition by horizontal transfer, in fact, by a plasmid-mediated event, as this operon was recently located on the virulence plasmid (26). The reported variability among virulence plasmids isolated from different *Salmonella* serotypes (11, 35) may explain why probes pef1 and pef2 did not produce perfectly matching hybridization patterns.

Distribution of fimbrial operons among *S. bongori* and *S. enterica*. Since 99% of clinical isolates belong to *S. enterica* subspecies I (43), previous studies on the distribution of fimbrial operons among *Salmonella* serotypes did not include a representative set of strains from *S. bongori* or other *S. enterica* subspecies (22, 53, 54). To obtain information on the distribution of fimbrial operons among *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, and VI, we used a set of 18 strains described by Reeves and coworkers (44). Probe agf1 detected homologs in *S. bongori* and in six subspecies of *S. enterica* (Fig. 3). This finding is consistent with the idea that *agfA* was present in a common ancestor of all *Salmonella* serotypes. CsgA, the major fimbrial subunit of Curli in *E. coli*, has 74% amino acid sequence identity with AgfA (17), which is in the range of sequence conservation found between proteins present in *E. coli* and *S. typhimurium* (50). Since sequences related to *agfA* were not detected in enterobacteria more distantly related to the genus *Salmonella* (22), it is likely that the *agf* operon was acquired by a common ancestor of *E. coli* and the genus *Salmonella*. Probes fim1, fim2, and fim3 (Fig. 1) detected homologs in all *Salmonella* serotypes tested, indicating that the *fim* operon entered a lineage ancestral to the genus *Salmonella* (Fig. 3). The *fim* operon has been mapped to 14 min on the *S. typhimurium* LT-2 genetic map (18), while genes encoding type 1 fimbriae of *E. coli* K-12 are located at 98 min (4). Differences in gene order and nucleotide sequence, as well as the different

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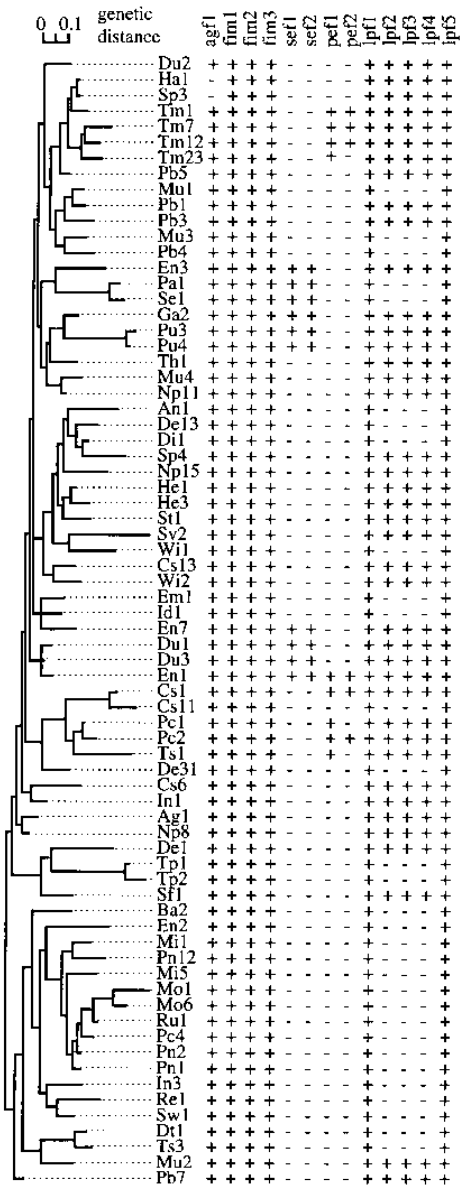

FIG. 2. Distribution of fimbrial operons among strains of *S. enterica* subspecies I (SARB collection). The left side shows the phylogenetic tree constructed by Boyd and coworkers (10). The distribution of fimbrial operons is shown on the right. *Salmonella* serotype abbreviations are as follows: Ag, *S. agona*; An, *S. anatum*; Ba, *S. brandenburg*; Cs, *S. choleraesuis*; De, *S. derby*; Di, *S. duisburg*; Dt, *S. decatur*; Du, *S. dublin*; Em, *S. emek*; En, *S. enteritidis*; Ga, *S. gallinarum*; Ha, *S. haifa*; He, *S. heidelberg*; Id, *S. indiana*; In, *S. infantis*; Mi, *S. miami*; Mo, *S. montevideo*; Mu, *S. muenchen*; Np, *S. newport*; Pa, *S. paratyphi* A; Pb, *S. paratyphi* B; Pc, *S. paratyphi* C; Pn, *S. panama*; Pu, *S. pullorum*; Re, *S. reading*; Ru, *S. rubislaw*; Se, *S. sendai*; Sf, *S. senftenberg*; Sp, *S. saintpaul*; St, *S. stanley*; Sv, *S. stanleyville*; Sw, *S. schwarzengrund*; Th, *S. thompson*; Tm, *S. typhimurium*; Tp, *S. typhi*; Ts, *S. typhisuis*; Wi, *S. wien*. +, hybridization signal; −, no hybridization signal.

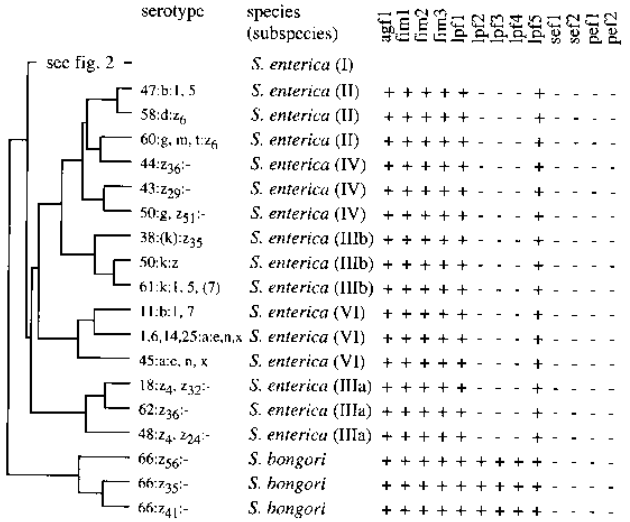

FIG. 3. Distribution of fimbrial operons among stains of *S. bongori* and *S. enterica*. The left side shows the phylogenetic tree constructed by Reeves and coworkers (44). The distribution of fimbrial operons is shown on the right. +, hybridization signal; −, no hybridization signal.

map positions of these fimbrial operons on otherwise colinear chromosomes (45), imply that they have distinct origins and were acquired independently. Probes lpf2, lpf3, and lpf4 (Fig. 1) gave hybridization signals with serotypes from *S. bongori* but did not detect any homologs in *S. enterica* subspecies II, IIIa, IIIb, IV, or VI. This distribution may be explained by acquisition in a lineage ancestral to the genus *Salmonella* and subsequent loss in a lineage ancestral to *S. enterica* subspecies II, IIIa, IIIb, IV, and VI (Fig. 3).

Comparison of the chromosomal regions upstream and downstream of *lpf* from *S. typhimurium* with corresponding regions in *E. coli* and different *Salmonella* serotypes. The genes *lpfABCDE* are located at 78 min on the *S. typhimurium* chromosome, and no homologs are present at the corresponding region of the *E. coli* K-12 chromosome. A sequence comparison revealed that these five genes are inserted between homologs of *E. coli* orf103 and orf102 at 78 min on the *S. typhimurium* chromosome (5). The complex phylogenetic distribution of the *lpf* operon among serotypes of *S. bongori* and *S. enterica* is indicative of repeated acquisition and loss. To clarify this point we analyzed the chromosomal areas surrounding the *lpf* operon in *Salmonella* serotypes lacking these genes by PCR amplification. A comparison of the nucleotide sequences from *E. coli* K-12 (GenBank accession number U00039) and *S. typhimurium* (5) identified conserved nucleotide sequences for the design of primers which anneal downstream of *lpfE* (within orf102 of *E. coli*) and upstream of *lpfA* (upstream of orf104 of *E. coli*) (Fig. 4A). With this primer pair, no product was obtained for *Salmonella* serotypes which contain *lpf*, since the conditions used for PCR did not allow amplification of the entire 7-kb region. However, all *Salmonella* serotypes lacking *lpf* yielded PCR products of a single size (about 1.2 kb). PCR amplification of the corresponding region from *E. coli* DH5α yielded a product of about 2.7 kb, the size predicted by the nucleotide sequence.

Further nucleotide sequence analysis of the *lpfA* upstream region showed that a 5′-truncated remnant of *E. coli* orf103 (94 bp of the 3′ end of orf103) was present in *S. typhimurium* (Fig. 4C). As compared to *E. coli* K-12, most of orf103 has been lost in *S. typhimurium* by a deletion of about 1,240 bp (Fig. 4B). It is not clear from these data whether this deletion occurred before (as shown in Fig. 4), after, or at the same time as the *lpf* operon entered the genome. The *lpf* operon has been inserted directly behind the stop codon of the 5′-truncated orf103 homolog in *S. typhimurium* (Fig. 4C). The PCR products obtained from three serotypes, *Salmonella paratyphi* A enzyme type Pal, *Salmonella typhi* enzyme type Tp2 and *Salmonella* II

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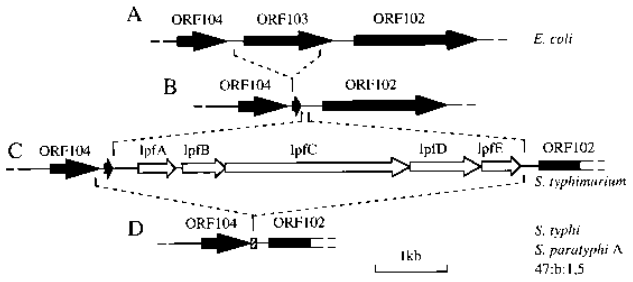

FIG. 4. Model for acquisition and loss of the *lpf* fimbrial operon. The region on the *S. typhimurium* chromosome carrying the *lpf* operon (C) and the corresponding regions from *S. typhi*, *S. paratyphi* A, serotype 47:b:1,5 (D), and *E. coli* (A) are shown. The positions of deletions or insertions are indicated by dashed lines. A hypothetical intermediate in these events is also shown (B). Genes with homology to those of *E. coli* are shown as black arrows. The position of a 78-bp sequence found only in *S. typhi*, *S. paratyphi* A, and serotype 47:b:1,5 is indicated by a hatched bar.

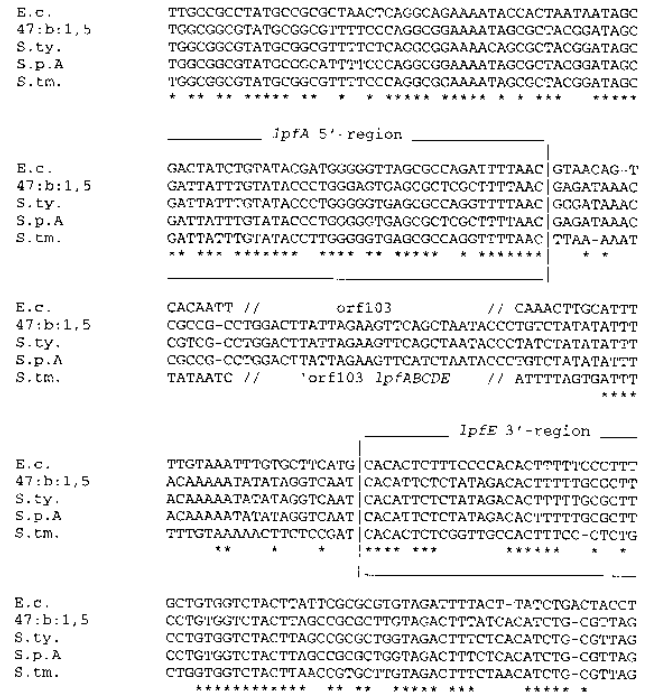

FIG. 5. Comparison of nucleotide sequences of corresponding areas from the *E. coli* (E.c.), the *S. paratyphi* A Pa1 (S.p.A), the *S. typhi* Tp2 (S.ty.), the *Salmonella* II 47:b:1,5 (47:b:1,5), and the *S. typhimurium* (S.tm.) chromosomes by using the program CLUSTAL. Nucleotides conserved in all five sequences are indicated by stars. Areas in the *E. coli* and the *S. typhimurium* sequences where additional genes are inserted are indicated by orf103 and `orf103 *lpfABCDE*, respectively.

serotype 47:b:1,5, lacking *lpf* were cloned into vector pCR II to produce plasmids pTY990, pTY989, and pTY991, respectively. Nucleotide sequencing revealed that the 5′-truncated remnant of orf103 was not present in these serotypes (Fig. 4D). A comparison with the *S. typhimurium* sequence showed a 6,057-bp deletion in *S. typhi*, *S. paratyphi* A, and serotype 47:b:1,5 which encompassed the 5′-truncated remnant of orf103 and the *lpf* operon (Fig. 4C, D). These findings imply that the *lpf* operon was first acquired by a lineage ancestral to the genus *Salmonella* and was subsequently deleted together with the 5′-truncated remnant of orf103 from *S. typhi*, *S. paratyphi* A, and serotype 47:b:1,5. The absence of orf103 also explains why the PCR products from *Salmonella* serotypes which lack the *lpf* operon were smaller than that obtained from *E. coli* K-12. The branching orders on the phylogenetic tree suggest that loss of the *lpf* operon from distantly related serotypes such as *S. typhi*, *S. paratyphi* A, and 47:b:1,5 occurred by independent events (Fig. 2, 3).

Interestingly, the deletion of the *lpf* operon in *S. paratyphi* A, *S. typhi*, and serotype 47:b:1,5 was accompanied by an insertion of 78 bp in each case (Fig. 5). This sequence showed no homology to any entries in current nucleotide databases; however, it was highly conserved among *S. paratyphi* A, *S. typhi*, and serotype 47:b:1,5. The identity of the deletion endpoints and the simultaneous acquisition of a conserved 78-bp nucleotide sequence imply that the loss of *lpf* in *S. typhi*, *S. paratyphi* A, and serotype 47:b:1,5 involved a common mechanism, presumably horizontal transfer following a single ancestral deletion event. The phylogeny of *Salmonella* serotypes suggests that the *lpf* operon was initially lost from a lineage ancestral to *S. enterica* subspecies II, IIIa, IIIb, IV, and VI. This deletion of *lpf* may then have been transferred into other serotypes by homologous exchange of horizontally transferred segments. Our finding that PCR products of identical size were obtained for all *Salmonella* serotypes which lack the *lpf* operon is consistent with this idea. Similar evolutionary mechanisms result in genetic transfer events which generate allelic variation at the *fliC* locus, thereby increasing serovar diversity within the genus *Salmonella* (31).

DISCUSSION

The phylogenetic relationships among *Salmonella* serotypes have been reconstructed by comparison of nucleotide sequences from homologous genes or by MLEE of homologous gene products (8, 9, 36, 37, 44, 49). These studies have provided the framework necessary for investigating how the acquisition of nonhomologous genetic material has contributed to the evolution of the genus *Salmonella*. In this study, we determined the phylogenetic distribution of fimbrial operons among *Salmonella* serotypes (Fig. 2, 3) in order to resolve the genealogy of the recombinational events which led to their acquisition. The *sef* fimbrial operon has a limited phylogenetic distribution, indicative of recent acquisition by two distantly related evolutionary lineages within *S. enterica* subspecies I. Similarly, the phylogenetic distribution of the *pef* operon provides evidence that it too was introduced recently into two lineages of *S. enterica* subspecies I. The *lpf* operon entered the genome earlier during evolution, in a lineage ancestral to the genus *Salmonella*. Like the *lpf* operon, the *fim* operon is not present in *E. coli* or related species, and its phylogenetic distribution indicates acquisition by an ancestor of the genus *Salmonella*. Finally, the *agf* operon is the most ancient of the *Salmonella* fimbrial operons and was most likely already present in a common ancestor of *E. coli* and the genus *Salmonella*. This sequence of recombinational events created new evolutionary lineages of *Salmonella* possessing different adhesive properties. Another factor contributing to the variety of fimbrial repertoires observed among *Salmonella* serotypes is the loss of adhesive properties. In the case of *lpf*, the entire operon has been deleted in several lineages of the genus *Salmonella*, leading to a scattered phylogenetic distribution (Fig. 2, 3). The comparative analysis presented here shows that horizontal gene transfer and deletion events have created unique combinations of fimbrial operons among *Salmonella* serotypes. Besides these mechanisms, diversity in adhesive properties is further increased by genetic changes that cannot

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be detected by hybridization analysis. For example, in the case of the *fim* operon encoding type 1 fimbriae, point mutations can abolish mannose-sensitive adhesion and result in expression of fimbriae with unspecified binding properties (so-called type 2 fimbriae) (19, 23, 24, 33, 41).

It is not clear which selective forces led to the development of the scattered phylogenetic distribution of *lpf*. It has been shown that expression of the *lpf* fimbrial operon can be advantageous in mice (7), but it could be a disadvantage in other host species. Colonization of these other hosts could have selected for variants that lost *lpf*. A similar situation may exist for the growth of *Salmonella* serotypes in chickens, a habitat that appears to select against type 1 fimbriation and flagellation. For example, the avian species-adapted serotypes *Salmonella gallinarum*, the agent of fowl typhoid, and *Salmonella pullorum*, which causes pullorum disease in chicks, are nonflagellate and carry point mutations in their *fim* operons that abolish mannose-sensitive adhesion (19, 24, 41). Similarly, *S. typhimurium* strains that are nonmotile and lack type 1 fimbriation are isolated primarily from cases of avian disease (23). As is the case with *lpf* and *fim*, the phylogenetic distribution of *sef* and *pef* could be seen as a result of adaptation to particular hosts. The *sef* operon was found to be present in two distantly related lineages within *S. enterica* subspecies I (Fig. 2). One of these phylogenetic groups included the avian species-adapted serotypes *S. gallinarum* and *S. pullorum* (32). The second lineage contained the common clone *S. enteritidis* En1, which is responsible for the recent worldwide increase in salmonellosis due to consumption of undercooked eggs and poultry (46). Interestingly, a *sefA* mutant of *S. enteritidis* was impaired in its ability to colonize chicks during the first week of infection (55), suggesting that acquisition of SEF-14 fimbriae may indeed have contributed to the adaptation of *Salmonella* serotypes to poultry. Similarly, the *pef* operon is present in two distantly related evolutionary lineages (Fig. 2), one of which includes *S. enteritidis* En1 and *S. choleraesuis* Cs1, two clones with worldwide distributions that represent 93 and 88%, respectively, of all isolates from these two serotypes (8). The second lineage contains strains of *S. typhimurium*. Serotypes *S. choleraesuis* and *S. typhimurium* are the most frequent isolates from pigs (48). These findings suggest that acquisition of specific fimbrial operons may have been one of the mechanisms by which *Salmonella* serotypes were able to expand their host range to include many domesticated animals. Because strains causing salmonellosis are usually acquired from animal sources (39), this adaptation to livestock may in turn be the reason *S. enterica* subspecies I accounts for more than 99% of human isolates.

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