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Recent Horizontal Transmission of Plasmids between Natural Populations of *Escherichia coli* and *Salmonella enterica*

E. FIDELMA BOYD AND DANIEL L. HARTL*

Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138

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Seventy-one natural isolates obtained from a *Salmonella* reference collection were examined for the presence of plasmids closely related to the *Escherichia coli* F plasmid. The collection consists of several serovars of the *S. enterica* Typhimurium complex, subspecies I, to which 99% of pathogenic salmonellae belong. Molecular genetic techniques of DNA hybridization, along with PCR and DNA sequencing, were used to examine the occurrence, distribution, and genetic diversity of F-like plasmids among *Salmonella* strains. The F plasmid genes examined were *finO*, *traD*, *traY*, and *repA*, which map at dispersed positions on the F plasmid of *E. coli*. Comparative sequence analysis of each of the four genes in *Salmonella* plasmids showed them to be homologous (in some cases, virtually identical) to those found in F plasmids of *E. coli* natural isolates. Furthermore, the frequency of F-like plasmids in *Salmonella* strains was approximately the same as that observed in the *E. coli* Reference Collection. However, in *Salmonella*, the distribution was confined predominately to the serovars Typhimurium and Muenchen. The unexpected finding of a shared pool of F-like plasmids between *S. enterica* and *E. coli* demonstrates the significant role of conjugation in the histories of these important bacterial species.

Salmonella enterica is an important human pathogen related to *Escherichia coli* (11, 12). *S. enterica* and *E. coli* are thought to have diverged from a common ancestor 140 million years ago, around the time of the origin of mammals (26). *E. coli* evolved as a commensal organism of mammals and birds, whereas *S. enterica* is a facultative intracellular pathogen, typically colonizing reptiles, birds, and mammals. Some serovars show a remarkable host species specificity; for instance, *S. enterica* serovars Typhi and Gallinarum are found exclusively in humans and birds, respectively (14).

Molecular genetic analysis has confirmed the division of *S. enterica* into seven distinctive groups and has identified an eighth group composed of several strains that were previously assigned to group IV (8, 9, 23, 24, 31). *Salmonella* Reference Collection A (SARA), a collection of natural isolates of *Salmonella* (1), contains serovars of a single major lineage of *S. enterica*, group I, among which the genetic diversity is equivalent to subgroup A of the *E. coli* Reference Collection (ECOR) (25). Included in the SARA collection are isolates of serovars Typhimurium, Heidelberg, Muenchen, Paratyphi, and Saintpaul; these isolates exemplify the full range of genetic variation in the Typhimurium complex as determined by multilocus enzyme electrophoresis (MLEE) (4).

The fertility factor F is a high-molecular-weight (~100 kb), low-copy-number conjugational plasmid that is notable for its ability to integrate into the host chromosome and to support conjugational transfer of chromosomal genes (15). Previously, Boyd et al. (10) demonstrated that there is a relatively high incidence of F-related plasmids among natural isolates of *E. coli*. Among the ECOR collection of isolates, 15% were found to possess F-related plasmids. Analysis of F plasmid genes showed them and, by inference, the plasmids themselves, to be mosaic in structure with different functional regions acquired from different sources. Recombination has been suggested as an important mechanism for generating genetic diversity in these plasmids (10). Also, plasmid transfer among strains within and between the major ECOR groups has been frequent, as evidenced by the fact that the evolutionary history of the plasmids is very different from that of their hosts (10), as judged by sequence analysis of chromosomal genes and MLEE (8).

Previous studies of plasmid diversity in natural isolates of *E. coli* and *Salmonella* were based primarily on plasmids with particular phenotypic attributes, in particular, a diverse set of plasmids involved in colicin resistance (1, 3, 27). Analysis of the colicin and immunity genes of these plasmids revealed an excess of amino acid replacement polymorphisms relative to silent polymorphisms in the immunity gene, suggesting selection for diversity (2, 28, 29). Furthermore, colicin gene sequences obtained from plasmids of *E. coli* and *S. enterica* serovar Typhimurium were very similar, suggesting overlapping plasmid pools (2).

The *E. coli* and *S. enterica* genomes have many features in common, including insertion sequences (IS) (6, 7, 22), repetitive extragenic palindromic sequences (24), and most chromosomal genes. Recent studies demonstrated that these divergent taxa both harbor several of the same classes of IS elements and, in the case of IS*1*, that there has been recent lateral transfer between these species (6, 7). It has been suggested that plasmids may play a major role in the transmission of IS elements among strains (18, 19, 21, 30). However, there has been no report of natural isolates of these species sharing F-related plasmids, which are known to harbor many of the IS elements that the species have in common.

In order to determine the evolutionary significance of F plasmid transfer between *E. coli* and *S. enterica*, we examined strains of subspecies I for F plasmid occurrence, distribution, and genetic diversity. We sequenced portions of four F plasmid genes: *finO*, which is involved in regulation of conjugation; *traD* and *traY*, which are involved in conjugational metabolism; and *repA*, a gene of the repFIB replicon found predominately in the IncFI group (5, 16, 17). Herein, we describe the isolation of F-like plasmids from *S. enterica* that show a recent common ancestor with the *E. coli* F plasmids. The important implication of this finding is that there is relatively frequent conjugational

* Corresponding author.

TABLE 1. List of *S. enterica* isolates with the F-like plasmid

Strain	Serovar	MLEE profile	Source	Locality	Date
SARA 1	Typhimurium	Tm 1	Human	Mexico	
SARA 2	Typhimurium	Tm 1		Laboratory strain	
SARA 4	Typhimurium	Tm 1	Horse	Rhode Island	1987
SARA 5	Typhimurium	Tm 1		Mongolia	
SARA 6	Typhimurium	Tm 2	Human	Ohio	
SARA 7	Typhimurium	Tm 3		Norway	
SARA 9	Typhimurium	Tm 7	Parrot	California	1987
SARA 10	Typhimurium	Tm 9	Opossum	California	1987
SARA 11	Typhimurium	Tm 10		Thailand	
SARA 12	Typhimurium	Tm 11	Horse	Louisiana	1987
SARA 13	Typhimurium	Tm 12		France	
SARA 14	Typhimurium	Tm 13		Panama	
SARA 15	Typhimurium	Tm 14	Dog	Texas	1987
SARA 20	Typhimurium	Tm 22		France	
SARA 21	Typhimurium	Tm 23	Heron	Oregon	
SARA 26	Saintpaul	Sp 3	Human	France	1988
SARA 63	Muenchen	Mu 1	Human	France	1988
SARA 65	Muenchen	Mu 1	Chicken	Florida	1987
SARA 67	Muenchen	Mu 1	Human	Mexico	

transfer between these species, despite their different niche specializations. The demonstration of overlapping plasmid pools also leads one to speculate on the importance of F plasmids as a mechanism for interspecies recombination between these genetically divergent taxa.

MATERIALS AND METHODS

Bacterial strains. The natural isolates of *S. enterica* analyzed were obtained from SARA (4). SARA consists of 72 strains of the Typhimurium complex; 71 natural isolates encompassing strains of the serovars Typhimurium, Heidelberg, Muenchen, Paratyphi, and Saintpaul; and an LT2 laboratory strain (SARA 2). The evolutionary relationships among the isolates have been inferred on the basis of MLEE (4).

PCR amplification. Primers for PCR and DNA sequencing were designed from a published sequence as described elsewhere (10). PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Inc., Chatsworth, Calif.).

Dot blot analysis. Total DNA was prepared from all 72 *Salmonella* isolates with G-Nome DNA isolation kits from Bio 101 (Vista, Calif.). *Salmonella* DNAs were transferred to a Hybond N+ membrane to test for similarity with five plasmid probes. Probes derived from each of the plasmid genes, *finO*, *traD*, *repA*, *traY* (F), and *traY* (R1) (10), were obtained by PCR and labeled by the random primer method with fluorescein-conjugated nucleotides and, after hybridization, were detected by the ECL (enhanced chemiluminescence) system of Amersham (Arlington Heights, Ill.).

DNA hybridization. All isolates that were positive by dot blot analysis were further examined by restriction digests of DNA with *Eco*RI. The products were separated in 0.6% agarose gels and transferred to a Hybond N+ membrane. Hybridization of probes [*finO*, *traD*, *repA*, *traY* (F), and *traY* (R1)] was carried out overnight at high stringency.

Nucleotide sequencing. DNA sequencing of PCR-amplified DNA was performed with Dye Terminator chemistry. A 370A DNA sequencer was used according to the manufacturer's instructions.

Computer analysis. DNA sequence data were assembled and edited with Sequencher programs (version 2.1.1.). Phylogenetic analysis was performed with the program MEGA (20). Maximum parsimony trees were constructed for *finO*, *traD*, *traY* (R1), and *repA* by the branch-and-bound method of tree construction.

Nucleotide sequence accession numbers. The nucleotide sequences of the genes described in this paper have been deposited in the GenBank database under the accession number U81622 to U81639.

RESULTS

Distribution of F plasmids. To assay the distribution of F plasmids among natural isolates of *S. enterica*, four F plasmid gene probes, *finO*, *traD*, *repA*, and *traY*, were used to identify homology. The *traY* genes from an F plasmid and the related R1 plasmid show both size and sequence polymorphisms and therefore two probes, designated *traY* (F) and *traY* (R1), were used for the *traY* gene. Only the DNA from SARA isolates that were positive by dot blot analysis was restricted with *Eco*RI and reprobed: 26 isolates in total. The SARA isolates examined in this manner were SARA 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 20, 21, 26, 49, 52, 62, 63, 65, 67, 68, 69, 71, and 72; an ECOR strain, ECOR 11, was included as a positive control. The DNA hybridization survey identified 19 *Salmonella* isolates that showed clear homology with the *E. coli* F plasmid genes (Table 1). With the *finO*, *traD*, *repA*, and *traY* (R1) probes, 13, 19, 15, and 18 SARA isolates, respectively, were positive (Fig. 1). The distribution of F-related plasmids in the SARA collection is summarized in Fig. 2. The majority of the plasmids are found in serovar Typhimurium isolates; 16 strains from this serovar gave a positive signal with at least two of the F plasmid gene probes (Fig. 2).

The probes yielded different degrees of hybridization with each of the strains. With the *finO* probe, SARA 7, 26, 63, 65, and 67 gave strong positive signals, whereas SARA 4, 5, 6, 9, 10, 11, 14, and 15 gave weak signals. Nineteen of the SARA isolates gave a strong signal with the *traD* probe (Table 1). However, SARA 49, 52, 62, 68, 69, 71, and 72 gave no signal with the *finO*, *traD*, *repA*, and *traY* (R1) probes; SARA 20 and 67 gave no signal with the *traY* (R1) probe; and SARA 21 and 26 gave no signal with *repA* probe. With the *repA* probe, SARA 7, 63, and 65 gave a strong positive signal whereas SARA 1, 2, 4, 5, 6, 9, 10, 11, 12, 13, 14, and 15 gave two signals each (Fig. 1).

PCR survey. The strains that were positive by Southern blotting were used as templates for PCR amplification. With the *finO*, *traD*, *repA*, and *traY* (R1) primers, totals of 5, 19, 3, and 4 SARA isolates respectively, supported amplification. The hybridization-positive SARA strains whose sequences could not be amplified were possibly too divergent in sequence for the primer sequences to amplify efficiently.

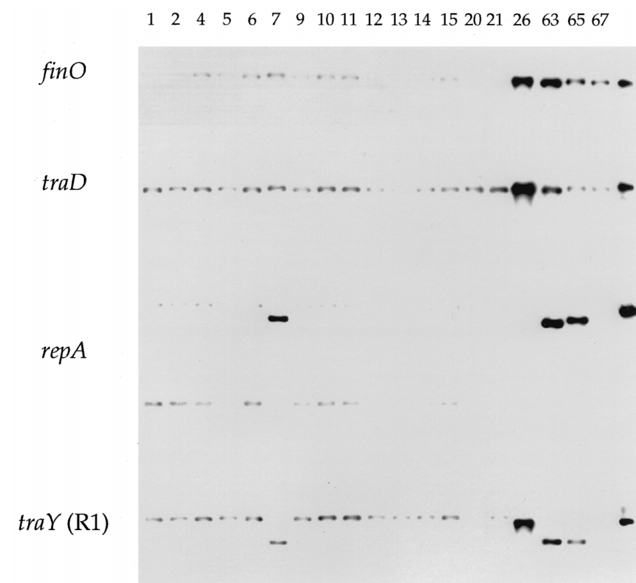

FIG. 1. DNA hybridization of natural isolates of *Salmonella* from SARA with the *finO*, *traD*, *repA*, and *traY* (R1) gene probes. DNA was digested to completion with *Eco*RI and hybridized with F plasmid gene probes prepared by PCR from *E. coli* K-12 F plasmids, except for *finO*, which was prepared from ECOR 62. SARA strains 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 20, 21, 26, 63, 65, and 67 are numbered above each lane; the last lane contains an *E. coli* strain, ECOR 62, as a positive control. With the *repA* probe, two signals (rows 3 and 4) were obtained for SARA strains 1, 2, 4, 5, 6, 9, 10, 11, 12, 13, 14, 15, 20, and 21.

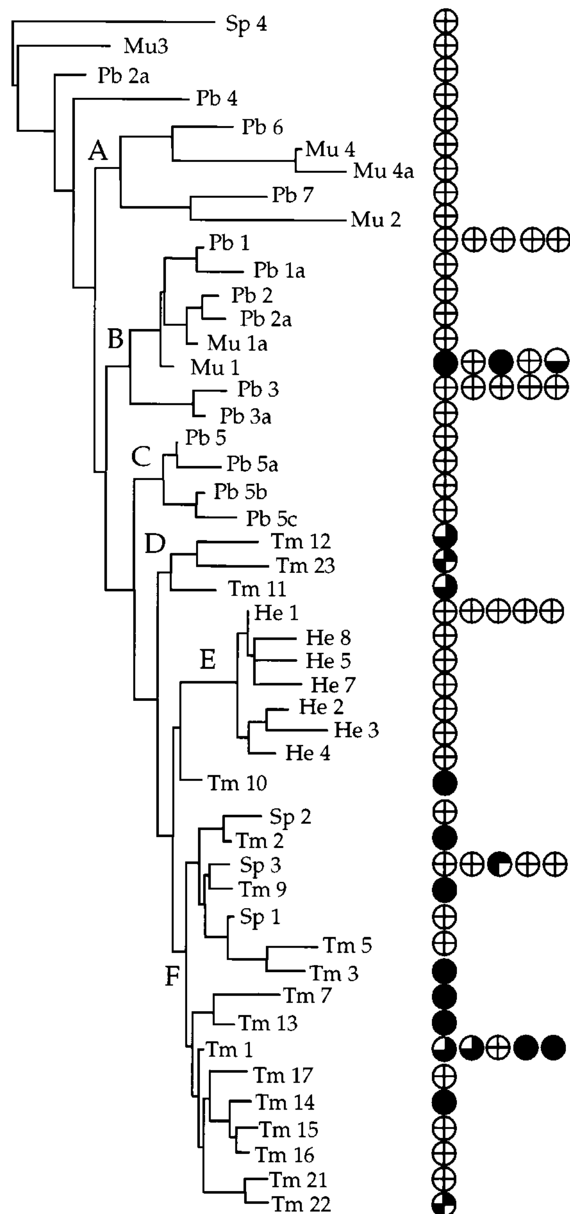

FIG. 2. Inferred evolutionary relationships among the 48 electrophoretic types represented by the 72 SARA strains based on MLEE, taken from data by Beltran et al. (4). Major phylogenetic groups are labeled with letters A to F. Plasmid distribution is indicated by shaded circles. Each circle is divided into four quarters representing the *traD*, *repA*, *traY* (R1), and *finO* genes, clockwise from 12 o'clock. Shaded areas indicate positive signal, and unshaded areas represent negative signal by DNA hybridization. Rows of circles indicate multiple SARA strains tested that are ordered consecutively; for example, Mu 1 is represented by five SARA isolates, SARA 63, 64, 65, 66, and 67. Abbreviations of serovars: Tm, Typhimurium; Sp, Saintpaul; He, Heidelberg; Pb, Paratyphi B; Mu, Muenchen.

Verification of plasmid-encoded loci. To determine the association of plasmid genes with plasmids, hybridization experiments were carried out with uncleaved genomic DNA with an extended electrophoresis time that separated the sheared chromosomal DNA from the closed circular DNA of the plasmids. After DNA transfer, the membranes were probed with *finO*, *traD*, *repA*, and *traY* gene probes. In the *Salmonella* strains and *E. coli* K-12 control, the hybridization band was clearly associated with a large plasmid separated from the main band formed by the bacterial chromosome. This was the case in all isolates except SARA 7; in this strain, the *finO*, *traD*, *repA*, and *traY* (R1) signals could not be resolved clearly from the chromosomal material.

Nucleotide sequence analysis. Among the five *finO* genes sequenced, there were 40 polymorphic sites in the 441-bp region sequenced, of which 15 were amino acid replacement sites (Fig. 3). The 523-bp region of the *traD* gene sequenced in six *Salmonella* strains had 110 polymorphic sites, 26 of which resulted in amino acid replacements (Fig. 3). In the *traY* (R1) gene from four *Salmonella* isolates, there were 13 polymorphic sites, including 3 replacements (Table 2). On the other hand, the *repA* genes sequenced from three *Salmonella* isolates had no polymorphic sites. All but one of the *finO* genes were very similar to those of *E. coli*; SARA 7 and SARA 65 shared near identity with the *finO* sequence from ECOR 47, and SARA 63 was very similar to ECOR 35. Among the SARA and ECOR *finO* sequences examined, the one recovered from SARA 67 was the most divergent. The *traD* sequences of SARA 7, 26, 63, and 65 were also very similar to those found in *E. coli*, with the exception of SARA 10, which had a highly divergent *traD* sequence with 68 unique polymorphic sites showing little similarity to any other *Salmonella* or *E. coli traD* sequences examined. At the amino acid level, a similar picture emerged for the TraD sequence of SARA 10, which had 20 unique amino acid replacement sites (Fig. 3). The *traY* (R1) nucleotide and amino acid sequences among the *Salmonella* were all very similar to those from *E. coli.*

Phylogenetic analysis. Maximum parsimony trees for *finO* and *traD* are shown in Fig. 4. Unweighted branch-and-bound analyses of maximum parsimony produced 45 trees (length 81) for the *finO* gene sequences and 90 trees (length 106) for the *traD* gene sequences. The trees constructed by this method had virtually the same topology as trees constructed by the neighbor-joining method. Sequences of the *finO* and *traD* genes from *E. coli* isolates are also shown for comparison. In the *finO* tree, two major groups can be distinguished. The *Salmonella finO* genes fall into both clades: SARA 7 is related to ECOR 47, and SARA 67 is related to ECOR 59 in group A, whereas SARA 63 and SARA 65 show similarity to ECOR 35 in group B (Fig. 4). The *traD* tree shows three major lineages, with SARA 7, SARA 63, and SARA 65 clustering together in lineage A and with SARA 10 and SARA 67 grouping together in lineage C (Fig. 4).

DISCUSSION

The extent to which *E. coli* and *S. enterica* share common pools of conjugational plasmids is unknown, as is the ancestral history of plasmids in these organisms. In order to test for the horizontal transmission of conjugational plasmids between *E. coli* and *S. enterica*, natural isolates from the SARA reference collection of *Salmonella* were analyzed for the presence of F-like plasmids. By comparative genetic analysis of nucleotide sequences, the distribution and evolutionary relationships of F plasmids within and between these species were elucidated, and the results were used to determine the mechanisms influencing the occurrence and maintenance of F-like plasmids in natural populations of enteric bacteria. We have observed an unexpectedly high prevalence of F-like plasmids in *Salmonella* strains. This might result from vertical transmission of F-like plasmids present in the common ancestor of *E. coli* and *S. enterica*. Alternatively, it could result from relatively recent horizontal transmission. In the former case, the F plasmid genes in *Salmonella* would be expected to be as divergent from homologous F plasmid genes in *E. coli* as the chromosomal

```
         ECOR42    I......R.. .......... ...E...... ..
```

FinO

```
                             111111111
                    11133334 4555688889 9123344444
                  1412312363 7036904577 9303801257

S. enterica
   SARA7          STPPKLAAAS NTENDDEDAK RCCSAAAADT
   SARA26         .......... .......... ..........
   SARA63         ......T... .......... ....V..E.I
   SARA65         ......T... .......... ....V..E.I
   SARA67         ..A.NV..T. P...N...S. C.S..T.EAI

E. coli
   ECOR9          ......T... .S........ ..S.V.SE.I
   ECOR11         ......T... .S........ ....V..E.I
   ECOR25         .........P ...S...... .........I
   ECOR27         .......... .......... .........I
   ECOR30         R......E.. ........PN ..F...SE.I
   ECOR71         .......... ..D....... .......E.I
   ECOR59         .......... TS..NEDES. .G.TQ.Q..I
   ECOR62         .......... .......... .......E.I
   ECOR35         ......T... .......... ..S.V..E.I
   ECOR39         ......T... .S......V. .......E.I
   ECOR47         .I........ .......... .........I
   ECOR48         ......T... .S........ ....V..E.I
   ECOR49         ......T... .S........ ..S.V..E.I
   ECOR50         ......T... .S........ ....V..E.I
   ECOR37         ...Q...... TS......V. ....V..E.I
   ECOR42         .......... .S........ ......TE.I
```

TraD

```
                         111 1111111111 11
                   1223456 6777799000 1122344566 77
                2348132860 2156901345 8908237539 05

S. enterica
   SARA67       -MLVVICKQK SHMIGTVVVS NEVDDDRRCG AV
   SARA65       ...I...... F....S.... .......... ..
   SARA63       ...I...... F....S.... .......... ..
   SARA26       .......... F......... ...E...... ..
   SARA10       .IF..LG.RQ FQTV.ACMTT DD...ALKLS TI
   SARA7        .IFI...... .....S.... .......... ..

E. coli
   ECOR9        T......... .......... .......... ..
   ECOR11       T..I...... .......... .......... ..
   ECOR27       I......... .......... .......... ..
   ECOR30       I...I..... ....SA.... .......... ..
   ECOR58       I......... .......... .......... ..
   ECOR70       I......... .......... ..I.E..... ..
   ECOR71       T......... .......... .......... ..
   ECOR59       II.....R.. .....S.... ..I.E..... ..
   ECOR62       I......... .......... ...E...... ..
   ECOR35       I..I...... .......... .......... ..
   ECOR47       I......R.. .......... .......... ..
   ECOR49       I..I...... .......... .......... ..
   ECOR50       I..I...... .......... .......... ..
```

FIG. 3. Distribution of the polymorphic amino acid positions among *finO* and *traD* genes from *E. coli* and *S. enterica* isolates. Horizontal numbers indicate codon positions (listed vertically). Standard single-letter amino acid abbreviations are used. Strain numbers on the left indicate the SARA and ECOR isolates examined.

genes are divergent. For homologous chromosomally encoded genes, such as the housekeeping gene malate dehydrogenase (*mdh*), the amount of nucleotide sequence variation between *E. coli* and *S. enterica* is, on average, 15%. However, within *E. coli*, pairs of strains differ, on average, at 1.1% of sites, and within *S. enterica*, they differ at 4.5% of sites (8).

We do find some F-like plasmid genes that are as divergent from those in *E. coli* as would be expected from vertical transmission. In particular, the *traD* and *finO* genes in two isolates, SARA 10 and SARA 67, have nucleotide and amino acid sequences that are highly divergent from their *E. coli* counterparts (Fig. 3). For example, among the 132 polymorphic nucleotide sites in the *traD* sequences, strains of *E. coli* and SARA 10 differed at an average of 13% of the sites. This level of divergence is consistent with that observed in homologous chromosomal genes between the two taxa. The *finO* gene of SARA 67 was less divergent, differing from the *finO* sequences in *E. coli* at an average of 5% of the sites. Since the *finO*, *repA*, and *traY* (R1) genes from SARA 10 were not amplifiable, the possibility exists that these genes may be at least as divergent as chromosomal genes.

On the other hand, some of the F plasmid genes give strong evidence of recent horizontal transmission. For example, all of the F plasmid genes from SARA 7, 26, 63, and 65 are highly homologous to their *E. coli* counterparts. The average pairwise differences for F plasmid genes between the species are 5.0% (*traD*), 3.4% (*finO*), 2.7% (*repA*), and 4.3% (*traY*). Within *Salmonella* strains, the differences are 7.6, 4.5, 0.0, and 4.0%, respectively; and within *E. coli*, they are 2.8, 4.0, 2.8, and 4.1%, respectively. The close similarity between F-plasmid genes from the two taxa suggests the occurrence of one or more recent horizontal transfer events between the species.

Consistent with the evidence for both vertical and horizontal transmission of F-like plasmids, comparison of host phylogeny with plasmid gene phylogeny revealed many inconsistencies. For example, SARA 63, 65, and 67 have identical MLEE profiles (Mu 1), and SARA 10 and SARA 26 are also closely related in the MLEE tree with profiles Tm 9 and Sp 3, respectively (Fig. 2) (4). However, with respect to both the *finO* and *traD* sequences, the F-like plasmid sequences in SARA 63, 65, and 67 do not group together. In the *finO* tree, SARA 7 clusters with ECOR 47 and SARA 67 groups with ECOR 59 in lineage A, whereas SARA 63 and 65 group with ECOR 35 in lineage B (Fig. 4). In the *traD* gene tree, SARA 7, SARA 63, and SARA 65 group together, whereas SARA 67 clusters with SARA 10 (Fig. 4). This pattern of closely related sequences for one gene but highly divergent sequences for another gene was also described for *E. coli* F plasmid genes and was interpreted as evidence for recombination among plasmid genes. Indeed, in the study of F-like plasmids in the ECOR isolates, intragenic recombination was readily detected in the plasmid genes (10). Recombination between plasmid genes evidently takes place at a much higher level than that observed in chromosomal genes, perhaps merely reflecting greater opportunities for homologous sequences to come into contact. Given the strong evidence for recombination among F plasmid genes in *E. coli*, it seems likely that intragenic recombination also plays a significant role in the evolution of F-like plasmids in *Salmonella.*

The evidence for recent horizontal transmission of F-like plasmids between *E. coli* and *S. enterica* was unexpected given the different ecological niches of these organisms. *E. coli* is a

TABLE 2. Nucleotide sequence polymorphisms in *S. enterica* from four F plasmid genes

Gene	No. of isolates	Total no. of sites	No. of polymorphic sites	No. of replacement sites	d_n/d_s ratio[a]
finO	5	441	40	15	0.15
traD	6	523	110	26	0.07
traY (R1)	4	171	13	2	0.07
repA	3	348	0	0	

[a] d_s and d_n are the average numbers of nucleotide differences per 100 synonymous sites and per 100 nonsynonymous sites, respectively, among all pairwise comparisons.

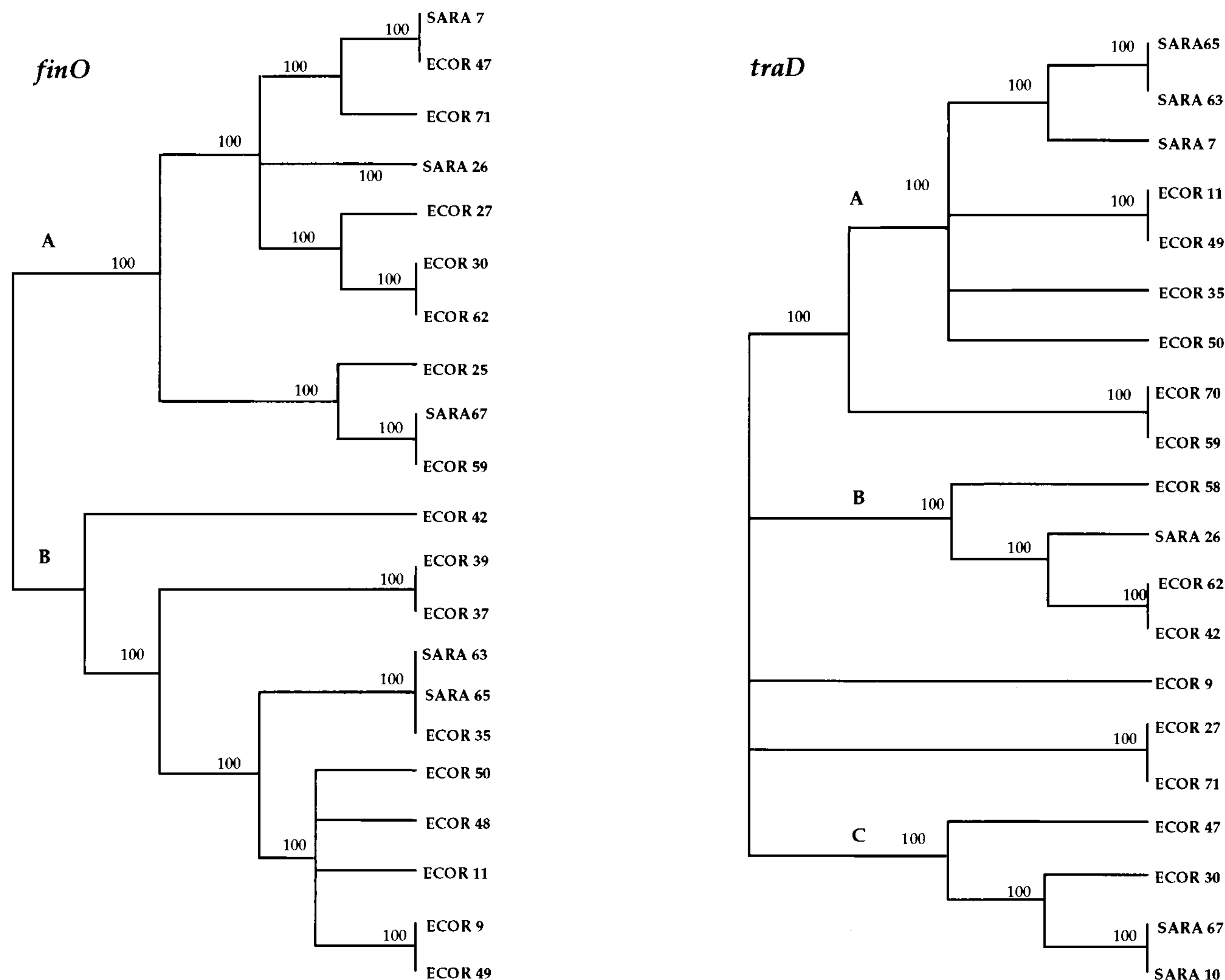

FIG. 4. Phylogenetic tree showing the relationships among strains of the SARA and ECOR collections containing *finO* and *traD* sequences. The trees are based on maximum parsimony analysis by the branch-and-bound method.

commensal organism of the intestines of mammals, whereas *Salmonella* is a facultative intracellular pathogen of both cold-blooded and warm-blooded organisms. The relatively high prevalence of F-like plasmids among *Salmonella* isolates is also surprising in view of the many natural barriers to recombination that exist, such as DNA restriction modification, which can reduce or eliminate the recovery of recombinants. Our data indicate that the occurrence of F-like plasmids in *Salmonella* is only a little less frequent than that found in *E. coli* natural isolates (10). Nevertheless, despite the evidence for limited genetic exchange between chromosomal genes of *E. coli* and *Salmonella* (13, 21, 32), horizontal transmission of F-like plasmids between these divergent taxa does occur at a level that is readily detected by DNA sequence analysis. Furthermore, the phylogenetic analysis suggests that the evolutionary dynamics of F-like plasmids is not strongly affected by the nature of the enteric bacterial host, at least for *E. coli* and *S. enterica.*

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