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NOTES

Mosaic Structure of the *smpB-nrdE* Intergenic Region of *Salmonella enterica*

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The *Salmonella enterica smpB-nrdE* intergenic region contains about 45 kb of DNA that is not present in *Escherichia coli*. This DNA region was not introduced by a single horizontal transfer event, but was generated by multiple insertions and/or deletions that gave rise to a mosaic structure in this area of the chromosome.

Since diverging from a common ancestor some 100 to 160 million years ago, the gene order on the chromosomes of the genera *Salmonella* and *Escherichia* has retained a high degree of conservation (12, 28). The relatedness of their genomes provides a unique opportunity to identify evolutionary changes that occurred after the lineages of the genera *Salmonella* and *Escherichia* split, and analysis of these alterations is likely to provide us with some interesting insights into the genomic archaeology of enteric pathogens.

Comparison of the colinear genetic maps of *Escherichia coli* K-12 and *Salmonella enterica* serotype Typhimurium (*S. typhimurium*) revealed 30 so-called loops, genomic regions larger than 0.6 map units (about 28 kb), which are restricted to only one of the two species (31, 32). It was concluded from this work that these differences in genome structure resulted either from acquisition of genetic material by phage- or plasmid-mediated horizontal transfer or from loss of DNA regions by deletion. The largest *S. typhimurium*-specific loop identified by Riley and coworkers is located at 60 to 61 centisomes in the intergenic region between *smpB* (6) and *nrdE* (18). The *smpB-nrdE* intergenic region of *S. typhimurium* contains an area of approximately 45 kb which is not present at the corresponding map position of the *E. coli* chromosome (Fig. 1) (10, 32, 33). Since the sequence for the entire 45-kb DNA region has not been determined, it is unclear whether this *S. typhimurium*-specific loop is contiguous or rather consists of noncontiguous insertions. It can be estimated from the size of the *S. typhimurium* loop in the *smpB-nrdE* intergenic region (roughly 1% of the genome) that it has the capacity to contain about 40 genes, some or all of which may determine properties that distinguish this organism from *E. coli*. To date, three phenotypic characteristics encoded by genes located in the *smpB-nrdE* intergenic region have been described in *Salmonella* serotypes: (i) expression of phase 2 (H2) flagellin, the structural subunit of which is encoded by *fljB* (16, 42); (ii) uptake of tricarboxylates via a periplasmic binding protein-dependent transport system encoded by the *tctCBA* operon (40, 41); and (iii) utilization of catecholate-type siderophores via IroN, an outer membrane receptor encoded by a gene located in the *iroA* locus (7, 8). In addition to the essential role of the *fljB* gene product for serotyping (21), some of the genes located in the *smpB-nrdE* intergenic region have proven to be valuable tools for PCR detection of *Salmonella* serotypes (5, 13, 39). Thus, tracing the evolutionary origin of the *smpB-nrdE* intergenic region is of interest for both the comprehension of bacterial speciation and the development and evaluation of methods to differentiate *Salmonella* serotypes from closely related organisms.

In order to investigate the ancestry of genetic material within the *smpB-nrdE* intergenic region, we determined its distribution among *Salmonella* serotypes by using a collection of 20 strains described by Reeves and coworkers (30). This collection represents the genetic diversity within the genus *Salmonella* and contains isolates of all phylogenetic lineages, including *Salmonella bongori* and *S. enterica* subspp. I, II, IIIa, IIIb, IV, and VI. The phylogenetic relatedness of these isolates has been investigated by multilocus enzyme electrophoresis (Fig. 2) (30). This information is useful for identification of the branch of the phylogenetic tree at which genetic material may have been acquired or lost (3, 4). It should be mentioned, however, that based on data obtained by multilocus enzyme electrophoresis or comparative sequence analysis, other investigators have reported dendrograms which differ from the phylogenetic tree shown in Fig. 2, in that some *S. enterica* subspecies are connected through different branch points (11). Thus, this fact should be considered when interpreting DNA hybridization data with only a single dendrogram.

Chromosomal DNA of *Salmonella* serotypes and of *E. coli* K-12 strain DH5α (14) was prepared as described previously (2) and restricted with *Eco*RI, and the fragments were separated on a 0.5% agarose gel. The DNA was then transferred onto a nylon membrane, and Southern hybridization was performed under nonstringent conditions as reported earlier (4). The overlapping inserts of the cosmids pTY908 and pTY2117 represent a DNA region of about 30 kb surrounding the *iroA* locus of *S. enterica* serotype Typhi (*S. typhi*) (8). Subclones of these cosmids (pTY972, pTY978, pTY911, pTY1, pTY930, and pTY9) have been reported previously (8) and were used to generate DNA probes specific for different areas of the *smpB-nrdE* intergenic region (Fig. 2). In addition, an internal frag-

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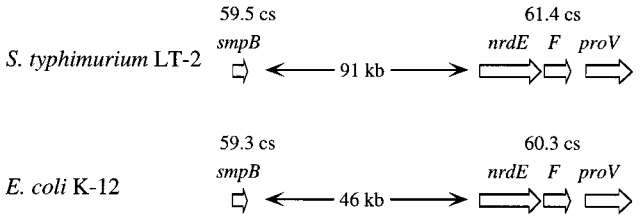

FIG. 1. Comparison of the *smpB-nrdE* intergenic regions of *E. coli* and *S. typhimurium*. According to the current physical map of *S. typhimurium* (upper half), the genes *smpB* and *nrdE* are separated by approximately 91 kb (33). In contrast, the distance between *smpB* and *nrdE* amounts to only about 46 kb in *E. coli* (lower half) (10). Genes are indicated by arrows. Map positions in centisomes (cs) of genes in *E. coli* and *S. typhimurium* are given above.

ment of the *S. typhimurium fljB* gene was PCR amplified with the primers 5′-GACTCCATCCAGGCTGAAATCAC-3′ and 5′-CGGCTTTGCTGGCATTGTAG-3′, the product was cloned into the vector pCR II (TA cloning kit; Invitrogen), and the resulting plasmid (pTY981) was used to generate a DNA probe specific for the H2 flagellar structural gene.

The results of Southern blot analysis are shown in Fig. 2 and are discussed below for each probe, reading from right to left. The probe generated from plasmid pTY9 hybridized with DNA from *E. coli* and all *Salmonella* serotypes tested. The nucleotide sequence determined for the insert of plasmid pTY9 showed 99% identity to 615 bp located upstream of the *S. typhimurium nrdE* gene (18), a region of the chromosome which is also present in *E. coli*. In contrast, a DNA region located about 4 kb upstream of the *nrdE* gene (pTY930) hybridized only with DNA from *S. typhi*, *S. bongori*, and one serotype of *S. enterica* subsp. IV. Nucleotide sequences were determined from the ends of the inserts of plasmids pTY930 and pTY941 (GenBank accession no. AF046859). Plasmid pTY941 contains DNA originating from an area located between the inserts of pTY1 and pTY930 (Fig. 2) (8). These nucleotide sequences showed homology to several genes located in the tail synthesis region of bacteriophage P2 and related phages, including *FI* (36), *FII* (36), *D* (19), *pinB* (37), and *ogr* (9) (Fig. 2). Since the region of the *S. typhi* chromosome which displayed homology to phage DNA was much smaller than the P2 genome (32 kb), it can be speculated that it resembles a defective prophage. It is not clear from hybridization analysis whether signals obtained from *S. bongori* or *S. enterica* subsp. IV serotypes resemble this defective prophage or originate from a different but closely related bacteriophage. It should be mentioned in this context that an attachment site (*atdA*) for bacteriophage P14 has been mapped close to *nrdE* in serotypes of *S. enterica* subsp. I belonging to serogroup C (*S. typhi* represents serogroup D) (33). The presence of a defective prophage in *S. typhi* and the map position of *atdA* in other *Salmonella* serotypes suggest that some of the genes located in the *smpB-nrdE* intergenic region may have been obtained via phage-mediated horizontal transfer.

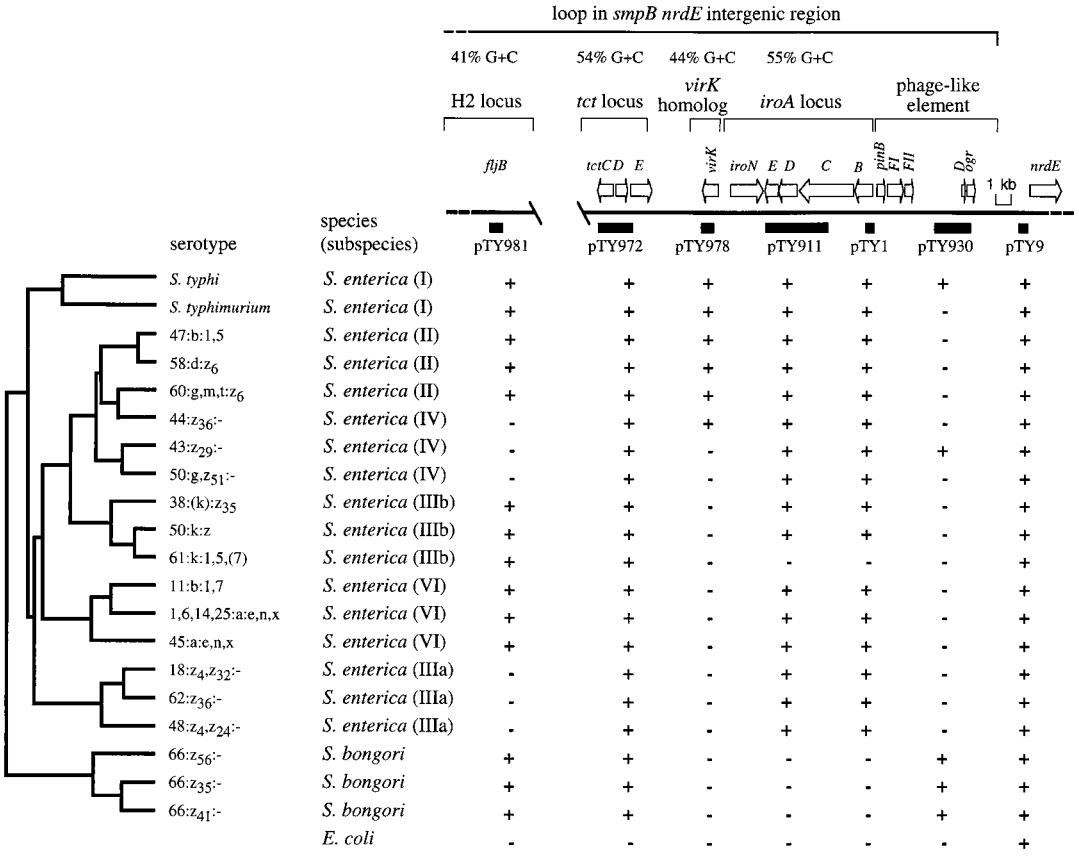

FIG. 2. Phylogenetic distribution of genes located in the *smpB-nrdE* intergenic region. The phylogenetic tree on the left was established by Reeves and coworkers (30). The top of the figure shows a map of the region from *S. typhi* AJB70. The positions of subcloned DNA fragments used as probes (black bars) and names of the corresponding plasmids (pTY9, pTY930, pTY1, pTY911, pTY978, and pTY972) have been reported previously (8). Results of hybridization with these DNA probes are shown below. +, hybridization signal; −, no hybridization signal. The positions and orientations of genes (*tctCDE*, *iroBCDEN*, and *nrdE*) or open reading frames with homology to genes described for other organisms (*virK*, *pinB*, *FI*, *FII*, *D*, and *ogr*) are indicated by arrows above the map.

Each of the *S. typhi* DNA probes generated from plasmids pTY981, pTY978, pTY911, and pTY1 hybridized with chromosomal DNA from *S. typhimurium* but not from *E. coli*, suggesting that the respective DNA fragments originate from the *S. typhimurium*-specific loop described by Riley and Krawiec (32). However, various areas of this loop differed with respect to their phylogenetic distribution within the genus *Salmonella*. Thus, this area of the chromosome may have suffered several insertions and/or deletions during divergence of *Salmonella* serotypes from a common ancestor (Fig. 2). In a previous study, the insert of plasmid pTY1 was shown to be present in all *S. enterica* strains tested (5). Using the strain collection shown in Fig. 2, plasmid pTY1 hybridized with all isolates of *S. enterica*, except serotype 61:k:1,5,[7], and this distribution was identical to that recently reported for pTY911 (7). These data imply that the entire *iroA* locus was likely acquired in a single horizontal transfer event soon after *S. enterica* branched from the *S. bongori* lineage (5). However, alternate scenarios that could explain the phylogenetic distribution of the *iroA* locus (e.g., deletion from *S. bongori* and *E. coli*) can currently not be ruled out. Subsequent loss of this DNA region was infrequent and was only detected in *S. enterica* subsp. IIIb serotype 61:k:1,5,[7]. The G+C content of the 10,837-bp DNA region containing *iroBCDE* and *iroN* is 55% (7), close to the overall G+C content of the *S. enterica* genome, which averages 52%. In contrast, the G+C content of the insert of plasmid pTY978, a DNA region located 1 kb upstream of *iroN*, averaged only 44% (GenBank accession no. AF029845) (Fig. 2). Previous studies have shown that only 4 of 87 regions sequenced from *Salmonella* serotypes have G+C contents lower than 45%, and this has been taken as evidence for their acquisition by lateral transfer (15, 35, 38). Since, during evolution, bacteria developed characteristic C+C contents, the atypical base composition of the insert of plasmid pTY978 supports the idea that this DNA region may have been obtained via horizontal transfer (1). Sequence analysis of pTY978 revealed an open reading frame whose deduced amino acid sequence showed 46% identity to VirK of *Shigella flexneri* (25). In *S. flexneri*, the *virK* gene is located on a mobile genetic element, namely the large virulence plasmid, which hints at a possible plasmid-mediated transfer of the *virK* homolog into the *S. enterica* genome. To assess the role of this gene in virulence, a *Sma*I-restricted kanamycin resistance cassette (KIXX; Pharmacia) was introduced into the *Stu*I site located within the open reading frame of the *S. typhi virK* homolog on plasmid pTY953 (8). The insert of the resulting plasmid (pTY1002) was restricted with *Xba*I-*Kpn*I and cloned into suicide vector pEP185.2 (20), and the resulting construct (pTY1003) was conjugated into *S. typhimurium* IR715. An exconjugant which was resistant to kanamycin but sensitive to chloramphenicol (the resistance encoded on pEP185.2) was designated AJB61. Insertion of KIXX into the *virK* homolog in strain AJB61 was confirmed by Southern blot analysis with a DNA probe derived from plasmid pTY978 (data not shown). The *virK* homolog was not required for *S. typhimurium* virulence, because identical oral 50% lethal doses (29) in 6-week-old BALB/c mice were determined for AJB61 and its parent, IR715. A DNA probe specific for the *virK* homolog (pTY978) hybridized only with isolates of *S. enterica* subsp. I, II, and IV. Thus, the phylogenetic distribution and the different G+C contents determined for the *iroA* locus and the *virK* homolog suggest that both DNA regions have different ancestries.

Sequence analysis (GenBank accession no. AF029846) revealed that the insert of pTY972 contained part of the *S. typhi tct* locus. A DNA probe derived from plasmid pTY972 hybridized with DNA from all *Salmonella* serotypes tested (Fig. 2), suggesting that the *tct* locus was obtained by a lineage ancestral to the genus *Salmonella*. Alternatively, the *tct* locus may have been lost by deletion from *E. coli* after its lineage split from that of the genus *Salmonella*. The G+C content of 54% determined for the *S. typhi tctDE* genes resembles the average *S. enterica* base composition. In contrast, the *S. typhimurium hin-fljB* region has a G+C content of only 41%, which is indicative of its acquisition by horizontal gene transfer (42). The *fljB*-specific DNA probe (pTY981) produced yet another hybridization pattern, because it was present in diphasic (expressing flagellar phase H1 and H2) but absent from monophasic (only expressing phase H1) serotypes of *S. enterica* (Fig. 2). Interestingly, a signal was also obtained with *S. bongori*, a species which contains only monophasic serotypes. Lack of phase H2 flagellar expression in *S. bongori* may thus be caused by mechanisms that escape detection by hybridization analysis, such as point mutations or small deletions in *fljB*. To explain the complex patterns of hybridization obtained with the *fljB*-specific DNA probe, several mechanisms should be considered. These include deletion events, acquisition of genetic material from other bacterial species by way of horizontal transfer, and/or recombination of horizontally transferred DNA regions among *S. enterica* subspecies, as proposed previously for the *fliC* locus (22).

It has been proposed recently that *Salmonella* pathogenicity islands 1 (SPI 1) and 2 (SPI 2), two large DNA regions that are present in *S. enterica* but absent from *E. coli*, were obtained through lateral transfer (17, 23, 24, 26). In *S. typhimurium*, SPI 1 is a 40-kb DNA region which is inserted between *fhlA* and *mutS*, two genes that are adjacent on the *E. coli* chromosome (24). Results from comparative sequence analysis of seven *inv/spa* genes located on SPI 1 which encode an invasion-associated type III excretion system support the idea that the entire pathogenicity island was initially acquired through horizontal transfer by a lineage ancestral to the genus *Salmonella* and subsequently transferred laterally from *S. enterica* subsp. IV to subsp. VII (11, 23). SPI 2 carries genes encoding a second type III excretion system which is required for growth in cells of the reticuloendothelial system (27, 34). Hybridization analysis revealed that the *ssa/spi* genes which encode this type III export apparatus are present in all subspecies of *S. enterica* but are absent from *S. bongori* and *E. coli* (17, 26). Evidence for acquisition of SPI 2 by horizontal transfer comes from the atypical G+C content (41%) of genes encoding the type III excretion system and the insertion of this pathogenicity island adjacent to the $tRNA^{Val}$ gene, a DNA region which may facilitate integration of newly acquired genetic material (17). Thus, in the case of SPI 1 and SPI 2, large DNA regions, each containing close to 30 genes that are related functionally, were apparently acquired through a single horizontal transfer event. In contrast to the rather homogeneous composition of pathogenicity islands, hybridization analysis of the *smpB-nrdE* intergenic region revealed that this area is composed of several DNA regions which differ with regard to their phylogenetic distribution and function (Fig. 2). These DNA regions may have been acquired independently from distinct sources, as suggested by differences in their G+C content. The *S. typhi smpB-nrdE* intergenic region is therefore apparently a mosaic of at least five DNA regions, each with a different ancestry: the H2 locus, the *tct* locus, a region encoding a *virK* homolog, the *iroA* locus, and a phage-like element. This information is relevant for evaluation of the specificity of PCR detection assays targeting *hin* (39), *tctC* (13), or *iroB* (5).

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