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Ferric Dicitrate Transport System (Fec) of *Shigella flexneri* 2a YSH6000 Is Encoded on a Novel Pathogenicity Island Carrying Multiple Antibiotic Resistance Genes

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Iron uptake systems which are critical for bacterial survival and which may play important roles in bacterial virulence are often carried on mobile elements, such as plasmids and pathogenicity islands (PAIs). In the present study, we identified and characterized a ferric dicitrate uptake system (Fec) in *Shigella flexneri* serotype 2a that is encoded by a novel PAI termed the *Shigella* resistance locus (SRL) PAI. The *fec* genes are transcribed in *S. flexneri*, and complementation of a *fec* deletion in *Escherichia coli* demonstrated that they are functional. However, insertional inactivation of *fecI*, leading to a loss in *fec* gene expression, did not impair the growth of the parent strain of *S. flexneri* in iron-limited culture media, suggesting that *S. flexneri* carries additional iron uptake systems capable of compensating for the loss of Fec-mediated iron uptake. DNA sequence analysis showed that the *fec* genes are linked to a cluster of multiple antibiotic resistance determinants, designated the SRL, on the chromosome of *S. flexneri* 2a. Both the SRL and *fec* loci are carried on the 66,257-bp SRL PAI, which has integrated into the *serX* tRNA gene and which carries at least 22 prophage-related open reading frames, including one for a P4-like integrase. This is the first example of a PAI that carries genes encoding antibiotic resistance and the first report of a ferric dicitrate uptake system in *Shigella*.

Pathogenicity islands (PAIs) are increasingly recognized as playing a vital role in bacterial virulence. PAIs are distinct virulence cassettes that often integrate into tRNA genes and encode bacteriophage-like integrases. Such islands may occupy large regions of the chromosome and often carry mobile elements, such as insertion sequences and transposons (25). PAIs have been found in many bacterial species, including *Yersinia* spp. (9, 13), enteropathogenic, enterohemorrhagic, and uropathogenic *Escherichia coli* (24, 38, 51), *Salmonella enterica* serovar Typhimurium (61), *Vibrio cholerae* (32), *Helicobacter pylori* (14), and *Shigella flexneri* (2, 42, 57, 71). Some strains of uropathogenic *E. coli* and *S. enterica* serovar Typhimurium may harbor at least five PAIs (19, 74). A variety of virulence determinants may be carried on PAIs, including genes encoding fimbriae, hemolysins (31, 64), type III secretion systems (15, 27), and iron uptake systems (13, 42, 71, 75). Various *Shigella* spp. produce the siderophores enterobactin and/or aerobactin, which are involved in iron uptake (34, 50). The aerobactin locus in *S. flexneri* was recently shown to be carried on the SHI-2 PAI (42, 71). This was the first report of an iron transport system being carried on a PAI in *Shigella*.

A number of PAI-like elements in *Shigella* spp. have been described. These include the SHI-2 PAI and a family of structurally related elements (42, 71) and the *she* PAI, which also belongs to a larger family of structurally related elements (2, 57). One of the characteristics of PAIs is their tendency to excise spontaneously from their sites of integration in the chromosome (26). In the *S. flexneri* 2a strain YSH6000, the spontaneous loss of multiple antibiotic resistance is accompanied by the deletion of an approximately 99-kb chromosomal region (56). The deletion of this region also coincides with a 50% decrease in contact hemolysis, a trait that correlates closely with virulence in *Shigella* spp. (56). These findings suggested that the 99-kb region is a deletable genomic element that carries multiple antibiotic resistance determinants. Preliminary sequence analysis of the 99-kb deletable element, which we have termed the multiple resistance deletable element (MRDE), demonstrated that the four antibiotic resistance determinants associated with the element are clustered within a 16-kb region (54) which we have termed the *Shigella* resistance locus (SRL). We recently found that the loss of multiple antibiotic resistance also occurs via a second type of spontaneous deletion event involving a distinct 66-kb element contained within the 99-kb MRDE (66). In the present study, we demonstrate that the 66-kb element is a PAI, termed the SRL PAI, that encodes a functional ferric dicitrate uptake system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown routinely with aeration at 37°C in either 2YT broth (40) or Luria-Bertani broth (LB) (5) with the addition of ampicillin (100 μg/ml), kanamycin (50 μg/ml), or tetracycline (12.5 μg/ml) when necessary.

Molecular techniques. Plasmid DNA was isolated using a modified alkaline lysis method (35), while genomic DNA was isolated as described previously (5). Restriction digests were carried out using enzymes supplied by Roche Molecular Biochemicals or New England Biolabs. Transformation of *E. coli* and *S. flexneri* strains was performed following electroporation (63) with a Bio-Rad gene pulser at 1.8 kV, 25 μF, and 200 Ω in 0.1-cm electroporation cuvettes.

RNA was extracted from *E. coli* and *S. flexneri* strains for expression analysis

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
S. flexneri		
YSH6000	Serotype 2a, wild-type Japanese isolate; Str^r Ap^r Cm^r Tet^r	58
YSH6000T	Derivative of YSH6000; MRDE deletant; Str^s Ap^s Cm^s Tet^s	44
SBA1366	Derivative of YSH6000; SRL deletant; Str^s Ap^s Cm^s Tet^s	66
SBA1415	*fecI*::*kan* allelic exchange into SBA1366	This work
E. coli		
DH5α	F^- ϕ80d *lacZ*Δ M15Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17 deo*^{r} thi-1 *supE441 gyrA96 relA1*	Bethesda Research Laboratories
AA93	Z1418 (F^- *araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi aroB*) Δ*fec*	49
SBA844	SBA845 with pSBA491	This work
SBA845	Z1418 Δ*fec* with pWSK129	This work
Plasmids		
pWSK29	pSC101-based low-copy-number vector; Ap^r Δ*lacZ*	73
pWSK129	pSC101-based low-copy-number vector; Kn^r Δ*lacZ*	73
pCACTUS	pSC101-based low-copy-number suicide vector with *sacB*, Cm^r	67
pUC4-KIXX	Source of Km^r from Tn*5*, Ap^r, Km^r	6
pSBA361	27.6-kb *Bam*HI fragment generated by marker rescue from YSH6000 cloned into pWSK29; Tet^r Ap^r	This work
pSBA491	8.9-kb *Hin*dIII fragment containing *fecIRABCDE* subcloned from pSBA361 into pWSK 129; Kn^r	This work
pSBA509	24-kb *Bam*HI fragment generated by marker rescue from YSH6000 cloned into pWSK129; Ap^r, Kn^r	This work

of the *fec* locus. Inocula were prepared by growing bacteria overnight in LB supplemented with antibiotics where necessary. Following centrifugation at 10,000 × *g* for 1 min, cells were washed in 1 ml of Fec medium (49) modified by the addition of 2′,2-dipyridyl (0.4 mM) and citrate (1 mM). Fifty milliliters of modified Fec medium was inoculated with 100 μl of each bacterial suspension and incubated with aeration until early exponential phase (4 h). Cells were centrifuged at 1,300 × *g* for 10 min, and the supernatant was discarded. RNA was extracted as described previously (62) and treated with DNase (Roche) to remove DNA contamination. RNA dot blots were probed with either a *fecA* DNA fragment or a *recA* fragment that served as a control for sample loading. Probes were derived by PCR amplification (*fecA*, 5′-GTTGTCGTCATAAGAGCGG-3′ and 5′-GCTCCCATTTCGCTCGGC-3′; *recA*, 5′-CTACGCACGTAAACTGG GCG-3′ and 5′-ACCGGTAGTGGTTTCCGGG-3′) and labeled with digoxigenin (Roche) as recommended by the manufacturer. The RNA concentration of matched strain pairs SBA844-AA93, YSH6000-YSH6000T, and SBA1366-SBA1415 was standardized by absorbance at 260 nm. The equal loading of RNA on membranes was confirmed by dot blot analysis with the *recA*-derived probe.

PCR and sequencing. Standard PCR, single strand-specific PCR (sspPCR), and inverse PCR were performed as described previously (47; *PCR Applications Manual*, 2nd ed., Roche Molecular Biochemicals; J. Novak and L. Novak, Promega Notes Magazine **61:**26–29, 1997). Long-range PCR was carried out with the Expand long-range PCR kit (Roche).

Nucleotide sequencing of the PAI in *S. flexneri* strain YSH6000 was carried out by sequencing genomic clones, inverse PCR products, sspPCR products, and a long-range PCR product. Sequence reactions were conducted using the BigDye system (PE Biosystems Inc.). Reaction products were analyzed on an Applied Biosystems model 373A DNA sequencing system. Sequence editing was carried out using Sequencher 3.0 for Macintosh. Sequence analysis and database comparisons were performed using BlastN and BlastX (3). Analysis of proteins was carried out using previously described web-based analysis tools (28, 29, 43, 46, 60).

Assays of growth under iron-limited conditions. Modified Fec medium (49) for growth under iron-limited conditions consisted of LB containing 0.4 mM 2′,2-dipyridyl, 1 mM citrate, and kanamycin when required. Inocula were prepared by growing strains overnight in 2.5 ml of LB or LB containing kanamycin for the maintenance of plasmids. To remove exogenous iron, bacteria were centrifuged at 10,000 × *g* for 1 min and resuspended in modified Fec medium. Following a second wash in modified Fec medium, bacterial suspensions were standardized by absorbance at 600 nm. Fifty milliliters of the modified Fec medium was inoculated with 0.1 ml of the standardized bacterial suspension. Aerated cultures were incubated at 37°C. Two-milliliter samples were taken over a 24-h period (2, 4, 6, 8, 12, and 24 h), and the absorbance at 600 nm was measured. Four cultures of each strain were grown simultaneously. Viable counts were also performed at 0 and 24 h to compare with absorbance readings.

Nucleotide sequence accession number. Nucleotide sequences have been deposited in GenBank under the accession number AF326777.

RESULTS

Identification and functional analysis of the *fec* iron transport locus in *S. flexneri* 2a. Sequencing of the regions surrounding the SRL using marker-rescued clones (described below) revealed a locus that was homologous to the ferric dicitrate transport (*fec*) genes located at min 97.3 of the *E. coli* K-12 genome (68). Like the *E. coli fec* locus, the *Shigella* locus consists of two operons carrying the regulatory genes, *fecI* and *fecR*, and the downstream structural genes, *fecABCDE*. The *S. flexneri fec* genes showed more than 99% nucleotide identity with the *E. coli* K-12 genes, but there were differences in the regions flanking the locus. The *E. coli* K-12 locus is flanked upstream by IS*1* and downstream by an IS*911* element that is insertionally disrupted by an IS*30* and a truncated IS*2*. In contrast, the *S. flexneri fec* locus was flanked downstream by an intact IS*911*. The sequence directly downstream of IS*911* is identical in *E. coli* and *S. flexneri*. Upstream of the *S. flexneri fec* locus were the first 61 bp of IS*1* followed by remnants of IS*3*, IS*629*, and IS*903*-like elements. Prior to this report, the *fec* locus had been identified only in *E. coli* strains (36, 53, 65, 72), although *fecA*, *fecD*, and *fecE* homologs had been observed in *H. pylori* (69). This is the first report of an intact ferric dicitrate transport system in a *Shigella* sp.

The ferric dicitrate iron transport system has been well characterized in *E. coli* K-12 (17). It is capable of maintaining the growth of *E. coli* under iron-limited conditions in the absence of other iron uptake systems. Because of the high similarity between the *S. flexneri* and *E. coli fec* loci, the function of the SRL PAI *fec* locus was tested in an *E. coli* Δ*fec* strain, AA93

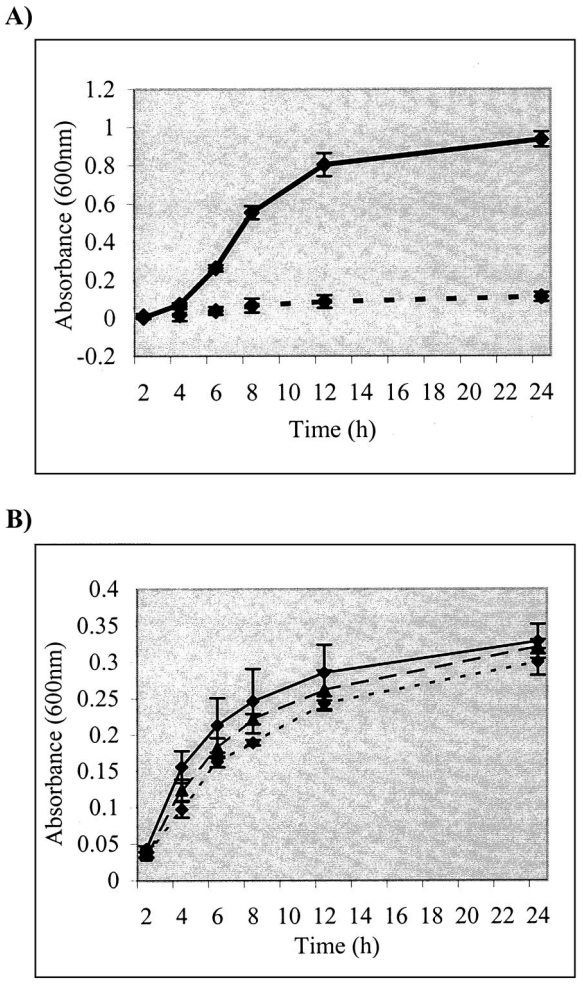

FIG. 1. Growth of *E. coli* (A) and *S. flexneri* (B) under iron-limiting conditions in medium supplemented with citrate. (A) SBA844 (solid line) is a Δ*fec* strain complemented with the *S. flexneri fec* locus, and SBA845 (broken line) is the Δ*fec* strain carrying an empty vector. (B) *S. flexneri* strains used were the wild-type SBA1366 (solid line), the *fecI*::*kan* strain SBA1415 (short dashes), and the MRDE deletant strain YSH6000T (long dashes). Error bars represent 2 standard deviations.

(49). In LB alone, all strains grew equally well (data not shown). However, the growth rate of AA93 under iron-limited conditions (LB supplemented with 0.4 mM 2,2′-dipyridyl and 1 mM citrate) was greatly reduced (Fig. 1A). Complementation of AA93 with the cloned *S. flexneri fec* locus (pSBA491), but not the cloning vector alone, restored its ability to grow under iron-limited conditions (Fig. 1A), demonstrating that the *S. flexneri fec* locus is functional.

Although the *fec* genes are functional in *E. coli*, it was necessary to establish their ability to function in *S. flexneri*. FecI is a member of the sigma 70 factor subclass that responds to extracytoplasmic stimuli and regulates extracytoplasmic functions (37). In *E. coli*, the *fecI* gene is essential for the transcription of the *fecABCDE* operon (48). Therefore, to study the function of the *fec* locus in *S. flexneri*, we inactivated the *fecI* gene by inserting the *kan* cassette from pUC4-KIXX into the unique *Xho*I site within *fecI*. To introduce the *fecI* mutation into the *S. flexneri* chromosome, a 5.1-kb PCR product containing *fecI*::*kan*, *fecR*, and the 5′ end of *fecA* was cloned into the suicide vector pCACTUS and subsequently introduced by electroporation into SBA1366 (YSH6000, SRL$^-$). A double-crossover mutant, SBA1415, was selected by growth at 42°C in the presence of sucrose. The mutation was confirmed by PCR using primers within, and external to, the pCACTUS construct (data not shown).

To test whether the Fec iron transport system had a role in the growth of *S. flexneri* 2a YSH6000 under iron-limited conditions, the *fecI*::*kan* mutant was compared to its parent (SBA1366) and strain YSH6000T, which has undergone a spontaneous excision of the 99-kb element. When strains were cultured under iron-limited conditions in medium supplemented with citrate, there was no significant difference in growth rate between any of the strains (Fig. 1B); neither mutation nor loss of the *fec* locus had any effect on the growth of *S. flexneri* 2a strain YSH6000.

There were several possible explanations for the lack of phenotypic difference between the wild-type strain and the *fecI* mutant, including the possibility that the *fec* locus is not transcribed in *S. flexneri* or that *fecI* may not be essential for transcription of the *fec* structural genes in *S. flexneri*. Both of these hypotheses were tested by RNA dot blot analysis of the expression of *fec* in YSH6000, YSH6000T, AA93, SBA844, SBA1366, and SBA1415 cells grown under iron-limited conditions (see Materials and Methods).

Although analysis with a *recA* probe confirmed that equal amounts of RNA were loaded for each pair on the dot blot, *fecA* mRNA was undetectable in the *S. flexneri fecI* mutant, SBA1415, but readily detectable in the isogenic parent strain, SBA1366, and in the wild-type strain YSH6000 (Fig. 2). As expected, *fecA* transcript was undetectable in the negative control strains YSH6000T and AA93, which do not carry the *fec* locus, but was detected in an AA93 strain complemented with the *S. flexneri fec* locus (SBA844). These results demonstrated that the *fec* locus is expressed in YSH6000 in a *fecI*-dependent manner and therefore suggest that this strain carries additional iron uptake systems that compensate for the *fec* mutation when grown in laboratory culture media.

Southern hybridization showed that the *fec* locus is present in a single copy in YSH6000 and absent in YSH6000T (data not shown). Therefore, if a second iron uptake system exists in YSH6000, it must belong to another class. Recently, aerobactin-mediated iron uptake systems were found on proposed

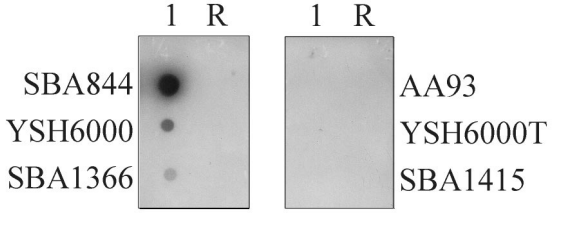

FIG. 2. Transcriptional analysis of *fecA* in *S. flexneri* and *E. coli*. An RNA dot blot compares matched strain pairs (*E. coli* SBA844-AA93 and *S. flexneri* YSH6000-YSH6000T and SBA1366-SBA1415) for transcription of *fecA* under iron-limited conditions. Lanes: 1, undiluted sample; R, samples treated with RNase to ensure that there was no DNase contamination. SBA844, YSH6000, and SBA1366 carry the *S. flexneri fec* locus, while AA93 and YSH6000T do not. SBA1415 carries the *fec* locus with a *kan* cassette inserted in *fecI*.

PAIs on the chromosome of several *S. flexneri* serotypes (42, 71). To test whether such a system existed in YSH6000, we examined this strain for the presence of *iucA*, the first gene in the aerobactin biosynthesis operon. A PCR product was amplified from strains YSH6000 and YSH6000T using primers designed from the SHI-2 PAI *iucA* region. The sequence of the PCR product from YSH6000T confirmed that an *iucA* gene identical to that from the SHI-2 PAI was present in YSH6000T. Therefore, it seems possible that an aerobactin locus and/or other types of iron uptake systems may have compensated for the loss of *fec* function in SBA1415.

The *fec* locus is present on a PAI. Iron transport systems have been identified on several PAIs, including the high-pathogenicity island (HPI) of *Yersinia* spp. and *E. coli* (10, 33, 59), *Salmonella* pathogenicity island 1 of *S. enterica* serovar Typhimurium (30), and PAI-VI$_{CFT073}$ in uropathogenic *E. coli*, which carries a putative iron transport system (23). Several of these systems, as well as the aerobactin iron transport system on the SHI-2 PAI of *S. flexneri* (42, 71), have roles in virulence. Based on the findings presented below, we demonstrated that the *S. flexneri fec* locus also is carried on a PAI that in addition carries the multiple antibiotic resistance genes of the SRL.

Southern hybridization analysis demonstrated that the *fec* locus is present in *S. flexneri* 2a YSH6000 but is absent in the spontaneous, antibiotic-sensitive derivative YSH6000T, suggesting that the *fec* locus and the SRL are physically linked. This was confirmed by DNA sequencing, which showed that the tetracycline and chloramphenicol resistance determinants characterized by Rajakumar et al. (57) are on the same *Bam*HI fragment as the *fec* locus (Fig. 3). We have found that the *fec* locus is present in a variety of *Shigella* strains. Thirty-five of 55 *Shigella* strains examined by high-stringency Southern analysis carried the *fecA* gene. Long-range PCR analysis of a single sample strain from each *Shigella* species with the same antibiotic resistance profile as strain YSH6000 confirmed that in each case the *fec* locus is linked to the tetracycline resistance gene, as it is in strain YSH6000 (data not shown). This suggests that the SRL PAI is present in all species of *Shigella*.

The antibiotic resistance determinants of strain YSH6000 delete spontaneously from the chromosome at a frequency of 10^{-5} to 10^{-6} (66), suggesting that the SRL, and therefore the closely linked *fec* locus, may be carried on a mobile element. To gain a better understanding of the element, the regions flanking the SRL were sequenced using plasmid clones obtained by marker rescue of the resistance determinants encoded by the SRL. *Bam*HI fragments of 27.6 kb, carried on pSBA361, and 24 kb, carried on pSBA509 (Fig. 3), were cloned by selection for tetracycline and ampicillin resistance, respectively. The remainder of the element was sequenced from DNA fragments derived by inverse PCR, sspPCR, and long-range PCR (Fig. 3).

Analysis of the DNA sequence showed that a large genetic element has inserted into the 3′ terminal region of the *serX* tRNA gene in YSH6000 (Fig. 4A). The element is bounded on the *serX*-distal side by a 14-bp direct repeat (DR) of the 3′-terminal 14-bp sequence of *serX* (Fig. 4A). The DNA sequences upstream of *serX* and downstream of the *serX*-distal DR are almost identical to sequences that are contiguous with the *serX* gene in *E. coli*, a species that is closely related to *S. flexneri*. Notably, the 3′ termini of tRNA genes commonly serve as integration sites for PAIs and prophages (25). In addition, the 3′ terminus of *serX* has sequence similarity to a P4 *att* site (Fig. 4B), implying that it may act as an integration site for prophage-like or PAI-like elements. The sequence of the genetic element revealed the presence of a P4 bacteriophage-like integrase gene 161 bp upstream of the 14-bp *serX*-distal DR. The integration of the element into the 3′ end of a tRNA gene, the presence of an integrase gene near one boundary of the element, and the recent finding that the element undergoes spontaneous, integrase-mediated, precise excision to restore the *serX* locus as it is organized in *E. coli* (66) led us to conclude that the element is a PAI, which we have termed the SRL PAI.

Interestingly, there was a significant discrepancy in the lengths of the 66.2-kb SRL PAI and the previously described 99-kb deletable element carrying multiple antibiotic resistance in YSH6000 (56). This discrepancy has been explained by the recent finding that the SRL PAI is entirely contained within a yet-larger distinct genetic element, termed the MRDE, which is flanked by IS*91*-like elements and is also capable of precise excision from the chromosome (66) (Fig. 5). This is similar to the *Y. pestis* 6/69 HPI, which is contained within a larger, deletable 102-kb chromosomal region and which also carries genes for hemin utilization. The boundaries of the 102-kb region are defined by two IS*100* elements which mediate the spontaneous deletion of the HPI and flanking chromosomal DNA in this strain (9).

Genetic organization of the SRL PAI. The SRL PAI is 66,257 bp in length (Fig. 3), beginning 161 bp upstream of the *int* gene and ending at the 3′ terminus of the intact *serX* gene. The SRL PAI contains 59 open reading frames (ORFs) (Table 2; Fig. 3), excluding the ORFs associated with insertion sequences, and has an average G+C content of 49.8 mol%. Although the overall G+C content is not significantly different from that of the *S. flexneri* chromosome (51 mol%), significant deviations occur in the regions homologous to Tn*2603*, Tn*10*, and the *fec* locus, which have G+C contents of 57, 39, and 58 mol%, respectively. This is consistent with the fact that Tn*2603* and Tn*10* are laterally acquired elements and suggests that the *fec* locus may also have been laterally acquired by the PAI relatively recently.

The deduced product of the *int* gene, located near the left boundary of the SRL PAI, has significant sequence similarity at the amino acid level to several integrase proteins from the P4 prophage Int family, including those from the CP4-57 cryptic prophage of *E. coli* (49% similar) and the *V. cholerae* PAI (52% similar). Integrases from the other *S. flexneri* PAIs, *she* PAI and SHI-2, showed 47 and 45% similarity to the SRL PAI Int, respectively. The putative Int protein encoded on the SRL PAI possesses a conserved motif (R, HXXR, Y) necessary for the function of P4-like integrases (1, 4), suggesting that the integrase may be functional.

Twelve ORFs on the SRL PAI have significant sequence similarity, ranging from 45 to 88%, to ORFs carried on the CP4-57 prophage (Fig. 3). Although the physical spacing of the SRL PAI ORFs differed from that of their homologues in CP4-57, their order and orientation are conserved, with the exception of *orf2* and *orf3* on the SRL PAI, which are inverted compared to their CP4-57 homologues, *yfjI* and *alpA*, respectively. A number of these SRL PAI ORFs, including *orf16*,

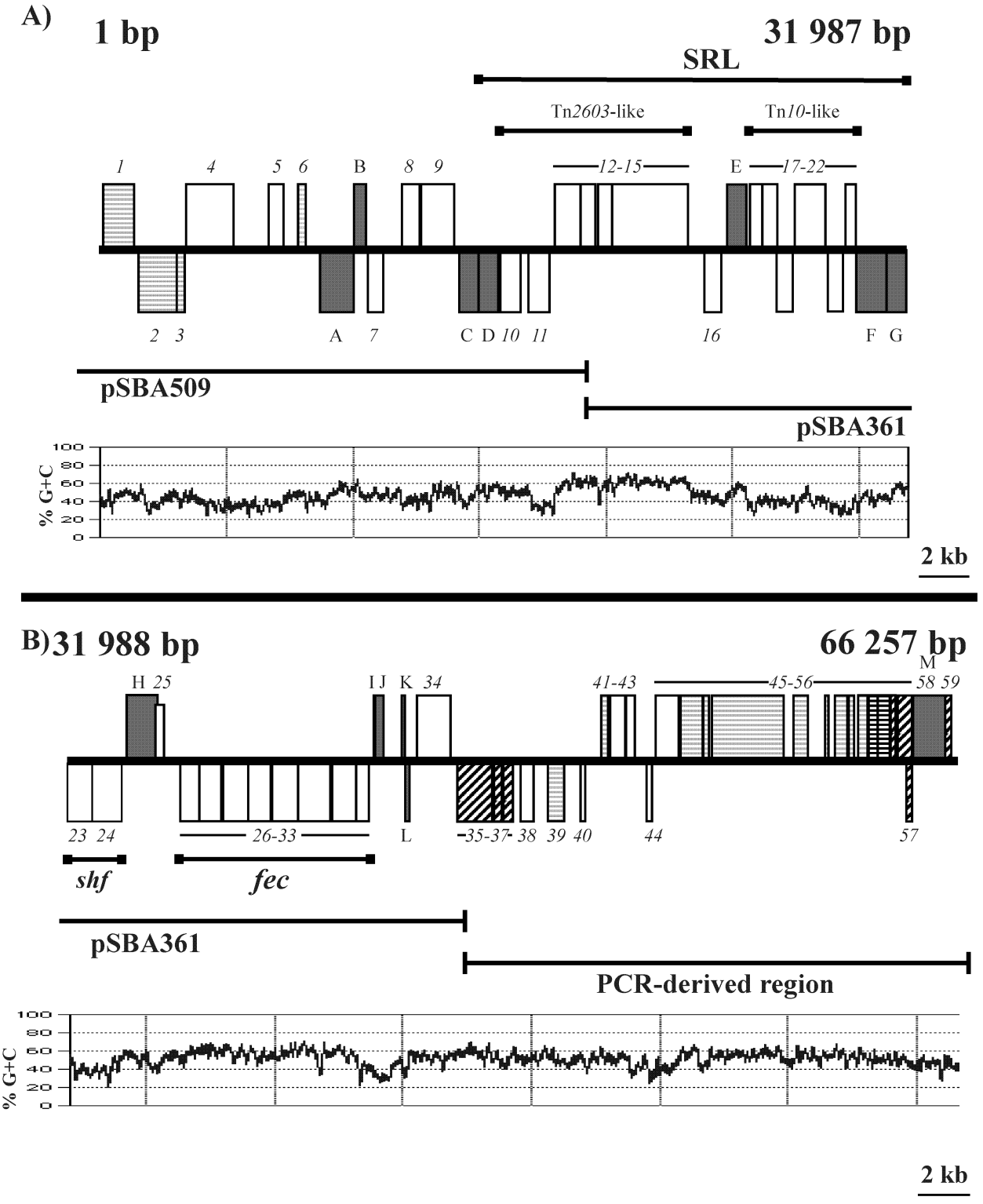

FIG. 3. Genetic organization of the SRL PAI. A map of the SRL PAI sequence from bp 1 to 31,987 (A) and 31,988 to 66,257 (B) is shown. The ORFs are represented by boxes above the line (forward orientation) or below the line (reverse orientation). The unlabeled open boxes represent ORFs of unknown function. IS elements (grey boxes) have been designated A through M (Table 3). Boxes with light grey horizontal lines represent the CP4 prophage-related ORFs, while boxes with diagonal black lines represent 933L prophage-related ORFs. Boxes with black horizontal lines represent ORFs that have homologs on both the 933L and CP4 prophages. Sequence data were derived from the genomic subclones pSBA509 and pSBA361 and PCR-derived products indicated below each map. The G+C content of the SRL PAI was plotted using a window size of 100 bp.

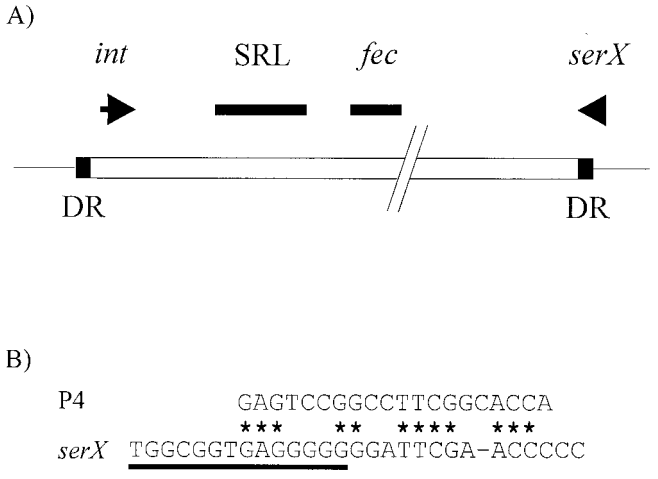

FIG. 4. Structure of the SRL PAI and the DR. (A) The SRL PAI is represented by the open box. The shaded boxes represent the 14-bp DR. The right-hand DR is the last 14 bp of *serX*. (B) The 14-bp DR and the potential *att* site. The lower sequence shows the 3′ end of the *S. flexneri* YSH6000 *serX* gene. The region representing the 14-bp DR is underlined. The upper sequence represents the P4 core *att* site. *, conserved nucleotide.

orf39, and *orf48*, are truncated in comparison to their CP4-57 homologues. Of the 12 ORFs homologous to CP4-57 ORFs, seven have homologues in CP4-44, another cryptic prophage in *E. coli* K-12 (8). Similarity between the SRL ORFs and ORFs on CP4-44 ranged from 87 to 99%. The SRL PAI also carries homologues of ORFs L0007 to L0015 from a third prophage, 933L, situated on the enterohemorrhagic *E. coli* (EHEC) locus of enterocyte effacement (LEE) PAI. Two of these ORFs, ORFs 53 and 54, are also common to CP4-44. Thus, a total of 22 SRL PAI ORFs, comprising just under 20% of the PAI sequence, appear to have a prophage origin. ORF 47 of the SRL PAI is homologous to autotransporter protein Antigen 43 (87% similarity), encoded by the cryptic prophage CP4-44, YpjA (37% similarity), encoded by a CP4-57 ORF of unknown function, and Sap (72% similarity), encoded by an ORF of unknown function on the *she* PAI of *S. flexneri* 2a.

The SRL PAI carries six intact insertion sequences and seven IS remnants that have undergone deletions (Table 3). IS elements and their remnants, including IS*1*, IS*200*, IS*600*, IS*629*, and IS*1328*, which are present on the SRL PAI, are commonly found in other PAIs (7, 21, 39, 71). The SRL PAI also carries an ORF, designated *shf*, that is almost identical to the previously described *shf* ORF carried on the virulence plasmids of *S. flexneri* serotype 2a strain YSH6000 (55) and serotype 5 (11). The *shf* ORFs have sequence characteristics that are common to some mobile elements, such as retroviral integrases and IS transposases. Close homologues of *shf* (>83% similarity at the protein level) are also found on the plasmids pAA2 of enteroaggregative and diffusely adhering *E. coli* (18) and pO157 of EHEC (12), as well as a chromosomal locus bearing two overlapping genes, *sat1* and *sat2*, in *S. enterica* serovar Typhimurium. In *Shigella* and *E. coli*, *shf* is part of a larger locus that includes a hexosyltransferase homologue, *capU*, an *msbB* homologue, and an IS*911* remnant (Fig. 6).

DISCUSSION

This study confirms our hypothesis that multiple antibiotic resistance in *S. flexneri* strain YSH6000 is encoded by a PAI. While our previous work suggested that such a PAI might be approximately 99 kb in length (56), we have shown that the SRL PAI is only 66.2 kb in length and is located on a distinct 99-kb element which, like the PAI, is capable of excision from the chromosome (66). Our conclusion that the antibiotic resistance determinants of the SRL are carried on a PAI is based on several characteristic sequence features and genetic properties common to other PAIs. These include a chromosomal insertion site in the 3′ terminus of a tRNA gene, the presence of short DRs at the boundaries of the element, the presence of a P4-like integrase gene near one end of the element, and the recent demonstration that the element undergoes precise, integrase-mediated excision from the chromosome (66). In addition, the SRL PAI typically contains a large number of IS elements and transposons. A striking feature of the PAI is the large number of ORFs that are related to the CP4 group of prophages and the 933L prophage, which is associated with the EHEC LEE PAI. Of particular interest is the almost complete conservation in the organization and orientation of homologous ORFs in the SRL PAI and CP4, suggesting very strongly that the SRL PAI shares a common ancestry with these prophages.

The SRL PAI represents the first example of a PAI that contains multiple antibiotic resistance genes. One other mobile genetic element that encodes antibiotic resistance, the SXT element of *V. cholerae*, has some features in common with the SRL PAI. The SXT element is capable of integrase-mediated, site-specific integration into and excision from the chromosome and carries all of the genes required for conjugative self-transfer to new hosts. However, although the SRL PAI undergoes site-specific, integrase-mediated excision from the chromosome, it does not appear to carry any of the genes required for conjugative transfer. Therefore, the SRL PAI appears to be quite a distinct type of genetic element. Whether the SRL PAI is capable of being transferred laterally to new hosts is unknown. However, the finding that the *fec* and SRL loci are linked in several tested strains from each of the four *Shigella* spp. suggests that the SRL PAI has disseminated throughout the genus *Shigella*.

In addition to antibiotic resistance, the SRL PAI encodes an iron uptake system. Iron is an essential nutrient in bacteria,

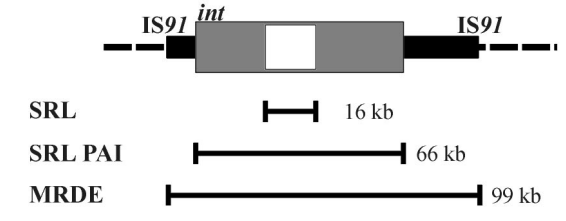

FIG. 5. Overview of the genetic organization of the *S. flexneri* 2a YSH6000 chromosome surrounding the SRL PAI. The three deletable elements in the region corresponding to min 23 of the *E. coli* chromosome are shown. The dashed line represents the chromosomal region identical to *E. coli*, while the solid line represents the 99-kb MRDE. The grey box denotes the SRL PAI, while the white box is the SRL. The MRDE is flanked by IS*91*-like sequences.

TABLE 2. ORFs contained within the SRL PAI

ORF	Gene	Position (bp)	Related protein[a]	% Similarity[b]	Protein accession no.
1	*int*	619–1836	Integrase CP4-57 (SlpA)	49	P32053 (U36840)
2	*orf2*	3535–2006	YfjI	45	P52124
3	*orf3*	3812–3507	AlpA (CP4-57)	66	P33997
4	*orf4*	3868–5730	No similarity		
5	*orf5*	7104–7697	No similarity		
6	*orf6*	8449–8745	YfjI (frameshift)	73	P52125
7	*orf7*	11582–10980	LysR-like transcriptional regulator	79	P39376
8	*orf8*	12300–12989	Hypothetical protein in LysR-AraE	63	P03813
9	*orf9*	13055–14353	DcuA (anaerobic decarboxylate transporter)	70	P04539
10	*aadA1*	16963–16172	AadA1 (streptomycin resistance)	95	P04826
11	*oxa-1*	17906–17076	Oxa-1 (ampicillin resistance)	99	P13661
12	*intI1*	18116–19129	IntI1	99	P09999
13	*tnpM*	19098–19682	TnpM	100	P04162
14	*tnpR*	19808–20368	TnpR	98	P04130
15	*tnpA*	20371–23337	TnpA	98	BAA78805.1
16	*cat*	24641–23982	Cat (chloramphenicol Resistance)	98	P00483
17	*orf17*	25759–26256	YdjB	79	BAA78832.1
18	*orf18*	26365–26949	JemC	79	AF162223
19	*tetR*	27553–26927	TetR	98	P04483
20	*tetA(B)*	27632–28837	TetA(B) (tetracycline resistance)	99	P02980
21	*tetC*	29543–28950	TetC	99	BAA78836
22	*tetD*	29631–30047	TetD	100	BAA78837
23	*orf23*	32594–31983	CapU (hexosyltransferase homolog)	99, 73	AAD34405, AB011549
24	*orf24*	33389–32622	Shf	93, 92.3	AF134403, U61977
25	*orf25*	34749–35096	Hypothetical ORF o137	98	S56511
26	*fecE*	36488–35721	FecE	100	P15031
27	*fecD*	37445–36492	FecD	100	P15029
28	*fecC*	38419–37442	FecC	100	P15030
29	*fecB*	39339–38437	FecB	98	P15028
30	*orf30*	40424–39384	FecA precursor	100	P13036
31	*fecA*	41707–40421	FecA	100	M63115
32	*fecR*	42747–41794	FecR	100	P23485
33	*fecI*	43265–42744	FecI	100	P23484
34	*orf34*	45173–46531	Putative periplasmic protein (*Campylobacter jejuni*)	52	AL139076
35	*orf35*	48155–46770	L0015	98	AAC31494
36	*orf36*	48552–48205	L0014	98	AAC31493
37	*orf37*	48950–48549	L0013	99	AAC31492
38	*orf38*	49805–49284	No similarity		
39	*orf39*	50940–50404	YfjJ	55	P52125
40	*orf40*	51855–51679	Hha/YmoA (*Yersinia enterocolitica*)	75, 75	P23870, P27720
41	*orf41*	52491–52769	Vis (P4)	53	—[c]
42	*orf42*	52858–53466	No similarity		
43	*orf43*	53637–53888	No similarity		
44	*orf44*	54534–54319	No similarity		
45	*orf45*	54667–55584	No similarity		
46	*orf46*	55669–56541	YfjP, YeeP	65, 97	P52131, P76359
47	*orf47*	56924–59761	Ag43 (Flu)	78	P39180
48	*orf48*	60145–60705	YfjQ	88	P52132
49	*orf49*	61390–61530	YfjX, KlcA	81, 51	P52139, P52603
50	*orf50*	61782–62273	YfjY, YeeS	80, 99	p52140, P76362
51	*orf51*	62336–62557	YeeT	98	P76363
52	*orf52*	62720–63094	YeeU, YfjZ	91, 75	P76364, P52141
53	*orf53*	63141–63515	L0007, YeeV, YpjJ	93, 87, 74	AAC31485.1, P76365, O46953
54	*orf54*	63512–64003	L0008, YeeW	96, 77	AAC31487.1, P76366
55	*orf55*	64015–64212	L0009	88	AAC31488.1
56	*orf56*	64297–64860	L0010	85	AAC31489.1
57	*orf57*	64878–64651	L0011	80	AAC31490.1
58	*orf58*	64929–65933	IS/328 transposase (*Y. enterocolitica*)	83	O56897
59	*orf59*	66204–66452	L0012	90	AAC31491.1

[a] Related proteins are from *E. coli* unless otherwise stated.
[b] At the amino acid level.
[c] —, data from reference 52

where it is a component of the electron transport system (45) and an essential cofactor for a variety of enzymes. While iron is readily available in the environment, in the human host it is stored in tissues, such as the liver, or chelated by extracellular proteins, such as transferrin and lactoferrin (22, 41). Intracellular pathogens, such as *Shigella*, can scavenge iron from within the cells they invade, but they must also obtain iron from the extracellular environment of the host. To achieve this they

TABLE 3. Insertion sequences on the SRL PAI

Fig. 3 label	Position(s)	IS element	Comment(s) (% nucleotide identity)
A	10421–9112	IS*629*	Intact (99)
B	10422–10905	IS*200*	First 118 bp truncated; transposase gene frameshift (98)
C	15323–14554	IS*600*	Deletion of bp 746–801; no intact ORFs (97)
D	16091–15324	IS*1*	Intact (99)
E	24865–25632	IS*1*	Intact (99)
F	31219–30060	IS*10*	Transposase truncated by 16 bp (99)
G	31987–31220	IS*1*	Intact (97)
H	33590–34827	IS*911*	Intact (92)
I	43448–43508	IS*1*	Truncated at bp 61
J	43507–43741 and 43749–43827	IS*3*	Internal deletion and truncation (87)
K	44562–44609 and 44621–44666	IS*903*-like	Remnant only; no intact ORFs (86)
L	44884–44718	IS*629*	Remnant only; last 163 bp (97)
M	64929–66203	IS*1328*	Truncated; transposase intact (83)

produce extracellular high-affinity, low-molecular-weight iron chelators, called siderophores (16, 45). *E. coli* and some *S. flexneri* and *Shigella boydii* strains produce the catechol siderophore enterobactin (50), while some *S. flexneri*, *S. boydii*, and *Shigella sonnei* strains also produce the dihydroxamate siderophore aerobactin (34). In the present study we have identified a third type of siderophore system, a ferric dicitrate system, in *S. flexneri* 2a. The *fec* system in YSH6000 is only the second example of iron uptake genes carried on a PAI in *Shigella*. However, iron uptake genes are also carried on PAIs in *S. enterica* serovar Typhimurium, *Yersinia* spp., and some pathogenic strains of *E. coli* (13, 33, 75).

Our work also describes the first example of a ferric dicitrate uptake system in the genus *Shigella*. Until now, this type of iron uptake system has been found only in the commensal strains *E. coli* B and *E. coli* K12, *E. coli* strains causing bovine mastitis (36), and EHEC O157:H7 strain EDL933 (65). Although it was demonstrated that the *fec* locus is functional and is expressed in *S. flexneri* strain YSH6000, we could not demonstrate any alteration in the growth rate of a *fecI* mutant strain grown in iron-limited culture media. This finding suggests that YSH6000 expresses additional iron uptake systems that are capable of compensating for the loss of Fec function. This is consistent with previous reports of siderophore production in *S. flexneri* and the presence of *iucA*, one of the genes involved in aerobactin synthesis, in strain YSH6000. Indeed the presence of multiple iron uptake systems in a single strain is not unusual. For example, *E. coli* strains may have up to five iron(III) transport systems (20). The possession of more than one iron uptake system may confer on bacteria a greater ability to survive in different niches outside or inside the host. Since the Fec system is expressed in nonpathogenic *E. coli* strains, which unlike *Shigella* spp. do not invade intestinal cells, its primary role in *S. flexneri* may be in the uptake of iron from the intestinal lumen, where exogenous citrate is available for the chelation of iron.

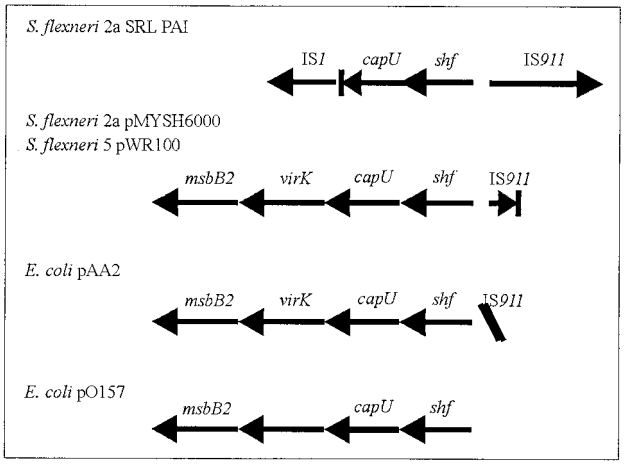

FIG. 6. Genetic organization of the *shf* locus in *S. flexneri* and *E. coli*, showing the extent of the conserved *shf* locus in several bacterial strains. pMYSH6000 is carried by YSH6000, the strain that contains the SRL PAI. pWR100 is borne by *S. flexneri* 5 strain M90T (70). pAA2 is from enteroaggregative *E. coli* strain O42 (18), and pO157 is carried by EHEC O157:H7 (12). *shf* and *capU* are conserved in all loci, although they are truncated in the *S. flexneri* chromosome due to an IS*1* insertion. An IS*911* is found upstream of three of the loci, although it is intact only on the *S. flexneri* SRL PAI chromosomal locus and is oriented in the reverse direction on pAA2 in enteroaggregative *E. coli*. In addition, the plasmid-borne loci exhibit a conserved organization with regard to *shf*, *capU*, *virK*, and *msbB2*, with pO157 having an alternative gene in place of *virK*.

In conclusion, our discovery of the SRL PAI in *S. flexneri* has revealed yet another type of genetic element that may be involved in the lateral transfer of antibiotic resistance. In addition, this element also encodes the first ferric dicitrate uptake system known to exist in *Shigella* spp. Future studies will address whether the SRL PAI is naturally mobilized to new bacterial hosts.

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