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Nested Deletions of the SRL Pathogenicity Island of *Shigella flexneri* 2a

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In this study, we determined the boundaries of a 99-kb deletable element of *Shigella flexneri* 2a strain YSH6000. The element, designated the multiple-antibiotic resistance deletable element (MRDE), had recently been found to contain a 66-kb pathogenicity island (PAI)-like element (designated the SRL PAI) which carries the *Shigella* resistance locus (SRL), encoding resistance determinants to streptomycin, ampicillin, chloramphenicol, and tetracycline. The YSH6000 MRDE was found to be flanked by two identical IS*91* elements present at the *S. flexneri* homologs of the *Escherichia coli* genes *putA* and *mdoA* on *Not*I fragment D. Sequence data from two YSH6000-derived MRDE deletants, YSH6000T and S2430, revealed that deletion of the MRDE occurred between the two flanking IS*91* elements, resulting in a single IS*91* element spanning the two original IS*91* loci. Selection for the loss of tetracycline resistance confirmed that the MRDE deletion occurred reproducibly from the same chromosomal site and also showed that the SRL PAI and the SRL itself were capable of independent deletion from the chromosome, thus revealing a unique set of nested deletions. The excision frequency of the SRL PAI was estimated to be 10^{-5} per cell in the wild type, and mutation of a P4-like integrase gene (*int*) at the left end of the SRL PAI revealed that *int* mediates precise deletion of the PAI.

Shigella spp., the causative agents of bacillary dysentery, are responsible for the deaths of more than 1.1 million people every year (28). Infections are transmitted via the fecal-oral route either as a result of person-to-person contact or through ingestion of contaminated food or water, and result in watery diarrhea which may progress to the bloody mucoid stools typical of bacillary dysentery (12). In developing countries individuals affected by acute diarrhea are commonly treated by oral rehydration and antimicrobial therapy. However, rehydration therapy alone provides little benefit to patients with dysentery caused by invasive enteropathogens such as *Shigella*, and the global importance of dysentery in developing countries has increased as a result of ineffective treatment (26). In addition, resistance to antibiotics such as tetracycline and ampicillin, which were once highly efficacious in treatment of shigellosis, has grown considerably in the past few decades (45). In many cases, resistance genes are found to reside on easily transferable R plasmids. However, chromosomally borne resistance genes have recently been identified in a number of studies (10, 15, 29, 41, 42), although the basis of chromosomal resistance has not been widely investigated.

Rajakumar et al. (42) have described a spontaneous 99-kb chromosomal deletion that results in multi-antibiotic susceptibility in *Shigella flexneri* 2a YSH6000. The resistance locus carried on the 99-kb element was found to encode resistance determinants to streptomycin, ampicillin, chloramphenicol, and tetracycline. These determinants have since been collectively designated the SRL, for *Shigella* resistance locus. The SRL exhibits similarity in sequence and organization to the antibiotic resistance loci of NR-1, an R plasmid commonly found in *Shigella*, and transposon Tn*2603* (41). Although the nature and exact location of the 99-kb multiple-antibiotic resistance deletable element (MRDE) harboring the SRL have not been determined, it has been mapped to a region of the chromosome on *Not*I fragment D bounded by the *S. flexneri* homologs of the *Escherichia coli* genes *ompA* and *pyrC* (42). The instability of the MRDE is reminiscent of the behavior of other large chromosomal regions in *E. coli* and *Yersinia pestis*, referred to as pathogenicity islands (PAIs) (7), suggesting that the MRDE may also be a PAI-like element.

The term "pathogenicity island" was first used to describe large unstable DNA regions in uropathogenic *E. coli* (UPEC) (7). However, the term is now used more generally to refer to sections of chromosome throughout a number of species that are often unstable and that frequently carry virulence genes (18). Since the introduction of the term PAI, islands have been identified in many species, including *E. coli*, *Yersinia* spp., *Helicobacter pylori*, *Salmonella* spp. and *S. flexneri* (20). In addition to virulence genes, PAIs also encode mobility elements such as integrases and insertion elements (IS elements) and commonly integrate into, or adjacent to, tRNA genes. These characteristics, which show remarkable similarity to those of bacteriophages, in conjunction with a G+C content that often differs from that of the host chromosome, have led to the suggestion that PAIs are acquired from different species via phage-mediated horizontal transfer (19). Indeed, it has recently been reported that the *Vibrio cholerae* PAI (VPI), which plays a role in the emergence of epidemic and pandemic cholera, is the genome of a prophage, and the VPIϕ has been shown to transfer to one VPIϕ-negative *V. cholerae* strain (24, 25).

In most cases, the instability of PAIs is due to their precise excision from the chromosome via recombination between

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
S. flexneri		
YSH6000	Wild-type *S. flexneri* 2a Japanese isolate; Sm^r Ap^r Cm^r Tc^r	46
YSH6000T	MRDE deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	38
S2430	MRDE deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	39
SBA1304	Wild-type *S. dysenteriae* 3 Japanese isolate	This study
SBA1363	Spontaneous SRL PAI deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	This study
SBA1365	Spontaneous MRDE deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	This study
SBA1366	Spontaneous SRL deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	This study
SBA1367	Spontaneous SRL PAI deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	This study
SBA1368	Spontaneous SRL deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	This study
SBA1369	Spontaneous MRDE deletant of YSH6000; Sm^s Ap^s Cm^s Tc^s	This study
AL11	YSH6000 insertional *int* mutant; Sm^r Ap^r Cm^r Tc^r Kn^r	This study
AL108	YSH6000 harboring pBRTp$^r\Delta$; Sm^r Ap^r Cm^r Tc^r Tp^r	This study
AL109	AL11 harboring pBRTp$^r\Delta$; Sm^r Ap^r Cm^r Tc^r Kn^r Tp^r	This study
AL110	AL11 harboring pAL66; Sm^r Ap^r Cm^r Tc^r Kn^r Tp^r	This study
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* Deo^r *thi-1 sunE441 gyrA96 relA1*	Bethesda Research Laboratories
S17-1(λ*pir*)	RP4-2(Tc^s::Mu)(Kn^s::Tn*7*) *recA* λ*pir* Sm^r Tp^r	36
JM109(λ*pir*)	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) λ*pir*	36
Plasmids		
pWSK29	pSC101-based low-copy-number vector; Δ*lacZ* Ap^r	47
pWSK129	pSC101-based low-copy-number vector; Δ*lacZ* Kn^r	47
pBRTp$^r\Delta$	4.8-kb pBR322-based cloning vector; Tp^r Tc^r	C. Sasakawa
pJP5603	R6K-based suicide vector; Kn^r	40
pSBA348	pWSK29 harboring 23-kb *Bam*HI fragment containing *solA-mdoA* region of YSH6000	K. Rajakumar
pSBA509	pWSK129 harboring part of SRL PAI and flanking regions	S. N. Luck
pSBA533	pWSK129 harboring 14-kb *Eco*RI subclone from pSBA509	S. N. Luck
pSBA574	pWSK29 harboring SBA1366 SRL deletion region; Ap^r	This study
pSBA575	pWSK29 harboring SBA1368 SRL deletion region; Ap^r	This study
pAL11	pJP5603 harboring 666-bp internal SRL PAI integrase gene fragment	This study
pAL64	pWSK29 harboring 1.3-kb PCR fragment containing *int*	This study
pAL66	pBRTp$^r\Delta$ harboring 1.3-kb *int* fragment	This study

identical sequences situated on either side of the element. PAIs are commonly arranged with short flanking direct repeats (DRs) of 9 to 20 bp which are analogous to phage *att* sites. These repeats are often identical to the 3′ sequence of the target tRNA gene, and upon PAI deletion only one copy of the DR remains on the chromosome (11). IS elements have also been found in the flanking region of some PAIs (19), and recombination between two flanking IS*100* elements has been shown to occur upon deletion of the high-pathogenicity island (HPI) from *Y. pestis* (5, 13).

In this study, we investigated the various types of deletion events leading to loss of multiple-antibiotic resistance in *S. flexneri* 2a strain YSH6000. We report here that the antibiotic resistance genes of the SRL are lost following at least three distinct events including the precise deletion of the MRDE and the independent deletions of the SRL and of a PAI-like element termed the SRL PAI, both of which are entirely contained within the larger MRDE.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown routinely at 37°C in Luria-Bertani (LB) medium (4) with the addition of ampicillin (100 μg/ml), kanamycin (50 μg/ml), trimethoprim (50 μg/ml), chloramphenicol (40 μg/ml), or tetracycline (10 μg/ml) when necessary.

Molecular biological techniques. Genomic DNA was isolated by small-scale preparation as described previously (4). Plasmid DNA was isolated by a modification of the alkaline lysis method (37). Standard cloning procedures using the vector pWSK29, pJP5603, or pBRTp$^r\Delta$ were employed. *E. coli* DH5α was transformed using the rubidium chloride method (16). DNAs from both plasmids and PCR products were prepared for sequence analysis using the PRISM Ready Reaction Dye Deoxy Terminator Cycle, and chromatograms were produced on an Applied Biosystems model 373A DNA sequencing system.

Selection of tetracycline-sensitive derivatives of YSH6000. Tetracycline-sensitive derivatives of YSH6000 were selected by plating dilutions of YSH6000 (grown in LB broth to a density of approximately 10^9 cells ml^{-1}) onto LB agar supplemented with fusaric acid (12 μg/ml), chlortetracycline (50 μg/ml), and 0.1 mM $ZnCl_2$ (31). Plates were incubated for 24 to 40 h at 37°C.

Southern hybridization. After electrophoresis, DNA was transferred to a charged nylon membrane (Roche) using a vacuum blotting apparatus (TE80 Transvac; Hoefer) or by capillary transfer in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0). Overnight hybridization and subsequent washings were performed under high-stringency conditions at 68°C as recommended in the Roche digoxigenin labeling and detection kit instructions. Probes were labeled by PCR amplification with digoxigenin as specified by Boehringer Mannheim. The 0.5-kb *phoH* probe was amplified using primers BAP507 (5′-ATAAACCCTTCCCGCTTCC-3′) and T3 (5′-AATTAACCCTCACTAAAGGG-3′) from a pSBA509 template. The 2.0-kb *csg* probe was amplified using primers *csgA* forward (5′-AAAGAATTCGCTCTGGCAGGTGTTGTTCC-3′) and *csgA* reverse (5′-AAAAAGTCGACTTAACCAAAGCCAACCTGAGTCACG-3′) from an SBA1304 template. The 1.0-kb *fec* probe was amplified using primers BAP914 (5′-GCTCCCATTTCGCTCGGC-3′) and BAP935 (5′-GTTGTCGTCATAAGAGCGG-3′) with a YSH6000 template. The 1.0-kb *int* probe was amplified using BAP1005 (5′-GCTGGATTGGGAACTTACC-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′) from a pSBA533 template.

PCR amplification of deletion point junctions. Amplifications were performed on chromosomal DNA with the following oligonucleotide primers: for mapping the MRDE deletion endpoint by inverse PCR, BAP499 (5′-CGGGAAGAATACCTGTTGATG-3′) and BAP531 (5′-TATTTGATGCTATGAAGAAGGGGG-3′); for the MRDE deletion region, BAP649 (5′-AGCGGCAGCGGTATTCAC-3′) and BAP530 (5′-TTAATCTCTTCTTCACTTCGCCCC-3′) or 470 (5′-TCCAGCCACCTTTAGCGG-3′); for the SRL deletion region, BAP1249 (5′-TATCCCGCTTGCCGTCGC-3′) and BAP694 (5′-AGCGGCAGCGGTATTCAC-3′); and for the PAI deletion region, BAP679 (5′-GTGCTGCTTTCGGTGTGC-3′) and BAP1157 (5′-GCCAGCATTTCAACAGGAGG-3′).

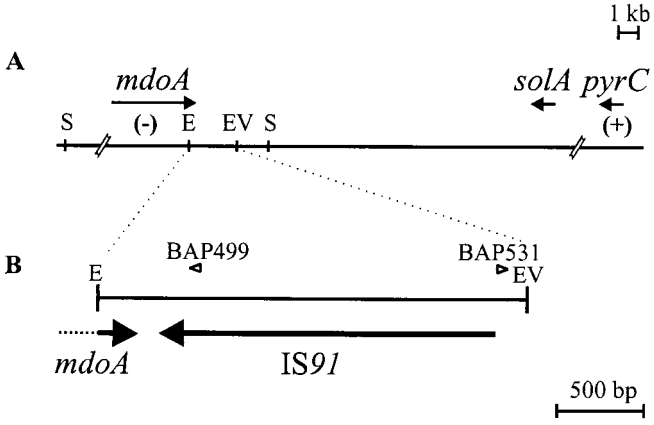

FIG. 1. Map of the right flank of the YSH6000 MRDE. (A) Schematic representation of the YSH6000 *mdoA-pyrC* region. (B) The 2.4-kb *Eco*RI/*Eco*RV fragment within pSBA348 bearing the right deletion endpoint of the MRDE. Chromosomal DNA is represented as a thin black line, and genes are represented as arrows. The presence (+) or absence (−) of regions as tested by Southern hybridization in MRDE deletants YSH6000T and S2430 is also shown. Open arrowheads indicate the positions of primers BAP499 and BAP531, which were used to amplify across the MRDE deletion region in YSH6000T by inverse PCR of *Sal*I-digested and religated genomic DNA. Abbreviations for restriction sites: E, *Eco*RI; EV, *Eco*RV; S, *Sal*I.

Mutant construction. Primers BAP1354 (5′-GCGGATTCCCCTGGCTTCG C-3′) and BAP1355 (5′-TTGGATTCAGGGGCGGGGGAAATGGG-3′) were used to amplify a 666-bp internal fragment of the integrase gene (bp 714 to 1380; GenBank accession no. AF326777), which was then ligated into the T-tailed *Hin*cII site of pJP5603. The recombinant plasmid carrying the *int* fragment, pAL11, isolated in JM109(λ*pir*), was transferred by conjugation into YSH6000 using the mobilizing strain S17-1(λ*pir*). Exconjugants carrying a single-crossover mutation of *int* were obtained by selection on kanamycin-ampicillin LB plates and confirmed by PCR and Southern hybridization.

Mutant complementation. A 1,344-bp product (bp 586 to 1930; GenBank accession no. AF326777) containing the entire *int* open reading frame (ORF) was amplified by PCR with primers BAP1636 (5′-TGGATGGGATCCCAGAG TGACGGGAATTAGC-3′) and BAP1637 (5′-ATGCCAGGATCCCATTACG AACTGGCATTG-3′). Both primers included *Bam*HI sites at the 5′ ends, and this product was cloned into the T-tailed *Eco*RV site of pWSK29, designated pAL64, and sequenced to confirm that no errors were incorporated. The 1.3-kb *Bam*HI fragment from pAL64 containing the *int* fragment was then cloned into the *Bam*HI site of pBRTp^{r}Δ, and clones were selected by sensitivity to tetracycline. The orientation of the *int* fragment was confirmed by restriction enzyme digestion to be the same as that of the interrupted Tc^r gene.

Computer analysis. Sequencing chromatograms were analyzed using the Sequencher program (GeneCodes Corporation, Ann Arbor, Mich.). Nucleotide sequence similarity searches of the databases were performed using the BlastN or BlastX program (2). Protein sequence alignments were performed in eclustalw, available on the Australian National Genomic Information Server (ANGIS [http://www.angis.org.au]).

RESULTS

Mapping the MRDE deletion point. The MRDE of *S. flexneri* 2a YSH6000 had previously been mapped to chromosomal *Not*I fragment D, between the *ompA* and *pyrC* genes (42). Southern analysis of the wild-type strain, YSH6000, and the spontaneous MRDE deletion strains, YSH6000T and S2430, with a series of probes derived from the *ompA-pyrC* region showed that the right MRDE endpoint lies within a 2.4-kb *Eco*RI-*Eco*RV fragment between *mdoA* and *solA* (Fig. 1A). Sequencing of this fragment revealed that this region contained the 3′ end of the *mdoA* locus and a 1,829-bp element showing 93% similarity at the nucleotide level to IS*91* of *E. coli* (Fig. 1B). Due to the high number of IS elements in *Shigella*, Southern hybridization could not be used to determine the deletion endpoint within this region. As the position of the left endpoint of the MRDE was unknown, inverse PCR with primers situated near the right deletion endpoint was employed to amplify a product which traversed the deletion site in strain YSH6000T. A PCR product of approximately 6 kb was amplified from *Sal*I-digested and religated YSH6000T genomic DNA using primers BAP499 and BAP531 (Fig. 1B). As the positions of these primers allowed the amplification of a maximum of 2 kb of right flanking region, this suggested that the amplified inverse PCR product extended across the MRDE deletion point and 4 kb into the left flanking region. Sequence analysis revealed that this fragment contained the right end of an IS*91* element followed by a sequence exhibiting high-level identity to the 3′ region of the *E. coli putA* gene, which is situated approximately 31 kb upstream of *mdoA* in *E. coli*. These data showed that a single copy of the IS*91* element spanned the deletion region in YSH6000T and suggested two possible explanations. There may have been a single IS*91* element adjacent to the *mdoA* locus that, after the deletion event, spanned the region between this locus and the *putA* sequence. Alternatively, an IS*91* element may have been present at each locus in YSH6000, and after the deletion event, only one copy of the element remained on the chromosome. To decide between these two alternatives, direct PCR and sequence analysis of both the left flanking region of the MRDE in YSH6000 and the deletion regions of YSH6000T and S2430 were carried out. Identical intact IS*91* elements were found at both flanks of the MRDE in YSH6000, confirming that two distinct IS*91* elements were present, one downstream of the *mdoA* locus and the other interrupting the *putA* sequence. The data also showed that an identical intact IS*91* was present at the deletion point in both S2430 and YSH6000T. The organizational similarity between *E. coli* and *S. flexneri*, together with the known sequence of the SRL PAI, which is located within this region (S. N. Luck, S. A. Turner, K. Rajakumar, H. Sakellaris, and B. Adler, submitted for publication), allowed us to propose a structure for the MRDE in YSH6000 and for the corresponding regions bearing the deletion points in YSH6000T and S2430 (Fig. 2A). This organization suggests that the MRDE was not acquired or did not evolve as a unit but is composed of a PAI that is situated within a distinct deletable region of the chromosome defined by two IS*91* elements.

Although the *S. flexneri* IS*91* showed considerable similarity to the *E. coli* IS*91*, some differences were noted. IS*91* is known to show absolute insertion specificity for the tetramer GAAC or CAAG, always inserting such that the right inverted repeat (IR_R) is adjacent to either of these target sequences (34). The left flanking IS*91* in YSH6000 also exhibits this specificity, but the right flanking IS*91* is inserted adjacent to the sequence CGAG (Fig. 2A), indicating that this element may have an insertion specificity different from that of the *E. coli* IS*91*. Additionally, the sequences showing similarity to the two major ORFs of IS*91*, ORF121, which is implicated in insertion specificity (6) and *tnpA*, the transposase ORF (33, 35), are shortened by 12 and 31 amino acids, respectively, at the carboxy termini compared with their *E. coli* homologs.

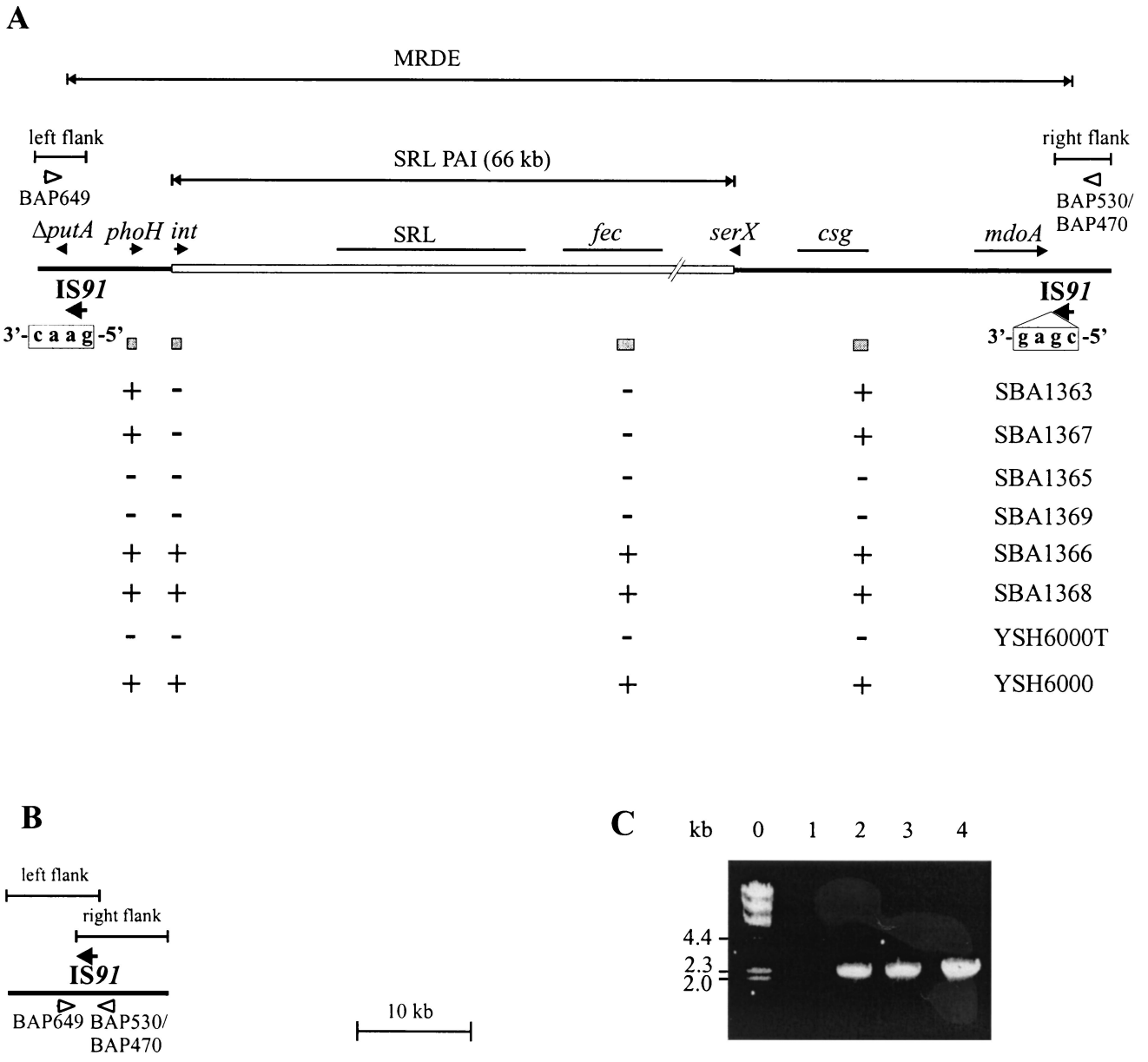

FIG. 2. Deletion of the YSH6000 MRDE. (A) Schematic representation of the genetic organization of the wild-type YSH6000 MRDE. The boundaries of the MRDE are defined by the two IS*91* elements, indicated by arrows. The SRL PAI is represented as an open rectangle. Genes and IS elements are represented as arrows, with truncations indicated by a capital delta. The *fec*, *csg*, and SRL loci are shown as thin black lines. The tetramers immediately downstream of the IS*91* elements at the left and right flanks of the MRDE are shown boxed below each element. Shaded boxes indicate the positions of probes used to determine the presence (+) or absence (−) of these regions within six streptomycin-, ampicillin-, chloramphenicol-, and tetracycline-sensitive strains, YSH6000T, and YSH6000. Open arrowheads represent primers BAP649 and BAP530/BAP470, which were used to amplify across the deletion endpoint. The physical distance between *putA* and *csg* is based on PCR and/or sequence analysis, while the distance between *csg* and *mdoA* was calculated based on the original sizing of the MRDE at 99 kb (42). Regions designated the MRDE left and right flanks, common to both the wild type and MRDE deletants, are indicated above the diagram. (B) Schematic representation of the resultant structure across the deletion point following loss of the MRDE element in YSH6000T, S2430, SBA1365, and SBA1369. (C) Detection of the MRDE deletion point. Shown are PCR products amplified using primers BAP649 and BAP470 (panels A and B). Lanes: 0, *Hin*dIII-digested λ DNA size markers; 1, YSH6000; 2, YSH6000T; 3, SBA1365; 4, SBA1369.

Spontaneous deletion of the MRDE. In order to assess whether the MRDE deletes reproducibly from the same point in the chromosome, we selected for the loss of tetracycline resistance by growth of YSH6000 on LB medium supplemented with fusaric acid. Colonies that grew on fusaric acid medium were confirmed for sensitivity to tetracycline and further tested for susceptibility to the antibiotics streptomycin, ampicillin, and chloramphenicol. Using this method, six Sm^s, Ap^s, Cm^s, and Tc^s strains were identified: SBA1363 and SBA1365 through SBA1369. PCR using the inwardly directed primers BAP649 and BAP470 was employed to confirm MRDE deletions, as the intact deletion region would be am-

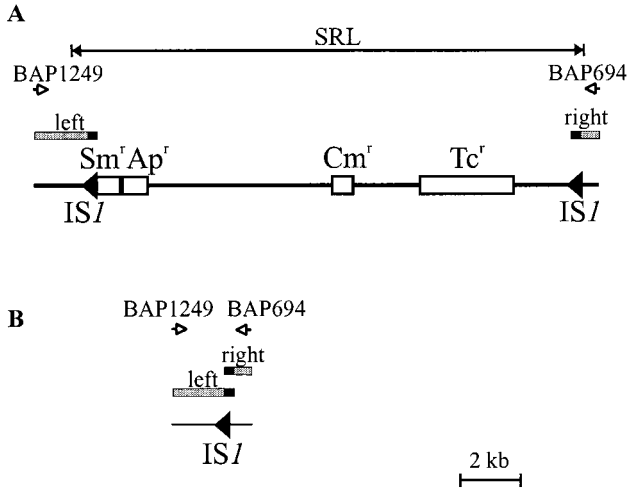

FIG. 3. Deletion of the SRL. (A) Diagram of the SRL in YSH6000. (B) Schematic representation of the structure across the deletion point following loss of the SRL element in SBA1366 and SBA1368. IS elements are represented as arrows, and chromosomal DNA is represented by thin black lines. Determinants encoding resistance to streptomycin, ampicillin, chloramphenicol, and tetracycline are represented as open boxes labeled Sm^r, Ap^r, Cm^r, and Tc^r, respectively. Left and right flanking regions common to both the wild type and SRL deletants are indicated by shaded boxes, with the solid part of each box corresponding to the identical 487 bp of each IS*1*.

plified only in $MRDE^-$ strains. Products were amplified in strains SBA1365 and SBA1369 (Fig. 2C, lanes 3 and 4); sequencing revealed that these strains each carried a single IS*91* element at the deletion point, confirming that the MRDE deletions in these strains were identical to those in YSH6000T and S2430 (Fig. 2B).

Novel deletions leading to loss of antibiotic resistance. As typical MRDE deletions could be confirmed by PCR in only two of the six multi-antibiotic-sensitive strains, the basis for antibiotic sensitivity in the remaining strains was investigated further. Pulsed-field gel electrophoresis of *Not*I-digested chromosomal DNA showed that in both SBA1365 and SBA1369, *Not*I fragment D carrying the MRDE underwent a deletion of the same size as those observed in YSH6000T and S2430 (data not shown). However, in the remaining strains a variety of smaller deletions were observed (data not shown). To localize these deletions more precisely, strains were analyzed by Southern hybridization with a series of probes corresponding to four regions within the MRDE (Fig. 2A). With the exception of SBA1365 and SBA1369, which had undergone deletion events identical to that in YSH6000T, the deletions were confined to the region between *phoH* and *csg* (Fig. 2A).

Deletion of the SRL. Sequence analysis of the region responsible for antibiotic resistance in *S. flexneri* YSH6000 revealed that the MRDE harbors a cluster of resistance genes, now called the SRL (Fig. 2A). The SRL shows significant similarity to the resistance region in the *Shigella* R plasmid NR-1 but also includes an *oxaI* cassette, encoding β-lactamase (41), therefore conferring resistance to the antibiotics streptomycin, ampicillin, chloramphenicol, and tetracycline. The SRL is 16.7 kb in length, including the two flanking 768-bp IS*1* elements (Fig. 3A). Each IS*1* contains an intact *insAB′* and Δ*insA-B′-insB* ORF, both of which have been implicated in transposition (30, 32), suggesting that they are still functional. However, the IS*1* elements were not identical; the left and right IS*1* elements showed 99 and 97% identity, respectively, to the *E. coli* IS*1* nucleotide sequence.

Although the profiles of SBA1366 and SBA1368 indicated that all probed areas were present (Fig. 2A), these strains were susceptible to all four antibiotics to which resistance was encoded by the SRL. For this reason it was considered that these two strains might harbor deletions of the resistance locus itself. Inward-facing primers, BAP1249 and BAP694, situated on either side of the SRL were used to amplify this region. These primers are separated by 18.5 kb in the wild type and thus do not result in the amplification of a product (Fig. 3A), but amplification of a 2.6-kb product occurred in both SBA1366 and SBA1368. The PCR products were cloned into *Eco*RV-digested, T-tailed pWSK29 and designated pSBA574 and pSBA575, respectively. Sequence analysis revealed that these fragments contained sequences identical to that flanking the left end of the SRL, a stretch of sequence identical to the left IS*1* element, followed directly by sequences identical to that flanking the right end of the SRL (Fig. 3B). These data indicate that the SRL was able to delete from the chromosome independently of the MRDE and that upon deletion, a single IS*1* element identical to the left IS*1* of the SRL spanned the deleted area. However, it is not possible to determine whether the IS*1* element spanning the deletion point has its origin entirely from the left IS*1* of the SRL or whether it is a composite of the left and right IS*1* elements, which share an identical sequence throughout bp 1 to 487 (Fig. 3).

Deletion of the SRL PAI. We have recently sequenced the region surrounding the SRL, revealing the presence of a 66-kb element which displays characteristics of a PAI. This element, designated the SRL PAI, is contained completely within the MRDE (Fig. 2A). The SRL PAI carries an integrase-like gene at the left boundary, contains numerous IS elements and phage-related sequences, and also encodes a ferric dicitrate transport system (*fec*) (Luck et al., submitted). The genetic organization in the wild-type strain YSH6000 showed the SRL PAI to be flanked by 14-bp DRs which correspond to the 3′ terminus of the tRNA gene *serX* (5′-GGGGGAGTGGCGG T-3′). The right flank of the PAI harbored an intact *serX*, but similarity to the *E. coli* sequence ended directly downstream of the 14-bp sequence. At the left end of the SRL PAI, the 14-bp repeat was followed by a stretch of sequence similar to that found downstream of the *serX* tRNA gene in *E. coli*, indicating that the PAI had inserted into the 3′ end of the *serX* gene (Fig. 4A). Although deletion of this element had not been previously reported, the Southern hybridization profiles of SBA1363 and SBA1367 suggested that the PAI might be capable of excision (Fig. 2A). Inward-facing primers situated on either side of *serX* were used to amplify a product spanning the right and left flanking regions of the PAI. A product of approximately 1.1 kb was amplified in both SBA1363 and SBA1367, and sequence analysis of this fragment revealed the presence of an intact *serX* gene, with only a single copy of the 14-bp repeat present. Upstream and downstream regions were identical to the left and right flanking regions of the PAI, demonstrating that the SRL PAI itself is capable of precise excision from the chromosome, leaving behind an intact *serX* gene (Fig. 4B).

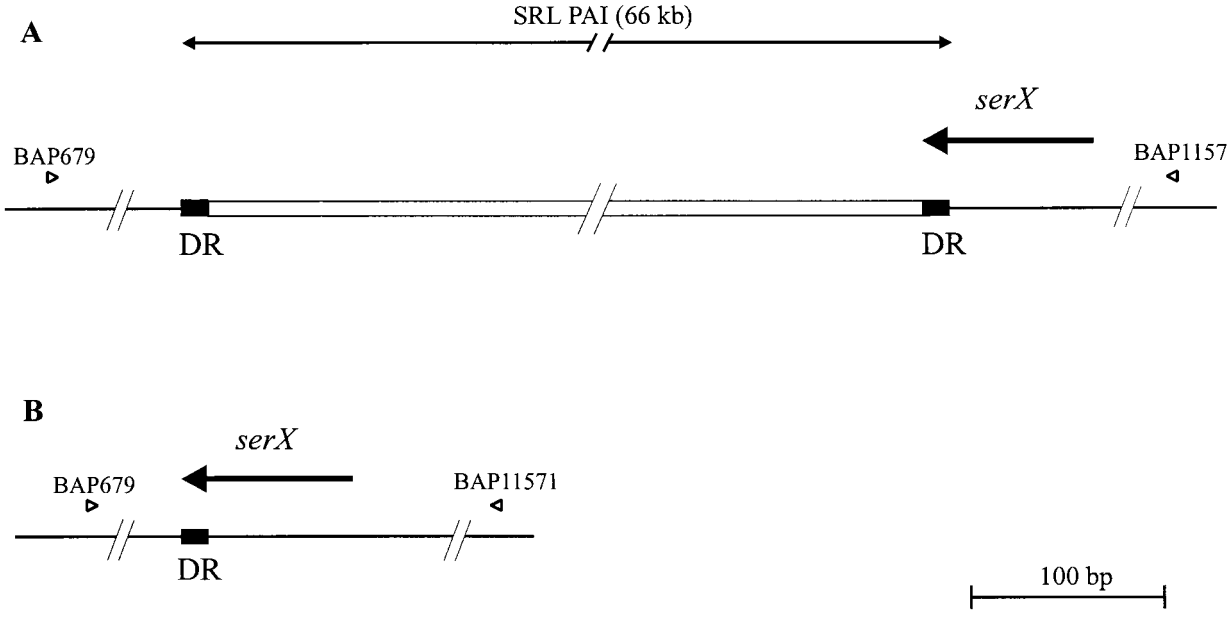

FIG. 4. Deletion of the SRL PAI. (A) Diagram of the genetic organization of the left and right SRL PAI flanking regions in YSH6000. (B) Schematic representation of the structure across the deletion point following loss of the SRL PAI in SBA1363 and SBA1367. Thin black line, chromosomal DNA; open rectangle, SRL PAI; arrows, genes; solid boxes, 14-bp DRs. Also shown are the locations of primers BAP1157 and BAP679, used to amplify across the SRL PAI deletion point.

Role of integrase in excision of the SRL PAI. Hacker et al. (18) have suggested that flanking repeats may act as targets for site-specific recombinases, facilitating integration and/or excision of PAIs. It was thought that the 14-bp repeat may therefore represent the core sequence of the SRL PAI attachment (*att*) site, acting in a manner similar to that of the *att* sites of site-specific bacteriophages (9). The presence of mobility genes encoding determinants, such as integrases, on PAIs had been noted previously (18, 19), but with the exception of the PAI-like *V. cholerae* SXT element (21), the role of integrases in the excision of integrated PAIs had not been demonstrated. The SRL PAI encodes an ORF at its left boundary that shows similarity at the amino acid level to several integrase proteins from the P4 prophage Int family (Luck et al., submitted). This ORF, designated *int*, encodes a putative protein of 405 amino acids and contains the highly conserved HXXR, Y motif necessary for integrase function (3). As the model of PAI deletion described above suggests the involvement of a site-specific recombinase, the role of the SRL PAI *int* in deletion of the element was investigated.

An insertion mutation in the *int* gene was constructed in *S. flexneri* 2a strain YSH6000 (see Materials and Methods), and the frequencies of spontaneous SRL PAI excision in YSH6000 and the *int* mutant strain, AL11, were compared. Spontaneous SRL PAI excisants were isolated by taking advantage of the selective properties of fusaric acid against tetracycline resistance encoded by the SRL PAI (see Materials and Methods). Tetracycline-sensitive derivative strains were further tested for susceptibility to the antibiotics streptomycin, ampicillin, and chloramphenicol, and PAI deletions were confirmed by PCR using primers to amplify a 1.1-kb product across the intact *serX* gene, as performed previously for SBA1363 and SBA1367 (Fig. 4). By comparing the number of PAI excisants to the total cell count, the PAI deletion frequency was estimated to be approximately 10^{-5} per cell in the wild-type strain YSH6000. However, precise excision of the SRL PAI was not detected at all in the mutant strain AL11 (detection limit = 1.1×10^{-7} per cell). Since deletion of the MRDE occurs at a rate of 10^{-6} per cell in both the wild type and AL11 (data not shown), the *int* mutation was responsible for at least a 10-fold decrease in the SRL PAI excision rate, suggesting that the integrase gene is essential for precise excision of the PAI.

To confirm that the loss of PAI excision in this strain was due to inactivation of the integrase, AL11 was complemented with pBRTp$^r\Delta$, carrying an intact *int* gene (pAL66). The resultant strain (designated AL110), YSH6000/pBRTp$^r\Delta$ (AL108), and AL11/pBRTp$^r\Delta$ (AL109) were tested for spontaneous SRL PAI excision using a PCR assay. Genomic DNA extracted from AL108, AL109, and AL110 was standardized for concentration and assayed for excision of the PAI using the inward-facing primers BAP1157 and BAP679 to amplify *serX* as described previously. Although PAI excision is an infrequent event, a PCR product could be detected using wild-type AL108 DNA (Fig. 5, lane 1), but as expected, no PCR product was detected when AL109 DNA was used as the template (Fig. 5, lane 2), confirming the previous findings that strains lacking a functional *int* gene are unable to undergo PAI deletion. An amplification product across the PAI deletion point was again detected using the *int*-complemented strain AL110, confirming that *int* was required for SRL PAI excision (Fig. 5, lane 3).

DISCUSSION

In this study we demonstrated three independent mechanisms for the deletion of the resistance locus of *S. flexneri* 2a YSH6000: deletion of the MRDE involving IS*91* elements, deletion of the SRL involving IS*1* elements, and deletion of the SRL PAI occurring via *int*-mediated recombination of 14-bp

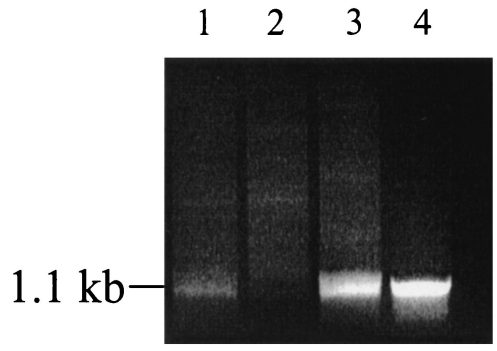

FIG. 5. Complementation of the *int* mutation. PCR products spanning the SRL PAI excision site were obtained by amplification using primers BAP1157 and BAP679 (Fig. 4). From 100-μl PCR mixtures, all was loaded for lanes 1 and 2, 75 μl was loaded for lane 3, and 25 μl was loaded for lane 4. Lanes: 1, AL108 (YSH6000/pBRTp^{r}Δ); 2, AL109 (AL11/pBRTp^{r}Δ); 3, AL110 (AL11/pAL66); 4, SBA1363 (YSH6000-derived spontaneous PAI deletant).

DRs located at each extremity of the element. In many ways, the different deletion events involving the SRL PAI resemble the variety of deletion events that the HPI undergoes in different species of *Yersinia*. Like the SRL PAI, in *Yersinia pseudotuberculosis* IP32637, the HPI deletes via recombination between flanking 17-bp DRs (8). However, like MRDE deletion, in *Y. pestis* or *Yersinia enterocolitica* Ye8081 deletion of the HPI is associated with loss of flanking chromosome either by homologous recombination of flanking IS*100* elements or by as-yet-undefined mechanisms, respectively (5, 13). To the best of our knowledge, the SRL PAI of *S. flexneri* 2a YSH6000 is the first PAI that has been observed to undergo both integrase-mediated and non-integrase-mediated excision in the same strain. It is only the second such element to be described which carries multi-antibiotic resistance determinants (the first was the SXT element of *V. cholerae* [20]).

The structuring of the three nested elements is itself unique, and although the MRDE was the first element to be described as carrying the SRL (41, 42), it seems unlikely that the entire 99 kb inserted en bloc into the *Shigella flexneri* 2a YSH6000 chromosome. Rather, it would appear that insertion of the IS*91* elements and insertion of the SRL PAI were distinct events. This hypothesis is supported by the occurrence of independent deletions involving the PAI and MRDE, and it also explains the remarkable sequence and organizational conservation of the regions surrounding the PAI and the corresponding region in the *E. coli* chromosome. Additionally, had the entire MRDE inserted into the *S. flexneri* chromosome, this would presumably have led to duplication of the regions between the *mdoA* locus and *putA*, a phenomenon which was not observed in YSH6000 (S. A. Turner, unpublished data).

We have shown that the *int* gene was not required for MRDE deletion, and the loss of one of the flanking IS*91* elements after deletion suggested that this element itself may be involved in excision of the MRDE. IS*91* is the prototype of a small, unique family of IS elements that are believed to propagate by a rolling-circle replication mechanism (30). IS*91* was originally isolated from a hemolysin-encoding plasmid of *E. coli* and has since been implicated in the spread of hemolysin (*hly*) genes (49, 50). *hly* genes have been reported on several PAIs in *E. coli* (17), and although no *hly* genes were discovered on the SRL PAI, it does carry an ORF showing similarity to a hemolysin expression-modulating protein of *E. coli* (Hha) (Luck et al., submitted). It is an intriguing possibility that *hly* determinants may have originally been present on the SRL PAI.

IS element-mediated deletion of adjacent DNA has been demonstrated for some IS elements (14). However, it has been reported that IS*91* is unable to cause deletions of adjacent DNA (6); thus, the deletion of the MRDE is probably not mediated by transposition of the elements themselves. It is likely that MRDE deletion is the result of *recA*-mediated homologous recombination between the two flanking elements, which would cause a looping out of the intervening region, resulting in a single residual copy of the IS*91* element on the chromosome. Such RecA-dependent "adjacent deletions" have been shown to occur for other IS elements (27), and work is currently under way to determine if the MRDE deletion is the result of RecA-dependent homologous recombination.

Deletion of the SRL also appears to involve IS elements. Two potentially functional IS*1* elements flank the SRL, although upon deletion, only a single IS*1* element remains on the chromosome. IS*1* is known to mediate deletions of adjacent DNA that end precisely at one boundary of the element (14). The loss of the SRL may be an example of such a deletion.

Alternatively, the SRL may delete via homologous recombination between the flanking IS*1* elements. As described previously, the YSH6000 SRL shares many similarities with the resistance determinant (r-det) of NR1, an archetypal resistance plasmid of *Shigella* (41). The r-det of NR1, which has a size of 23.3 kb and is flanked by DRs of IS*1*, is also a transposable unit (48). Using phage P1 as a carrier, the IS*1*-flanked r-det (Tn*2671*) was shown to move both to the site of the resident IS*1* in the P1 genome and to another region of the P1 genome (22). It was proposed that the former mechanism would involve an intermediate circular form of the r-det carrying a single copy of the IS*1* that excises from NR1 via homologous recombination between the flanking IS*1* elements (22). The organization of the YSH6000 SRL deletants suggests that a similar recombination event may have taken place between the two flanking SRL IS*1* elements, thus leaving a single copy of an IS*1* element on the chromosome. Interestingly, after mobilization to P1, Tn*2671* was subsequently mobilized to *E. coli* recipients and then to the genome of phage P7, demonstrating the existence of a natural mechanism for spread of antibiotic resistance genes (23). This highlights the potential of the SRL to spread to other genomes, independent of mechanisms involving the MRDE or SRL PAI deletions.

In this study we have demonstrated that the SRL PAI of *S. flexneri* 2a strain YSH6000, which is flanked by 14-bp DRs, excises from the 3′ end of the *serX* tRNA gene via mechanisms that resemble the site-specific recombination exhibited by some prophages. The absence of detectable precise deletion of the SRL PAI in the *int* mutant suggests that recombination between the flanking DRs is *int*-mediated site-specific recombination, a mechanism similar to that observed for some phages. Phage transduction has previously been implicated in the mobility of some PAIs (e.g., the *V. cholerae* VPI and the staphylococcal PAI SaPI), and bacteriophage proteins encoded on PAIs are often assumed to have played a role in the original

mobilization of the elements (20). However, with the exception of the SXT element of *V. cholerae* (21), a role for phage-like integrases in the excision of integrated PAIs has not been demonstrated. Here, the *S. flexneri* 2a strain YSH6000 SRL PAI integrase was shown to be required for the precise excision of the element, confirming that the integrase is functional and plays an important role in the deletion of the SRL PAI.

The SRL PAI is one of a growing number of PAIs that appear to delete precisely through site-specific recombination of short flanking DRs. It is probable that, like phages, these PAIs originally integrated into the 3′ termini of tRNA genes, with the DR sequence acting in a manner similar to the core of *att*B sites during phage integration (9). Other elements in this category may include PAI I_{536} and II_{536} of UPEC, with 16- and 18-bp DRs, respectively (7), the *she* PAI of *S. flexneri* with 22-bp DRs (1), and the HPI of *Y. pseudotuberculosis* with 17-bp DRs (8). Additionally, these five PAIs also carry sequences near one end of the element exhibiting similarity to the P4 family of phage-like integrases (1, 8, 17). Indeed, many other PAIs have been found to possess sequences with similarity to integrases and other phage-related ORFs. Interestingly, the insertion site of the HPI has been found to contain a region showing partial similarity to the P4 *attB* site (8). The 3′ end of *serX* near the SRL PAI integration site also possesses a sequence that matches 12 of the 20 bp of the P4 *attB* (Luck et al., submitted). The *she* PAI in *S. flexneri* and the HPI of *Y. pseudotuberculosis* have been found inserted in at least two different *phe* and *asn* tRNA genes possessing the target DR of each, respectively (8; K. Al-Hasani, B. Adler, K. Rajakumar, and H. Sakellaris, submitted for publication). Similarly, the SRL PAI has been found inserted in both *serX* and a paralog of this gene, *serW*, in *Shigella* (S. A. Turner, unpublished data), indicating that, like the chromosomal integration of phages, there is a high level of insertion specificity for these PAIs.

In this study, the SRL PAI was shown to have a deletion rate of approximately 10^{-5}, which is consistent with those of PAI I_{536} and II_{536} (10^{-4} to 10^{-5}) (18), the HPI (10^{-4}) (8), and the *she* PAI (10^{-5} to 10^{-6}) (43). Other similarities include flanking DRs, the presence of integrases, and evidence of site-specific deletion. In light of evidence presented here that the *int* gene is required for precise excision of the SRL PAI, the shared features of these PAIs provide a strong argument that integrase-dependent, site-specific recombination is likely to be a common mechanism of excision among these elements. Indeed, the P4-like integrase from the *Y. pestis* HPI has been shown to act in a site-specific manner (44), while in the HPI of *Y. enterocolitica*, interruption of the integrase gene and loss of conservation in the flanking 17-bp DRs are thought to be responsible for the "stabilization" of this element in the chromosome. In the HPI of *Y. pseudotuberculosis*, the integrase gene and DRs are intact, probably explaining why this element undergoes precise excision from the chromosome (5). It is noteworthy that although the *int* sequences of both PAI I_{536} and II_{536} of UPEC are thought to be nonfunctional, these PAIs delete from the chromosome at a rate similar to that of the SRL PAI (17, 18), implying that other recombinases may mediate the excision of these elements.

Further evidence for the importance of integrases is found in the *V. cholerae* SXT element, which exhibits many similarities to PAIs, including mobility, insertion into a specific chromosomal site, and the presence of 17-bp DRs flanking the inserted element. The *int* gene of the SXT element is necessary for excision and/or production of an extrachromosomal circular form of the element, which is required for the transfer of SXT to both *V. cholerae* and *E. coli* recipients (21). Recently Rakin et al. provided evidence that the integrase from *Y. pestis* HPI promotes both excision and integration of a minimal integrative HPI module into the *asn* tRNA target site (44). These findings demonstrate that the *int* of the SRL PAI and other PAIs may play equally important roles not only in excision but also in acquisition and dissemination of the PAI-like elements on which they reside.

Importantly, from a clinical perspective, these elements pose a substantial risk given their ability to alter dramatically both the virulence and the antibiotic susceptibility profile of a pathogen.

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REFERENCES

1. **Al-Hasani, K., K. Rajakumar, D. Bulach, R. Robins-Browne, B. Adler, and H. Sakellaris.** 2001. Genetic organization of the *she* pathogenicity island in *Shigella flexneri* 2a. Microb. Pathog. **30:**1–8.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Argos, P., A. Landy, K. Abremski, J. B. Egan, E. Haggard-Ljungquist, R. H. Heoss, M. L. Kahn, B. Kalionis, S. V. L. Narayana, L. S. Pierson III, N. Sternberg, and J. M. Leong.** 1986. The integrase family of site-specific recombinases: regional similarities and global diversity. EMBO J. **5:**433–440.
4. **Ausubel, F. A., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1993. Current protocols in molecular biology. Greene Publishing and Wiley Interscience, New York, N.Y.
5. **Bach, S., C. Buchrieser, M. Prentice, A. Guiyoule, T. Msadek, and E. Carniel.** 1999. The high-pathogenicity island of *Yersinia enterocolitica* Ye8081 undergoes low-frequency deletion but not precise excision, suggesting recent stabilization in the genome. Infect. Immun. **67:**5091–5099.
6. **Bernales, I., M. V. Mendiola, and F. de la Cruz.** 1999. Intramolecular transposition of insertion sequence IS*91* results in second-site simple insertions. Mol. Microbiol. **33:**223–234.
7. **Blum, G., M. Ott, A. Lischewski, A. Ritter, H. Imrich, H. Tschape, and J. Hacker.** 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect. Immun. **62:**606–614.
8. **Buchrieser, C., R. Brosch, S. Bach, A. Guiyoule, and E. Carniel.** 1998b. The high-pathogenicity island of *Yersinia pseudotuberculosis* can be inserted into any of the three chromosomal *asn* tRNA genes. Mol. Microbiol. **30:**965–978.
9. **Campbell, A. M.** 1992. Chromosomal insertion sites for phages and plasmids. J. Bacteriol. **174:**7495–7499.
10. **Casalino, M., M. Nicoletti, A. Salvia, B. Colonna, C. Pazzani, A. Calconi, K. A. Mohamud, and F. Maimone.** 1994. Characterization of endemic *Shigella flexneri* strains in Somalia: antimicrobial resistance, plasmid profiles, and serotype correlation. J. Clin. Microbiol. **32:**1179–1183.
11. **Dozois, C. M., and R. Curtiss III.** 1999. Pathogenic diversity of *Escherichia coli* and the emergence of "exotic" islands in the gene stream. Vet. Res. **30:**157–179.
12. **DuPont, H. L., M. M. Levine, R. B. Hornick, and S. B. Formal.** 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. **159:**1126–1128.
13. **Fetherston, J. D., P. Schuetze, and R. D. Perry.** 1992. Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. Mol. Microbiol. **6:**2693–2704.
14. **Galas, D., and M. Chandler.** 1989. Bacterial insertion sequences, p. 109–162. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
15. **Gebre-Yohannes, A., and B. S. Drasar.** 1990. Plasmid profiles of antibiotic-resistance *Shigella dysenteriae* types 2, 3, 4, 6, and 7 isolated in Ethiopia during 1976–85. Epidemiol. Infect. **105:**65–72.
16. **Glover, D. M. (ed.).** 1985. DNA cloning: a practical approach, vol. 1. IRL

Press Ltd., Oxford, United Kingdom.
17. **Hacker, J., G. Blum-Oehler, B. Janke, G. Nagy, and W. Goebel.** 1999. Pathogenicity islands of extraintestinal *Escherichia coli*, p. 59–76. *In* J. B. Kaper and J. Hacker (ed.), Pathogenicity islands and other mobile virulence elements. American Society for Microbiology, Washington, D.C.
18. **Hacker, J., G. Blum-Oehler, I. Muhldorfer, and H. Tschape.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089–1097.
19. **Hacker, J., and J. B. Kaper.** 1999. The concept of pathogenicity islands, p. 1–12. *In* J. B. Kaper and J. Hacker (ed.), Pathogenicity islands and other mobile virulence elements. American Society for Microbiology, Washington, D.C.
20. **Hacker, J., and J. B. Kaper.** 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. **54:**641–679.
21. **Hochhut, B., and M. K. Waldor.** 1999. Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. Mol. Microbiol. **32:**99–110.
22. **Iida, S., and W. Arber.** 1980. On the role of IS*1* in the formation of hybrids between the bacteriophage P1 and the R plasmid NR1. Mol. Gen. Genet. **177:**261–270.
23. **Iida, S., C. Hanni, C. Echarti, and W. Arber.** 1981. Is the IS*1*-flanked r-determinant of the R plasmid NR1 a transposon? J. Gen. Microbiol. **126:**413–425.
24. **Karaolis, D. K. R., and J. B. Kaper.** 1999. Pathogenicity islands and other mobile virulence elements of *Vibrio cholerae*, p. 167–187. *In* J. B. Kaper and J. Hacker (ed.), Pathogenicity islands and other mobile virulence elements. American Society for Microbiology, Washington, D.C.
25. **Karaolis, D. K. R., S. Somara, D. R. Maneval, Jr., J. A. Johnson, and J. B. Kaper.** 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature **399:**375–379.
26. **Khan, M. U.** 1985. Fourteen years of shigellosis in Dhaka: an epidemiological analysis. Int. J. Epidemiol. **14:**607–613.
27. **Kleckner, N.** 1989. Transposon Tn*10*, p. 227–268. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
28. **Kotloff, K. L., J. P. Winickoff, B. Ivanoff, J. D. Clemens, D. L. Swerdlow, P. J. Sansonetti, G. K. Adak, and M. M. Levine.** 1999. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull. W. H. O. **77:**651–665.
29. **Ling, J. M., P. C. Shaw, K. M. Kam, A. F. Cheng, and G. L. French.** 1993. Molecular studies of plasmids of multiply-resistant *Shigella* spp. in Hong Kong. Epidemiol. Infect. **110:**437–446.
30. **Mahillon, J., and M. Chandler.** 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. **62:**725–774.
31. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145:**1110–1112.
32. **Matsutani, S.** 1994. Genetic evidence for IS*1* transposition regulated by InsA and the δInsA-B′-InsB species, which is generated by translation from two alternative internal initiation sites and frameshifting. J. Mol. Biol. **240:**52–65.
33. **Mendiola, M. V., and F. de la Cruz.** 1992. IS*91* transposase is related to the rolling-circle-type replication proteins of the pUB110 family of plasmids. Nucleic Acids Res. **20:**3521.
34. **Mendiola, M. V., and F. de la Cruz.** 1989. Specificity of insertion of IS*91*, an insertion sequence present in alpha-haemolysin plasmids of *Escherichia coli*. Mol. Microbiol. **3:**979–984.
35. **Mendiola, M. V., Y. Jubete, and F. de la Cruz.** 1992. DNA sequence of IS*91* and identification of the transposase gene. J. Bacteriol. **174:**1345–1351.
36. **Miller, V. C., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
37. **Morelle, G.** 1989. A plasmid extraction procedure on a miniprep scale. Focus **11:**7–8.
38. **Nakata, N., C. Sasakawa, N. Okada, T. Tobe, I. Fukuda, T. Suzuki, K. Komatsu, and M. Yoshikawa.** 1992. Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. Mol. Microbiol. **6:**2387–2395.
39. **Okada, N., C. Sasakawa, T. Tobe, M. Yamada, S. Nagai, K. A. Talukder, K. Komatsu, S. Kanegasaki, and M. Yoshikawa.** 1991. Virulence-associated chromosomal loci of *Shigella flexneri* identified by random Tn*5* insertion mutagenesis. Mol. Microbiol. **5:**187–195.
40. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118:**145–146.
41. **Rajakumar, K., D. Bulach, J. Davies, L. Ambrose, C. Sasakawa, and B. Adler.** 1997. Identification of a chromosomal *Shigella flexneri* multi-antibiotic resistance locus which shares sequence and organizational similarity with the resistance region of the plasmid NR1. Plasmid **37:**159–168.
42. **Rajakumar, K., C. Sasakawa, and B. Adler.** 1996. A spontaneous 99-kb chromosomal deletion results in multi-antibiotic susceptibility and an attenuation of contact haemolysis in *Shigella flexneri* 2a. J. Med. Microbiol. **45:** 64–75.
43. **Rajakumar, K., C. Sasakawa, and B. Adler.** 1997. Use of a novel approach, termed island probing, identifies the *Shigella flexneri she* pathogenicity island, which encodes a homolog of the immunoglobulin A protease-like family of proteins. Infect. Immun. **65:**4606–4614.
44. **Rakin, A., C. Noelting, P. Schropp, and J. Heesemann.** 2001. Integrative module of the high-pathogenicity island of *Yersinia*. Mol. Microbiol. **39:**407–415.
45. **Sack, R. B., M. Rahman, M. Yunus, and E. H. Khan.** 1997. Antimicrobial resistance in organisms causing diarrheal disease. Clin. Infect. Dis. **24**(Suppl. 1)**:**S102–S105.
46. **Sasakawa, C., K. Kamata, T. Sakai, S. Y. Murayama, S. Makino, and M. Yoshikawa.** 1986. Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri*. Infect. Immun. **51:**470–475.
47. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195–199.
48. **Womble, D. D., and R. H. Rownd.** 1988. Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. **52:**433–451.
49. **Zabala, J. C., F. de la Cruz, and J. M. Ortiz.** 1982. Several copies of the same insertion sequence are present in alpha-hemolytic plasmids belonging to four different incompatibility groups. J. Bacteriol. **151:**472–476.
50. **Zabala, J. C., J. M. Garcia-Lobo, E. Diaz-Aroca, F. de la Cruz, and J. M. Ortiz.** 1984. *Escherichia coli* alpha-haemolysin synthesis and export genes are flanked by a direct repetition of IS*91*-like elements. Mol. Gen. Genet. **197:** 90–97.