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Molecular Epidemiology of the SRL Pathogenicity Island

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The Shigella resistance locus (SRL), which is carried on the SRL pathogenicity island (PAI) in *Shigella flexneri* 2a YSH6000, mediates resistance to the antibiotics streptomycin, ampicillin, chloramphenicol, and tetracycline. In the present study, we investigated the distribution and structural variation of the SRL and the SRL PAI in 71 *Shigella* isolates and 28 other enteric pathogens by PCR and Southern analysis. The SRL and SRL-related loci, although absent from the other enteric pathogens evaluated in this study, were found to be present in a number of *Shigella* isolates. SRL PAI markers were also present in the majority of strains carrying the SRL and SRL-related loci. PCR linkage studies with six of these strains demonstrated that the SRL is carried on elements similar in structure and organization to the YSH6000 SRL PAI, consistent with the hypothesis that the SRL PAI may be involved in the spread of multiple-antibiotic resistance in these strains.

Shigella spp. are a common cause of bacillary dysentery and are responsible for the deaths of more than 1 million people annually, the majority of which occur in developing countries (23). Infection is transmitted via the fecal-oral route and is characterized by excretion of stools containing white cells and blood (9). Shigellosis is one of the few enteric infections for which antimicrobial therapy is clearly effective. Although treatment with antibiotics has not been shown to reduce the rate of mortality (41), it does reduce both the duration and the severity of illness and the duration of pathogen excretion (14), which are important considerations in the prevention of further transmission. However, over the past several decades treatment has become increasingly difficult due to emerging resistance to most of the widely used antibiotics (22, 41).

Resistance in *Shigella* to multiple antibiotics such as sulfonamides (SULs), streptomycin (STR), ampicillin (AMP), chloramphenicol (CHL), and tetracycline (TET) was first reported in Japan shortly after their introduction as therapeutic agents for the treatment of shigellosis. In the majority of cases, resistance was found to be borne on conjugative R plasmids, and the inherent mobility of R plasmids was thought to explain the rapid increase in the number of multiply resistant *Shigella* strains observed during the 1950s (49). NR1, the archetypal *Shigella* R plasmid, which encodes resistance to mercury, SULs, STR, CHL, and TET, was isolated during that period (30, 51). Recently, in addition to plasmid-borne resistance, there have been several reports of chromosomally borne resistance (7, 10, 25). However, the basis of chromosomal resistance in *Shigella* has remained largely unexplored.

In 1997, Rajakumar et al. (36) reported on the identification of a chromosomal multiantibiotic resistance locus in *Shigella flexneri* 2a strain YSH6000. This locus, which has since been designated the *Shigella* resistance locus (SRL), encodes resistance to STR, AMP, CHL, and TET and exhibits both organizational and sequence similarity to corresponding regions of NR1. However, the SRL also exhibits several differences from NR1, including the absence of the mercury and SUL resistance determinants, a 17.5-kb deletion between the CHL and TET resistance determinants, and the precise insertion of a β-lactamase-encoding *oxa-1* cassette into the Tn*21*-borne integron In2 (26, 36). Although insertion of the *oxa-1* cassette into this location of Tn*21* has been demonstrated experimentally (45) and is observed in the closely related plasmid-borne transposon Tn*2603* (32), the unique combination and placement of resistance determinants distinguishes the SRLs even from these close relatives (36).

The SRL was originally reported to reside on a 99-kb deletable element which has since been designated the multiple-antibiotic-resistance deletable element (MRDE) (37, 46). Recent studies have revealed that the 16.7-kb IS*1*-flanked SRL is borne on a 66-kb pathogenicity island (PAI), designated the SRL PAI (26). The SRL is lost from *S. flexneri* 2a strain YSH6000 via three distinct but separate mechanisms involving deletion of the SRL itself, integrase-mediated deletion of the PAI, and deletion of the MRDE (an IS*91*-flanked element encompassing the SRL PAI and some surrounding chromosomal DNA). All deletions result in the loss of the SRL from the chromosome (46). As the IS*1*-flanked r determinant of NR1 and PAIs are mobile in other systems, it was considered that there was potential for dissemination of SRL within *Shigella* via one of these mechanisms (46). In this study, we present evidence that the resistance conferred by the SRL is widespread among clinical *Shigella* isolates and that dissemination of the SRL may be mediated by the SRL PAI.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. The strains were routinely grown at 37°C in Luria-Bertani (LB) medium (3), with the addition of AMP (100 μg/ml), STR (25 μg/ml), CHL (40 μg/ml), or TET (10 μg/ml) when necessary.

Molecular biological techniques. Genomic DNA was isolated by use of a small-scale preparation as described previously (3). Plasmid DNA was isolated by a modification of the alkaline lysis method (28).

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TABLE 1. Bacterial strains

Strain	Relevant characteristics (no. of strains in group)	Reference or source[a]
Shigella spp.		
YSH6000	Wild-type *S. flexneri* 2a Japanese Str^r Amp^r Chl^r Tet^r isolate	42
YSH6000T	MRDE deletant of YSH6000	29
SBA 1363	Spontaneous SRL PAI deletant of YSH6000	46
SBA 1367	Spontaneous SRL PAI deletant of YSH6000	46
S. flexneri	*S. flexneri* 1a (3)	CS
	S. flexneri 1b (3)	CS, DL
	S. flexneri 2a (12)	CS, DL
	S. flexneri 2b (5)	CS, DL
	S. flexneri 3 (1)	CS
	S. flexneri 3a (4)	CS, DL
	S. flexneri 3b (1)	CS
	S. flexneri 3c (1)	CS
	S. flexneri 4 (1)	DL
	S. flexneri 4a (3)	CS, DL
	S. flexneri 4b (1)	CS
	S. flexneri 5 (2)	CS
	S. flexneri 6 (4)	CS, DL
	S. flexneri X (1)	CS
	S. flexneri Y (1)	CS
S. sonnei	*S. sonnei* (11)	CS, DL
S. boydii	*S. boydii* 1 (1)	CS
	S. boydii 2 (1)	CS
	S. boydii 3 (1)	CS
	S. boydii 4 (2)	CS
	S. boydii 7 (1)	CS
	S. boydii 8 (2)	CS
S. dysenteriae	*S. dysenteriae* 1 (1)	CS
	S. dysenteriae 3 (4)	CS
	S. dysenteriae 4 (1)	CS
	S. dysenteriae 5 (1)	CS
	S. dysenteriae 6 (1)	CS
	S. dysenteriae 9 (1)	CS
Pathogenic *E. coli*	EIEC (12)	RRB, CS
	EHEC (3)	RRB
	EPEC (3)	RRB
	ETEC (3)	RRB
	EAEC (3)	RRB
Salmonella spp.	*S. enterica* serovar Typhimurium (2)	RRB
Yersinia spp.	*Y. enterocolitica* (2)	RRB

[a] CS, C. Sasakawa, University of Tokyo, Japan; DL, D. Lightfoot, University of Melbourne, Parkville, Australia; RRB, R. Robins-Browne, Royal Children's Hospital, Parkville, Australia.

Selection of TET-sensitive derivatives of SBA1299 and SBA1391. TET-sensitive derivatives were selected by plating dilutions of cultures grown in LB broth to densities of approximately 10^9 CFU ml^{-1} onto LB agar supplemented with fusaric acid (12 μg/ml), heat-inactivated chlortetracycline (50 μg/ml), and 0.1 mM $ZnCl_2$ (27). The agar plates were incubated for 24 to 40 h at 37°C.

Southern hybridization. After electrophoresis, DNA was transferred to a charged nylon membrane (Roche) by using a vacuum blotting apparatus (TE80 Transvac; Hoefer) or by capillary transfer in 20× SSC (750 mM NaCl plus 75 mM sodium citrate [pH 7.0]). Overnight hybridization and subsequent washings were performed under high-stringency conditions at 68°C, as recommended in the instructions supplied with the Roche digoxigenin labeling and detection kit. Probes were labeled by PCR amplification with digoxigenin as specified by Roche. The 1.0-kb *int* probe and the 1.0-kb *fec* probe were amplified as described previously (46). The 0.2-kb *oxa-1* probe was amplified with primers BAP1553 and BAP1559 (Table 2).

PCR amplification and linkage of SRL PAI markers. PCR amplifications were performed with chromosomal DNA by using the *Taq* DNA polymerase system, as specified by Roche. PCR amplifications of DNA fragments of over 5.0 kb were performed with Expand Long Template PCR buffer system 3, as specified by Roche. Colony PCRs were performed with single colonies added directly to the PCR mix, with an initial 8 min of incubation at 94°C

Separation of plasmid and chromosomal DNAs. Plasmid and chromosomal DNAs were separated by using a modification of the Eckhardt in-well lysis method (35). Approximately 10^7 to 10^8 cells were resuspended in lysozyme mixture (7% Ficoll, 20% sucrose, 1 U of RNase per ml, and 1 mg of lysozyme per ml in Tris-borate-EDTA [TBE]) and loaded into the wells of 0.4 and 0.8% agarose gels. The samples were then overlaid with a sodium dodecyl sulfate mixture (1% sodium dodecyl sulfate and 5% sucrose bromophenol blue in TBE) and electrophoresed for 30 min at 5 mA, followed by electrophoresis for 2 h at 100 mA.

Computer analysis. Searches of databases for nucleotide sequences with sequence similarities were performed with the BlastN or BlastX program (2). Sequence data were analyzed with the Sequencher program (GeneCodes Corporation, Ann Arbor, Mich.).

RESULTS

Distribution of the SRL among *Shigella* isolates. To determine whether the combination of drug resistance encoded by the SRL was present in other *Shigella* species, 71 clinical *Shigella* isolates including representatives from 27 different serotypes were tested for their ability to grow in the presence of STR, AMP, CHL, and TET. Of these strains, 31 were found to be resistant to all four antibiotics, including strains from each *Shigella* species (strains listed in Table 3). To establish whether resistance to these antibiotics was due to the presence of the SRL or other resistance loci, PCRs linking the coding region of *aadA1* (Str^r) with *oxa-1* (Amp^r), the coding region *oxa-1* with *catA* (Chl^r), and the coding region *catA* with *tetA*(B) (Tet^r) (Fig. 1A) were performed with all isolates exhibiting resistance to the four antibiotics. These linkages enabled a distinction to be made between the SRL and the closely related resistance locus of NR1, as the NR1 locus does not contain the *oxa-1* cassette and carries an additional ~17.5 kb between the *cat* and *tetA*(B) sequences (36). Physical linkage of all four determinants was demonstrated in the majority (22 of 31) of strains (Table 4), indicating conservation of the genetic organization of the YSH6000 SRL in these isolates. With the exception of one strain in which no linkages were demonstrated, all strains exhibited linkage of two or three resistance determinants (Table 4), suggesting the presence of a locus either that was identical to or that shared some similarity with the organization of the YSH6000 SRL.

Distribution of the SRL PAI markers among *Shigella* isolates. In order to discover whether the SRL-related loci were present as part of the SRL PAI or as independent loci, strains were tested for the presence of three SRL PAI markers. Probes corresponding to the integrase gene (*int*), which has been shown to be necessary for deletion of the SRL PAI (46) (Fig. 1A, region A), and *fecA*, a critical component of the *fec* iron transport locus (47) (Fig. 1A, region B), were used in a high-stringency Southern analysis of the 31 Str^r Amp^r Chl^r Tet^r strains (Table 3). Additionally, primers amplifying a region internal to *orf58* of the SRL PAI (26), which has no database matches at the nucleotide level, were used to analyze these strains by PCR (Fig. 1A, region C; Table 3).

The results indicated that sequences homologous to the SRL PAI *int* were present in all but 2 of the 31 Str^r Amp^r Chl^r Tet^r strains, and with one exception, multiresistant strains that carry the *int* marker also carry the *orf58* marker (Table 3). Interest-

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TABLE 2. Primers used in this study

Target DNA	Primer name	Primer sequence (5′-3′)	Size of PCR product (kb)
orf58	BAP2022	CGCTGTTAAGGTAAATCCC	0.2
	BAP2023	AGCGAGCCATAAAGTGTGC	
aadA1 + oxa-1	BAP2024	TGACGGGCTGATACTGGG	1.3
	BAP1553	AAAACCCCCAAAGGAATGGAG	
oxa-1 + cat	BAP1559	GCTACTTTCGAGCCATGC	7.0
	BAP2026	CGGTGAGCTGGTGATATGG	
cat + tetA	BAP2025	TTACGCCCCGCCCTGCCAC	4.0
	BAP1622	CCCCTAACCAACCGAACC	
int + rox	BAP1012	TGGGCGGATTATGTGATG	2.2
	BAP1620	CCGGAATTCCTCTTCCGCTTGTGTGCC	
rox + orf6	BAP1621	GCGCGGATCCCAGCAGCAGCATTTTCCG	6.0
	BAP1377	AAGGAGTGGCATCGTTAGC	
orf6 + oxa-1	BAP1388	AACACGGTATTATTCTGGGC	10.0
	BAP1553	AAAACCCCCAAAGGAATGGAG	
tetC + fecD	BAP1013	GCATAAACCAGCCATTGAG	8.3
	BAP514	GCGCTGCTGACCCGACTGG	
fecD + orf34	BAP936	AGACAAACCACGGCGCAC	9.4
	BAP563	GCTCACCAGACGCGTAAAC	
orf34 + orf41	BAP1440	CCTGGCGGCACAACCTAC	7.8
	BAP1619	GCGCGGATCCTCGGATGACACACGCCCC	
orf41 + orf45	BAP1618	CCGGAATTCTAGCTTACTCTGGCAAATCC	3.6
	BAP1552	ATGCCAATCACGGGTTCG	
orf45 + orf48	BAP1531	TTATGATCCCCCGGAACG	6.9
	BAP1689	AGACACGGGGTTCCAGGG	
orf48 + orf58	BAP1691	GGCGCTGGCACAGGCGGC	4.4
	BAP2023	AGCGAGCCATAAAGTGTGC	
int + ⇓ serX[a]	BAP679	GTGCTGCTTTCGGTGTGC	1.1
	BAP689	CCGGGCAGTACGTGCAGC	
int + ⇓ serW[b]	BAP1462	ATACTCCACCCGCCCACC	0.4
	BAP689	CCGGGCAGTACGTGCAGC	
orf58 + ⇑ serX[c]	BAP2022	CGCTGTTAAGGTAAATCCC	2.2
	BAP1157	GCCAGCATTTCAACAGGAGG	
orf58 + ⇑ serW[d]	BAP2022	CGCTGTTAAGGTAAATCCC	2.2
	BAP1783	TGTTTTACCGCCTGATGGG	
csgA	csgAUp	AAAGAATTCGCTCTGGCAGGTGTTGTTCC	1.8
	csgADn	AAAAAGTCGACTTAACCAAAGCCAACCTGAGTCACG	

[a] ⇓ *serX*, sequence downstream of *serX*.
[b] ⇓ *serW*, sequence downstream of *serW*.
[c] ⇑ *serX*, sequence upstream of *serX*.
[d] ⇑ *serW*, sequence upstream of *serW*.

ingly, SBA1298 and SBA1388, the only two Str[r] Amp[r] Chl[r] Tet[r] *int*-negative strains identified, were among the strains that showed the least similarity to the SRL in SRL linkage studies (Table 4). Thus, in the majority of strains that contain the *int* marker, resistance to STR, AMP, CHL, and TET (100% of strains; in 28 of 29 strains the resistance is SRL related) and the *orf58* marker (97% of strains) are also present, suggesting that the resistance determinants in these strains are borne on elements similar to the SRL PAI. Moreover, the *int* marker was absent from the other 40 strains, indicating a perfect correlation between the presence of *int* and resistance to the four antibiotics (data not shown). Interestingly, the *orf58* marker was present in 24% of the 42 strains negative for the *int* marker (data not shown). The majority of strains that possessed the *int* marker were also positive for the *fecA* marker (69%) (Table 3). However, 45% of the *int*-negative strains also possessed the

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TABLE 3. Distribution of SRL PAI markers in Str^r Amp^r Chl^r Tet^r *Shigella* isolates

Species (no. of strains)	Presence of SRL PAI marker:			Linkage of *int* to[a]:	
	int	*fec*	*orf58*	⇓*serX*	⇓*serW*
S. flexneri 3c (1)	−	−	−	—	—
S. flexneri 2a (1)	−	+	−	—	—
S. flexneri 3a (1)	+	+	+	−	−
S. boydii 8 (1)	+	−	+	−[b]	−
S. boydii 4 (1)	+	+	+	−[b]	−
S. flexneri 1b (1)	+	+	+	−[b]	−
S. flexneri 2a (1)	+	+	+	−[b]	−
S. flexneri 6 (1)	+	+	+	−[b]	−
S. boydii 8 (1)	+	−	+	−	+
S. dysenteriae 3 (2)	+	−	+	−	+
S. sonnei Form I (1)	+	+	−	−	+
S. dysenteriae 3 (2)	+	+	+	−	+
S. flexneri 2a (1)	+	+	+	−	+
S. flexneri 3b (1)	+	+	+	−	+
S. flexneri 6 (1)	+	+	+	−	+
S. sonnei form I (1)	+	+	+	−	+
S. flexneri 2a (5)	+	−	+	+	−
S. flexneri 1a (1)	+	+	+	+	−
S. flexneri 2a (1)	+	+	+	+	−
S. flexneri 2b (1)	+	+	+	+	−
S. flexneri 4 (1)	+	+	+	+	−
S. flexneri 4a (2)	+	+	+	+	−
S. flexneri 2b (2)	+	+	+	+	+

[a] +, PCR linkage was demonstrated; −, PCR linkage was not demonstrated; ⇓*serX* and ⇓*serW*, linkage of *int* to sequences downstream of *serX* and *serW*, respectively; —, not tested.
[b] Linkage of *int* to sequences downstream of *serX* was negative, but linkage of *orf58* to sequences upstream of *serX* was positive.

fecA marker (data not shown). These data and the finding that *fec* is a chromosomal locus in the closely related strain *Escherichia coli* K-12 (19) make it difficult to assess whether *fecA* is consistently carried on the SRL PAI in *int*-positive strains.

The SRL PAI is widespread among Str^r Amp^r Chl^r Tet^r *Shigella* isolates. In order to examine whether the presence of the SRL PAI markers in the Str^r Amp^r Chl^r Tet^r isolates indicated the presence of an intact, ordered set of genes representing the SRL PAI, a representative of each *Shigella* species was chosen for further characterization. Genomic DNA from the following strains was analyzed by PCR for physical linkage of 13 loci spanning the YSH6000 SRL PAI: SBA1299 (*S. flexneri* 1a), SBA1303 (*S. sonnei* form I), SBA1308 (*S. boydii* 4), and SBA1391 (*S. flexneri* 4a), which are positive for the *int*, *fecA*, and *orf58* markers, and SBA1304 (*S. dysenteriae* 3) and SBA1386 (*S. boydii* 8), which are positive for the *int* and *orf58* markers but negative for the *fecA* marker (Fig. 1). In strains SBA1299, SBA1308, and SBA1391, physical linkage of all 13 loci was demonstrated, revealing the presence of elements that appear to have a structure similar to that of the YSH6000 SRL PAI (Fig. 1). All loci with the exception of *orf45* and *orf48* were also linked in strain SBA1303 (Fig. 1). As *orf45* and *orf48* sequences bearing the amplifying primer binding sites were present in SBA1303 (data not shown), it was considered likely that the insertion of additional DNA may account for the failure to amplify this region. Although the PCR extension times were increased to allow the amplification of 12- to 14-kb products, no products were amplified, implying that either the loci are not linked or these regions exceed 14 kb. In SBA1304 and SBA1386, which were negative for the *fecA* marker, all loci except for a large region extending from *tetC* to *orf48* were linked (Fig. 1). Thus, it appears that absence of the *fecA* marker may correspond to the absence of a larger region in both SBA1304 and SBA1386. Hence, despite minor differences in linkage patterns and sizes, these data demonstrate the presence of elements in all four *Shigella* species that have structures similar to those of the *S. flexneri* 2a strain YSH6000 SRL PAI.

To test whether these SRL PAI-like elements were plasmid or chromosomally borne, Eckhardt gel electrophoresis was performed with these six strains and DNA was analyzed by

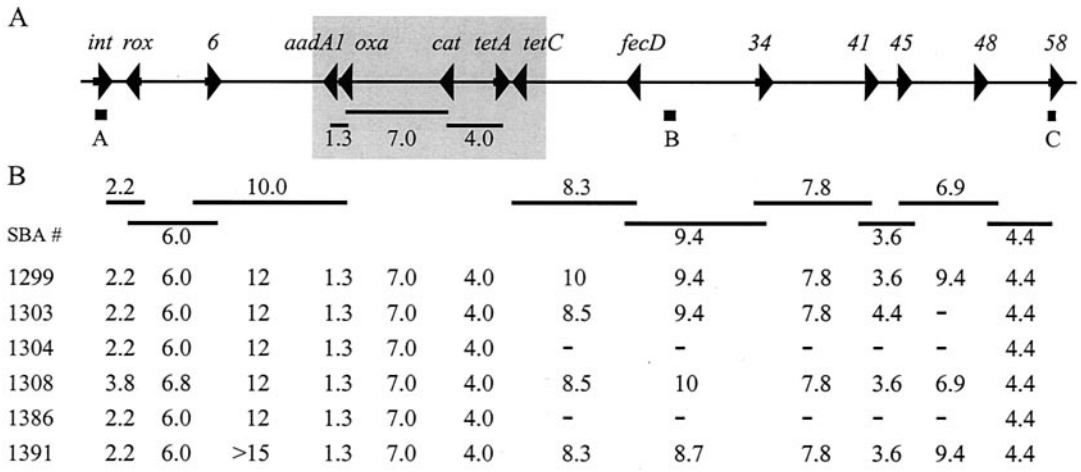

FIG. 1. Schematic representation of the genetic organization of the SRL PAI in strain YSH6000. (A) The SRL PAI is represented as a thin black line, with the positions and directions of relevant genes and open reading frames represented as arrows. The SRL is indicated by the large shaded box. Thick lines represent the regions amplified in linkage analysis of the SRL, with the corresponding numbers indicating their sizes (in kilobases) in the YSH6000 SRL PAI. Small black boxes represent the positions of the markers that were probed by Southern analysis or PCR amplification: box A, *int*; box B, *fecA*; and box C, *orf58*. (B) Thick black lines represent the regions amplified in linkage analyses of SBA1299 (*S. flexneri* 1a), SBA1303 (*S. sonnei* form I), SBA1304 (*S. dysenteriae* 3), SBA1308 (*S. boydii* 4), SBA1386 (*S. boydii* 8), and SBA1391 (*S. flexneri* 4a), with the corresponding numbers indicating their sizes (in kilobases).

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TABLE 4. Linkage of resistance determinants in Str^r Amp^r Chl^r Tet^r *Shigella* isolates

Linkage[a]			No. of strains (n = 31)
aadA1 + *oxa-1*	*oxa-1* + *cat*	*cat* + *tetA*	
−	−	−	1
−	−	+	1[b]
+	−	−	1[c]
+	−	+	3
+	+	−	3
+	+	+	22

[a] +, PCR linkage was demonstrated; −, PCR linkage was not demonstrated.
[b] Strain SBA1298.
[c] Strain SBA1388.

Southern hybridization with a *cat* gene-specific probe (data not shown). The results confirmed a chromosomal location for the *cat* gene (and, therefore, the SRL PAI-like elements) in each of the six *Shigella* isolates. In contrast, strains SBA1298 (*S. flexneri* 3c) and SBA1388 (*S. flexneri* 2a), in which resistance to STR, AMP, CHL, and TET was not thought to be related to the presence of the SRL PAI because of the absence of an *int* gene, possessed a plasmid-borne *cat* gene.

Chromosomal deletion of the SRL locus. The SRL PAI is known to undergo precise, integrase-mediated deletion from the chromosome in *S. flexneri* 2a strain YSH6000. This deletion can be identified by selection of strains on fusaric acid medium, followed by PCR amplification of the deletion point junction (46). To determine whether the elements in SBA1299, SBA1303, SBA1304, SBA1308, SBA1386, and SBA1391 were capable of undergoing similar deletions, these strains were also grown on fusaric acid medium. However, only strains SBA1299 (*S. flexneri* 1a) and SBA1391 (*S. flexneri* 4a) were found to be fusaric acid sensitive and therefore to be suitable for selection of the loss of the PAI by this method. Tet^s derivatives of SBA1299 and SBA1391 obtained by this selection method were subsequently tested for their susceptibilities to the antibiotics STR, AMP, and CHL. All SBA1299 derivatives were found to be Str^r and were thought to possess some additional determinant other than the SRL *aadA1* gene conferring this resistance. Thus, 10 Str^r Amp^s Chl^s Tet^s SBA1299 derivatives and 2 SBA1391 Str^s Amp^s Chl^s Tet^s derivatives were considered potential PAI deletants and were therefore selected for PCR characterization. However, amplification of *serX*, at the SRL PAI deletion point junction, was not successful for any of the SBA1299 and SBA1391 derivatives, suggesting that precise deletion of the element had not occurred.

Each derivative was tested by PCR for the presence of the markers *int*, *fecA*, and *orf58*. Nine SBA1299 derivatives and both of the SBA1391 derivatives were negative for all of these markers and were therefore consistent with PAI deletants. The deletion derivatives were further tested by PCR for the presence of the chromosomally borne *csgA* gene, which is approximately 7 kb downstream of *orf58* in YSH6000. This gene was absent from all nine SBA1299 derivatives and both of the SBA1391 derivatives, indicating that DNA flanking the PAI-like element was also deleted with the PAI markers. In YSH6000, imprecise deletion of the SRL PAI, which removes flanking chromosomal DNA including the *csgA* gene, occurs from the *mdoA* and the *putA* loci due to the presence of flanking IS*91* elements on the chromosome (46). The deletion of this region, known as the MRDE, was tested by PCR amplification across the deletion point junction identified in YSH6000. This amplification was positive for four of the SBA1299 derivatives and both of the SBA1391 derivatives, demonstrating that deletion events similar to MRDE deletion in YSH6000 had occurred in at least six of the derivative strains (data not shown). In strains in which the MRDE deletion point junction was not amplified, it is likely that loss of the PAI markers occurs by another type of deletion mechanism. As insertion sequence (IS) elements have been implicated in deletion of part or all of the SRL PAI in strain YSH6000 (46), it is possible that additional IS elements present in SBA1299 may be involved in the deletion of the SRL PAI from these strains.

Distribution of the SRL PAI among other members of the family *Enterobacteriaceae*. The dissemination of the SRL PAI throughout many *Shigella* species, in conjunction with the report that the *Yersinia* high-PAI (HPI) is present in different members of the family *Enterobacteriaceae* (43, 44), prompted the investigation of several other enteric pathogens for the presence of the SRL PAI. Twenty-eight members of the family *Enterobacteriaceae* including representatives of enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), *Salmonella enterica* serovar Typhimurium, and *Yersinia enterocolitica* were tested for antibiotic resistance. Among the 28 strains, only a single *Y. enterocolitica* strain was found to have a Str^r Amp^r Chl^r Tet^r phenotype. However, PCR linkage of the SRL determinants was not achieved in this strain, suggesting that the resistance profile was not due to the presence of the SRL.

Isolates were also tested for the presence of *int* and *fecA* by Southern hybridization and for the presence of *orf58* by PCR. *fecA* was detected in one EIEC, two ETEC, and three EAEC strains, while *orf58* was detected in four EIEC strains and one EHEC strain. Regions hybridizing to the *int* probe were found in two EHEC strains and one EPEC strain (data not shown). Recent sequencing of the EHEC O157:H7 EDL 933 genome revealed the presence of a 1,203-bp *orf*, present on identical O islands (O islands 43 and 48), whose sequence is nearly identical to that of the SRL PAI *int* gene (33). These genes share 97% nucleotide sequence identity over the first 1,189 bp but diverge abruptly after that point. PCR amplification with primers designed to anneal to this divergent region confirmed that the *int*-positive EPEC and EHEC strains identified in this study yielded an amplification product with primers specific for the EHEC integrase gene but not with primers specific for the SRL PAI *int* gene (data not shown).

Chromosomal insertion sites of the SRL PAI-related elements. The YSH6000 SRL PAI was found to be inserted into the 3′ end of the tRNA gene *serX*, and the *int* gene was situated adjacent to the 14-bp direct repeat with a sequence identical to that of the 3′ terminus of the *serX* gene (26). By pairing a primer situated in the *int* gene with one downstream of *serX*, the presence of the SRL PAI in this position may be confirmed by PCR. This method was used to test the 29 *int*-positive *Shigella* strains (Table 3) and revealed that in 13 of these strains, *int* was linked to *serX*. *E. coli* possesses a paralog of *serX*, *serW*, at minute 20. At the nucleotide level, *serW* displays 100% identity to *serX* and thus also contains the 14-bp se-

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quence thought to be targeted by the SRL PAI integrase (26, 46). As it has been noted that the HPI and the *she* PAI are also found to be inserted into different paralogs of the *asn* and *phe* tRNA genes, respectively (1, 6), the *Shigella* isolates were also tested for linkage of *int* to a sequence downstream of *serW*. Ten strains were found to be positive for this association (Table 3). Six strains were tested for the linkage of *orf58* at the right end of the PAI to sequences upstream of both tRNA genes, but linkages could not be demonstrated. Five strains exhibited linkage of *orf58* to sequences upstream of *serX* (Table 3). In the remaining one strain, both *int* and *orf58* were not found to be linked to either the *serX* or the *serW* sequence. Interestingly, in the two *int*-positive EHEC strains, *int* was linked to *serX* and *serW*, and in the *int*-positive EPEC strain, *int* was linked to *serX* (data not shown).

DISCUSSION

In this study we have demonstrated that loci showing similarity to the SRL, which mediates resistance to STR, AMP, CHL, and TET, are widespread among *Shigella* isolates of all four species. Additionally, the *S. flexneri* 2a strain YSH6000 SRL PAI *int* and *orf58* markers were found to be correlated with these loci, suggesting that the SRL PAI may be linked to the dissemination of the SRL throughout *Shigella* species. PCR linkage analysis revealed that the SRL PAI is essentially conserved in organization in these strains, although some variation was observed. Interestingly, the *fec* locus, which is present on the YSH6000 SRL PAI, is not always present in strains that carry the SRL-related loci and other SRL PAI markers. PCR linkage analysis of two such strains revealed that although the majority of the PAI organization and structure was conserved, the absence of the same region which spans the entirety of the *fec* locus and some surrounding sequences was observed in both isolates. Thus, it appears that the *fec* locus may have been lost from these strains or that these elements may represent a *fec*-negative predecessor of the SRL PAI. The absence of the same *fec*-carrying regions from these strains and the fact that the locus is flanked with phage and IS-related sequences (26), together with the presence of *fec* markers in PAI-negative *Shigella* isolates and its sporadic distribution throughout *E. coli* (15, 19, 34; this study), may suggest that the *fec* locus is independently mobile. Interestingly, *orf58*, which was found to be present in 97% of *int*-positive *Shigella* strains, was also present in 11% of *int*-negative strains. As *orf58* exhibits some similarity to the IS*1328* transposase of *Y. enterocolitica* (26), it is possible that the presence of this marker in *int*-negative strains may be due to its independent mobility.

Structural variation is not unique to the SRL PAI and is also found at one end of the *Yersinia* HPI (38) and the *Shigella she* PAI (1). Interestingly, unlike the *she* PAI, which has a great deal of diversity in structure, there appear to be few structural SRL PAI variants in *Shigella*, on the basis of the results obtained with the markers tested. The extraordinarily high degree of correlation between the presence of the *int* locus and the presence of SRL-related loci is dissimilar to the case for the *Vibrio cholerae* PAI-like SXT element, in which the presence of *int* sequences of the element was found to be associated with the presence of a variety of different multiresistance gene clusters (16). Interestingly, previous studies have demonstrated that the IS*1*-flanked YSH6000 SRL is capable of independent excision from the SRL PAI, a phenomenon which might allow its independent spread (46). However, thus far there have been no SRL-positive isolates that do not carry the SRL PAI *int* marker, suggesting that the SRL PAI acquired the SRL before widespread dissemination or that there is a strong selective pressure to maintain the SRL as part of the SRL PAI.

In this study, by using primers specific for the gene encoding the EHEC *int* O islands 43 and 48, which are remarkably similar to the SRL PAI *int*, sequences were amplified from two Str^s Amp^s Chl^s Tet^s EHEC isolates and one Str^s Amp^s Chl^s Tet^s EPEC isolate. The identical O islands 43 and 48 do not encode antibiotic resistance but show the same insertion specificity, and like the SRL PAI in YSH6000, they are also flanked by the same 14-bp direct repeat which corresponds to the 3′ ends of the *serX* and *serW* tRNA genes (33). As we have determined that the SRL PAI may also occupy both of these sites in the *Shigella* chromosome, the considerable similarity between these integrases appears to be sufficient to confer the insertion site specificities of both elements. Excluding this *int* sequence similarity, the EHEC O islands show no nucleotide sequence similarity to the SRL PAI. However, analysis of open reading frames revealed that the EHEC O islands and the SRL PAI share a common backbone, and on the basis of this similarity, it appears likely that both the SRL and *fec* regions were acquired as distinct elements by the SRL PAI. It is interesting that although O islands 43 and 48 exist in some *E. coli* strains, the SRL-encoded multiple antibiotic resistance was never correlated with these markers in the *E. coli* strains investigated. The SRL PAI markers *orf58* and *fecA* were also present in some *E. coli* isolates in this study. However, as discussed above, *orf58* shows some similarity to an IS element transposase and the *fec* locus is a common chromosomal marker in *E. coli*; therefore, their presence in these strains was not unexpected. Hence, although several enteric pathogens possess markers of the SRL PAI, no one strain contained more than a single marker, suggesting that the SRL PAI is not present in the strains tested in this study. Thus, although elements bearing resemblance to the YSH6000 SRL PAI are relatively common in *Shigella*, it appears that similar elements are not present in the other enteric pathogens. Such a genus-specific distribution is in contrast to the HPI, which is found to be widely distributed throughout the members of the family *Enterobacteriaceae* (8, 21, 43, 44, 52). It is possible that the early and extensive use of antibiotics for the treatment of *Shigella* infections may have influenced the distribution of the SRL PAI. It would be interesting to determine whether SRL PAI-related elements were present in enterobacterial strains isolated before the introduction of antibiotic therapy, as it has been noted that conjugative plasmids which presumably gave rise to R plasmids were quite common in enterobacteria of the preantibiotic era (18).

It is generally believed that PAIs have been transferred horizontally (11–13, 24). However, such transfer has been demonstrated only for the PAI-like *V. cholerae* self-transmissible conjugative SXT element (17), the *V. cholerae* VPI, and the staphylococcal SaPI (20, 40). Although horizontal transfer of the SRL PAI has not been demonstrated, previous studies have shown that it undergoes site-specific, integrase-dependent deletion from the chromosome (46). Additionally, this element appears to be widespread throughout *Shigella*, appears to be

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absent from some *Shigella* and *E. coli* strains, and may occupy at least two distinct chromosomal loci. Together, these data support the hypothesis that the SRL PAI is, or was, mobile at some point. If this hypothesis is correct, PAIs may be an additional mechanism of antibiotic resistance spread.

It is interesting that the SRL itself incorporates components of plasmid-, transposon-, and integron-encoded resistance determinants, being composed of loci that resemble the NR1 plasmid r determinant, various transposons (including Tn*2670*, Tn*21*, and Tn*10*), and the Tn*21*-borne integron In2, which bears an additional *oxa-1* gene cassette (26, 36). Indeed, a clinical study of isolates of the family *Enterobacteriaceae* noted that in addition to integron-borne antibiotic resistance, an association existed between the presence of integrons and non-integron-borne resistance genes. This genetic linkage of integrons with non-integron-borne factors encoding resistance to older antibiotics such as CHL and TET (50) is exemplified by the SRL and suggests that similar loci may be more common throughout the family *Enterobacteriaceae*.

Multiantibiotic resistance was originally reported to be plasmid borne (49) and is still considered to be the primary form of resistance in *Shigella* isolates. However, we have demonstrated linkage of the SRL PAI *int* to the chromosomal markers *serX* and *serW* in the majority of strains that carry the SRL-related loci, suggesting that these resistance determinants are not plasmid borne but reside on the chromosome. Further characterization of six such strains illustrated that the PAI-borne SRL was indeed situated on the chromosome. Additionally, there have been increasing reports of chromosomally linked determinants of resistance to multiple antibiotics, especially AMP, CHL, TET, and STR (7, 10, 25). A study from Somalia reported that all but 3 of 112 *S. flexneri* strains isolated between 1983 and 1989 carry chromosomally linked determinants for resistance to the antibiotics AMP, CHL, TET, and spectinomycin (7). Both spectinomycin resistance and STR resistance are conferred by the *aadA1* cassette (36). Similarly, a study from Tanzania reported that the common combination of resistance to AMP, CHL, and TET was not directly related to plasmid profiles and that AMP resistance was usually due to the presence of *oxa-1* (31). Previously, *oxa-1*-mediated AMP resistance was less common in gram-negative bacteria than resistance mediated by TEM β-lactamases (39). Importantly, these data indicate that chromosomal loci bearing some resemblance to the PAI-borne SRL not only are present in the Japanese and Australian isolates investigated in this study but also may be prevalent in a wider range of *Shigella* strains distributed globally.

The SRL PAI is the first PAI to be linked with antibiotic resistance in a wide variety of bacterial strains. However, resistance to the antibiotics sulfamethoxazole, trimethoprim, and STR are encoded on the PAI-like SXT element (48), while *Salmonella* genomic island 1, which has been detected in a few *Salmonella* serovars, also encodes multidrug resistance (4, 5). Thus, dissemination of antibiotic resistance genes on PAIs and PAI-like elements may be an important mechanism of horizontal transfer not only among *Shigella* isolates but also within other bacterial populations. The clinical implications of such transfer are potentially serious, as selection for these elements poses the danger not only of increasing the prevalence of multiantibiotic resistance but also of changing the virulence profiles of such strains.

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