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The aerobactin iron transport system genes in *Shigella flexneri* are present within a pathogenicity island

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Summary

Genes encoding the synthesis and transport of aerobactin, a hydroxamate siderophore associated with increased virulence of enteric bacteria, were mapped within a pathogenicity island in *Shigella flexneri*. The island, designated SHI-2 for *Shigella* pathogenicity island 2, was located downstream of *selC*, the site of insertion of pathogenicity islands in several other enteric pathogens. DNA sequence analysis revealed the presence of multiple insertion sequences upstream and downstream of the aerobactin genes and an integrase gene that was nearly identical to an *int* gene found in *Escherichia coli* O157:H7. SHI-2 sequences adjacent to *selC* were similar to sequences at the junction between *selC* and pathogenicity islands found in *E. coli* O157:H7 and in enteropathogenic *E. coli*, but the junctions between the island and downstream *yic* genes were variable. SHI-2 also encoded immunity to the normally plasmid-encoded colicins I and V, suggesting a common origin for the aerobactin genes in both *S. flexneri* and *E. coli* pColV. Polymerase chain reaction and Southern hybridization data indicate that SHI-2 is present in the same location in *Shigella sonnei*, but the aerobactin genes are not located within SHI-2 in *Shigella boydii* or enteroinvasive *E. coli*. *Shigella dysenteriae* type 1 strains do not produce aerobactin but do contain sequences downstream of *selC* that are homologous to SHI-2. The presence of the aerobactin genes on plasmids in *E. coli* pColV and *Salmonella*, on a pathogenicity island in *S. flexneri* and *S. sonnei* and in a different chromosomal location in *S. boydii* and some *E. coli* suggests that these virulence-enhancing genes are mobile, and they may constitute an island within an island in *S. flexneri*.

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Introduction

The acquisition of iron is a problem common to human bacterial pathogens. At least two different strategies are used by bacteria to compete with the host for the limited supply of this essential element. One mechanism is the expression of receptors for host iron complexes such as transferrin, lactoferrin and haemoglobin, which enable some pathogens to use these as a source of iron (Morton and Williams, 1990; Hanson *et al*., 1992; Cornelissen and Sparling, 1994). Another mechanism is the synthesis and secretion of siderophores, low-molecular-weight, high-affinity, iron-binding compounds that can remove iron from host sources and facilitate its uptake by the bacterium (Neilands *et al*., 1987; Crosa, 1989).

Among the *Enterobacteriaceae*, iron uptake by direct utilization of host sources and via siderophores has been observed. Growth on haem or haemoglobin as the sole iron source occurs in some isolates of *Yersinia* spp. (Hornung *et al*., 1996), *E. coli* (Law *et al*., 1992; Torres and Payne, 1997) and *Shigella* (Wyckoff *et al*., 1998). Siderophores are produced by most of these bacteria, but there is variation in the type of siderophore produced. Analysis of the *Shigella* spp., which multiply within colonic epithelial cells and produce dysentery, and clinical isolates of *E. coli*, which produce diseases ranging from mild diarrhoea to septicaemia and meningitis, revealed the presence of two different siderophore-mediated iron transport systems. The catechol siderophore enterobactin is produced by *E. coli* (Rogers, 1973; Earhart, 1996) and by some, but not all, *Shigella* (Perry and San Clemente, 1979; Payne *et al*., 1983). A second siderophore, aerobactin, is synthesized by *Shigella flexneri* and *Shigella boydii* (Lawlor and Payne, 1984). This hydroxamate is also synthesized by some *Shigella sonnei* and *E. coli* clinical isolates (Payne, 1988).

Mapping of several iron transport loci suggests that horizontal transmission of the genes has occurred. In *S. dysenteriae* type 1, genes encoding the haem transport system are contained on a 9.1 kb region located between two open reading frames (ORFs) of the *E. coli* K-12 map (Wyckoff *et al*., 1998). These genes are present in two distantly related lineages of the *E. coli* and *Shigella* group but not in other, more closely related strains, suggesting that there was more than one occurrence of acquisition or loss of these genes (Torres and Payne, 1997; Wyckoff *et al*., 1998). An iron acquisition system of *Yersinia* spp.

that maps to the high pathogenicity island has also been found in some pathogenic *E. coli* (Bearden *et al*., 1998; Buchrieser *et al*., 1998; Schubert *et al*., 1998). Similarly, the aerobactin genes have characteristics of transmissible elements. They are found on plasmids, including pColV (Williams, 1979) and F1*me* (Colonna *et al*., 1985) in certain strains of *E. coli* and *Salmonella*, respectively, but are chromosomal in *Shigella* spp. (Lawlor and Payne, 1984) and in other *E. coli* and *Salmonella* isolates (McDougall and Neilands, 1984; Marolda *et al*., 1987).

This study was undertaken to map the aerobactin genes in *Shigella* and analyse the chromosomal region in which they are found in order to understand better the mechanism for the distribution and possible transmission of these genes among pathogenic bacteria.

Results

Genetic organization of the S. flexneri *aerobactin island*

Cosmid clones containing the aerobactin biosynthesis and transport genes have been isolated previously from SA100 (Lawlor *et al*., 1987; Marolda *et al*., 1987). To characterize the genes surrounding this locus, the cosmid DNA was subcloned and the sequence determined. Analysis of the DNA sequence (GenBank accession no. AF097520) indicated the genetic organization shown in Fig. 1. Based on the similarities between this region and pathogenicity islands described below, we designated this 30 kb *S. flexneri* region SHI-2 for *Shigella* pathogenicity island 2.

The first gene within this cluster, *int*, has homology to the bacteriophage P4-like integrases (Table 1). The SHI-2 *int* is almost identical to the *int* gene recently described in a pathogenicity island, termed LEE for locus of enterocyte effacement, of *E. coli* O157:H7 (Perna *et al*., 1998). However, the homology between SHI-2 and *E. coli* O157:H7 LEE ends immediately 3′ of the *int* genes (Fig. 2B), indicating that these two strains do not contain the same island. Because the *S. flexneri int* gene has homology to the integrase associated with retronphage φR73 (Inouye *et al*., 1991) (Table 1), the strain was analysed for the presence of retronphage msDNA. No msDNA was detected in SA100, although it was detected in strains known to carry the retronphage (data not shown).

Downstream of *int* are a number of open reading frames (ORFs) with homology to insertion sequences and transposases (Fig. 1, Table 1). These include copies of IS*1*,

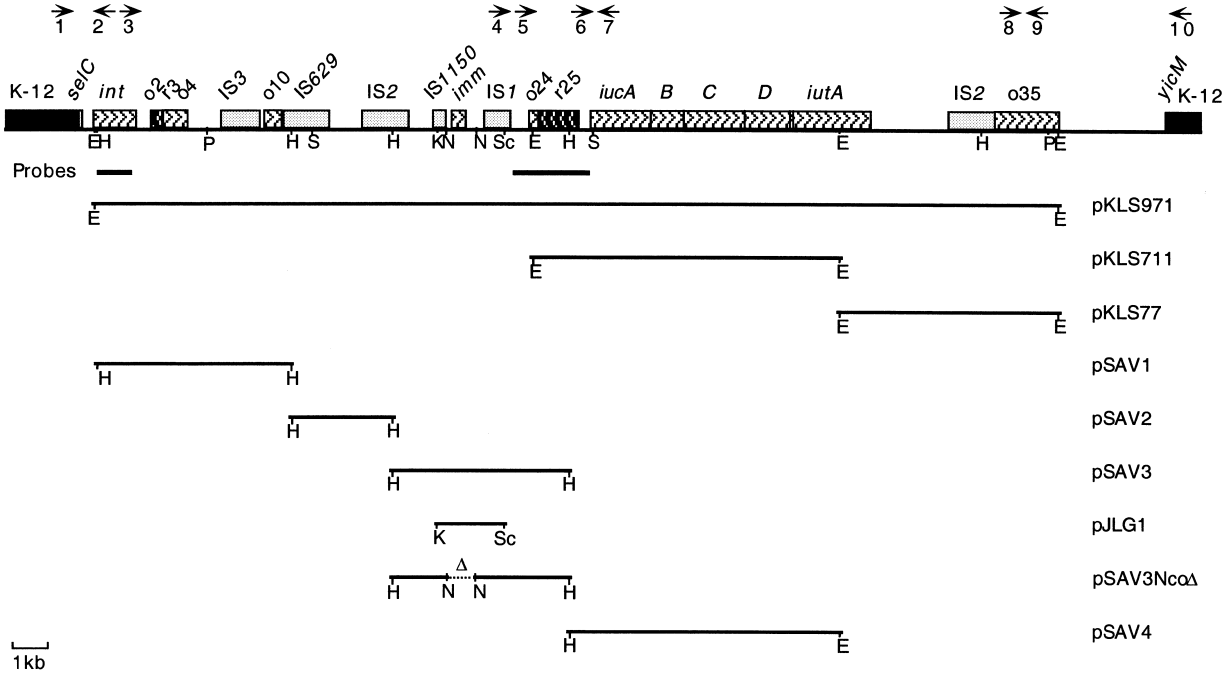

Fig. 1. Map of the *S. flexneri* pathogenicity island. The sequence of the DNA surrounding the aerobactin genes in *S. flexneri* SA100 was determined and the locations of the open reading frames inferred from the sequence analysis. The solid black boxes indicate sequences present in *E. coli* K-12 and *S. flexneri*. The patterned boxes indicate the size and position of ORFs within the island. o indicates reading frames whose direction of transcription is left to right, and r indicates those on the opposite strand. The DNA sequence between o35 and *vicM* has not been determined. The following letters below the line indicate relevant restriction sites: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; K, *Kpn*I; N, *Nco*I; Sc, *Sac*I. Only the *Eco*RI and *Hin*dIII sites are shown in full. The two bars below the map indicate the probes used for Southern hybridization. The numbered arrows indicate the approximate positions of primers used for PCR amplification of the various junction fragments. Primer 10 is in *yicK*, which is found upstream of *yicM* in *E. coli* K-12 but is not present *in S. flexneri*.

Table 1. ORFs within the *S. flexneri* pathogenicity island.

ORF or insertion sequence	Location[a]	Length (bp)	Similar sequences[b]	Percentage nucleotide (protein) identity
orf1(*int*)	257–1465	1209	O157:H7 *int* (Perna *et al.*, 1998)	89 (95)
			Retronphage ϕR73 *int* (Inouye *et al.*, 1991)	65 (69)
orf2	1880–2377	498	None	
rorf3	2203–1895	309	None	
orf4	2235–2921	687	None	
IS*3*	3867–4976	1109	*S. dysenteriae* IS*3*	80
orf10	5098–5613	516	None	
IS*629*	5655–6961	1306	*S. sonnei* IS*629* (Ohtsubo and Matsutani, 1990)	99
IS*2*	7899–9231	1332	*E. coli* IS*2* (Ghosal *et al.*, 1979)	98
IS*1150* OrfB	10014–10381	367	*E. coli* IS*150* OrfB	(59)
orf21 (*imm*)	10459–10854	396	None	
IS*1*	11379–12130	751	*E. coli* IS*1F*	99
orf24	12660–12926	267	None	
rorf25	14072–12882	1191	Tetracycline resistance antiporter (Hillen and Schollmeier, 1983)	(6, [34% over a 52-amino-acid region])
orf26	13168–13410	243	None	
orf27	13720–14145	426	pColV-K30 aerobactin promoter region	93
rorf28	14091–13843	249	pColV-K30 aerobactin promoter region	93
orf29–orf32 (*iucA,B,C,D*)	14500–20200	5700	Aerobactin biosynthesis genes *iucA-iucD* (Lawlor and Payne, 1984)	>90
orf33 (*iut*)	20280–22480	2200	pColV-K30 aerobactin receptor gene *iutA* (Krone *et al.*, 1987)	87 (86)[c]
IS*2*	24430–25762	1332	*E. coli* IS*2*	98
orf35	26040[d]	>1100	SFMD precursor protein (SWISSPROT P77468)	(13)

a. Location is given as nucleotide position, numbering from the first base in the island 3′ of *selC*.
b. Homology based on BLASTN and BLASTX database analyses.
c. Homology based on sequence of 800 bp of 3′ end of the ORF.
d. The end-point of this ORF has not been determined.

IS*3*, IS*629*, part of IS*1150* and two copies of IS*2,* one on each side of the aerobactin locus (Fig. 1, Table 1). The genes for aerobactin synthesis and the aerobactin receptor were mapped within this region, and sequencing within this operon indicated that the *S. flexneri* DNA sequence was essentially the same as that of the pColV-K30 aerobactin genes (Table 1). To determine whether the presence of aerobactin genes in the *Shigella* chromosome

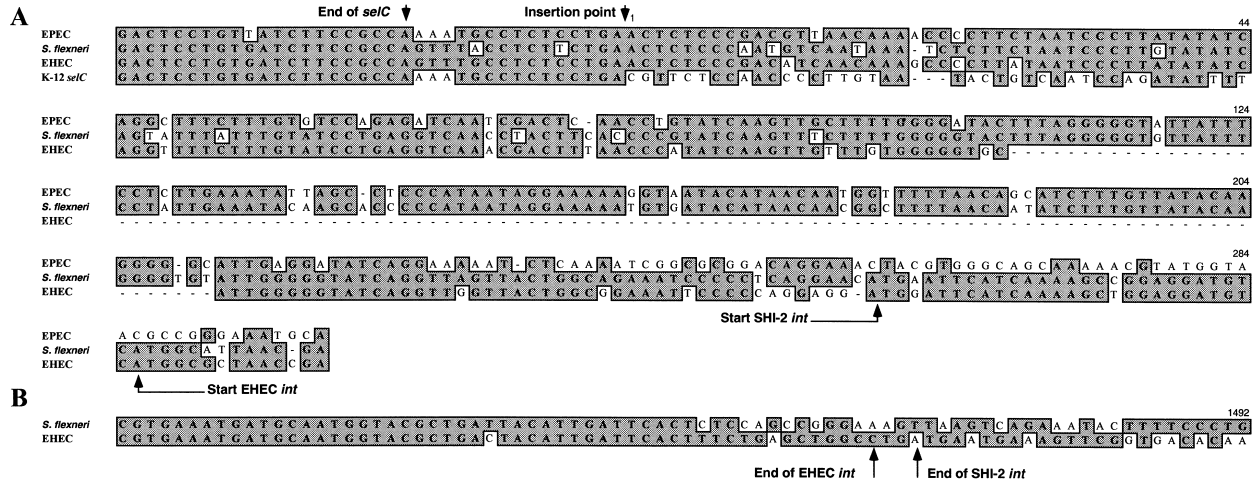

Fig. 2. A. Comparison of the junctions between *selC* and the pathogenicity island of *S. flexneri* SA100 and the LEEs from EPEC (McDaniel *et al.*, 1995) and EHEC (Perna *et al.*, 1998) strains. The 3′ end of the *selC* sequence of *E. coli* K-12 is also shown. The insertion point is considered the point at which the sequences in the strains containing islands diverge from *E. coli* K-12 and is shown as nucleotide 1 in the SA100 island sequence.
B. Comparison of the 3′ end of the *int* sequences of SHI-2 and the EHEC LEE.

Table 2. Conservation of sequences flanking *iuc* in aerobactin-producing strains[a].

	Amplification with primer pairs[b]			
Strain	6,7	5,7	4,7	8,9
S. flexneri SA100, M90T	+	+	+	+
S. sonnei PB66	+	+	−	+
S. boydii 0-1392	+	+	−	−
E. coli pColV-K30	+	−	−	−
E. coli 1107-81	+	−	−	−

a. The presence of aerobactin genes was determined by Southern hybridization and confirmed by bioassays (Lawlor and Payne, 1984; Lawlor *et al.*, 1987) for aerobactin synthesis and transport.
b. Location of primer sequences shown in Fig. 1. + indicates amplification of a DNA fragment of the size predicted from the SA100 sequence; − indicates no amplification.

is the consequence of insertion of the ColV plasmid, the sequences flanking the aerobactin operons were compared. Polymerase chain reaction (PCR) and sequence analysis of the DNA flanking the aerobactin operons showed that *S. flexneri* differs from pColV-K30 (Fig. 1, Table 2); there was no PCR amplification of sequences upstream of pColV *iucA* when primers derived from the *S. flexneri* sequence more than 1 kb upstream were used (Table 2). Amplification with a primer derived from the *iucA* sequence (Fig. 1, primer 7) and a primer beginning 426 bases upstream of the *iucA* start codon (primer 6) produced a 640 bp product in SA100 and pColV (Table 2). Primer pairs 5,7 and 4,7 amplified the expected 1879 bp and 2829 bp fragments in *S. flexneri*, but no products were observed with either primer pair when pColV DNA was used (Table 2). These PCR results are in agreement with earlier studies using Southern hybridization that showed conservation of the aerobactin genes, but not the flanking DNA, in *S. flexneri* and *E. coli* pColV (Lawlor *et al.*, 1987; Marolda *et al.*, 1987).

Association between aerobactin and colicin immunity genes

Although the genes adjacent to the *S. flexneri* aerobactin genes are not identical to those of pColV, there is a common feature to both regions. Both pColV and sequences upstream of the *S. flexneri* aerobactin operon encode immunity to colicin V (Table 3). The *S. flexneri* cosmid (pKLS971) and subclones pSAV3 and pJLG1 (Fig. 1) encode immunity to colicins V and Ib and to a colicin produced by *S. flexneri* SA100 (Table 3). An ORF, designated *imm*, is required for protection against the colicins; deletion of an *Nco*I fragment encompassing this gene (Fig. 1, pSAV3NcoΔ) eliminates protection against ColV, ColIb or the *S. flexneri* colicin (Table 3). Unlike the plasmid-encoded ColV and ColI immunity genes, however, the *S. flexneri* gene encoding colicin immunity is not closely linked to the colicin synthesis genes. The *S. flexneri* cosmid pKLS971, which contains ≈ 10 kb upstream and downstream of the immunity gene, was tested for colicin production, but no detectable colicin was produced by *E. coli* strains carrying this cosmid (data not shown). The colicin encoded by *S. flexneri* has not been characterized but, like colicins V and I, its receptor is the Cir protein. A *cir* mutant, JK458, was resistant to colicins V and I and the *S. flexneri* colicin, while the parent Cir^+ strain JK360 was sensitive to all three (Table 3).

Additional aerobactin-producing strains of *Shigella* were tested for sensitivity to these colicins (Table 3). *S. boydii* 0-1392, like *S. flexneri* SA100, was not sensitive to colicins V and Ib or to the *S. flexneri* colicin. This may reflect the presence of immunity genes in *S. boydii*, or this strain may lack the receptor for these colicins. The *S. sonnei* strain, PB66, was sensitive to all three colicins and lacked sequences homologous to *S. flexneri imm* (data not shown). Therefore, there is a linkage between aerobactin genes and a colicin immunity gene in *S. flexneri* and pColV strains, but not in the other strains tested.

Association between SHI-2 and the selC *tRNA gene*

To obtain additional evidence about the possible mechanism by which the aerobactin genes might have spread

Table 3. Identification of genes within *Shigella* spp. and *E. coli* encoding immunity or resistance to colicins.

	Sensitivity to[a]		
Strain	Colicin V	Colicin Ib	*S. flexneri* colicin
RM1058	+	+	+
RM1058/pKLS971	−	−	−
RM1058/pSAV3	−	−	−
RM1058/pSAV3ΔNco	+	+	+
RM1058/pJLG1	−	−	−
S. sonnei PB66	+	+	+
S. boydii 0-1392	−	−	−
JK354 (Cir^+)	+	+	+
JK458 (Cir^-)	−	−	−
LG1315 (pColV K-30)	−	−	−

a. Strains producing the indicated colicins were stabbed into agar and overlaid with the strain to be tested for sensitivity; + indicates a zone of inhibition > 5 mm around the stab.

within the *Enterobacteriaceae*, the junction between the aerobactin region and sequences common to the *S. flexneri* and *E. coli* K-12 chromosomes was analysed. The junction at the 5′ end of the island was found to be immediately downstream of the *selC* gene (Fig. 2). This location is the site of insertion of several pathogenicity islands in other enteric pathogens, including the LEE in enteropathogenic (EPEC) (McDaniel *et al*., 1995) and O157:H7 enterohaemorrhagic (EHEC) *E. coli* (Perna *et al*., 1998), Pai I of uropathogenic strains (Blum *et al*., 1994) and SPI-3 in *Salmonella enteritidis* (Blanc-Potard and Groisman, 1997). Comparison of the junction sequences among the *E. coli* and *Shigella* strains (Fig. 2A) indicates that the SHI-2 junction has homology to the EPEC and EHEC LEEs. The sequences immediately downstream of *selC* in *S. flexneri* are most closely related to those of the EPEC LEE, while the first gene in the island, *int*, is homologous to the EHEC *int* gene (Fig. 2A). The EHEC LEE appears to contain a deletion in the sequence between *selC* and *int* compared with the EPEC and *S. flexneri* islands (Fig. 2A). The *S. flexneri* DNA downstream of the *int* gene was not homologous to the sequence downstream of the EHEC *int* (Fig. 2B) or to any other sequences in the DNA database. Thus, this DNA sequence analysis indicates that the mechanism of insertion of the LEEs and SHI-2 may have common features, but the genes within the islands are distinct.

The junction at the 3′ end of the island was also analysed and compared with other islands inserted at *selC*. The presence of LEE in EPEC and EHEC strains is associated with deletions in *yicK* and *yicL*, which map downstream of *selC* in *E. coli* K-12 (McDaniel *et al*., 1995; Perna *et al*., 1998). *S. flexneri* also lacks *yicK* and *yic*L sequences, but the deletion in *Shigella* appears to be larger than in the EPEC or EHEC and extends into *nlpA*, the gene downstream of *yicL* (Table 4 and Fig. 1).

Presence of SHI-2 in other Shigella *and* E. coli *strains*

To determine whether SHI-2 was present in other *Shigella* and *E. coli* and to determine whether the chromosomal aerobactin genes are always found within SHI-2, we used PCR amplification and Southern hybridization of selected island and junction fragments. The results of these assays, which are shown in Tables 2 and 4 and Fig. 3 and discussed below, suggested the genomic organizations depicted in Fig. 4.

The presence of the aerobactin genes in *Shigella* and *E. coli* isolates was determined initially by Southern hybridization with an *iuc* probe, and by chemical and bioassays for aerobactin synthesis and transport (Table 2). Three primer pairs that amplify the DNA sequences upstream of the aerobactin operon in *S. flexneri* (Fig. 1, primer pairs 4,7, 5,7 and 6,7) and one that amplifies downstream sequence (Fig. 1, primer pair 8,9) were used to analyse the sequences flanking the aerobactin operon in those strains that contained aerobactin genes. As expected, the primer pair located within the aerobactin genes (pair 6,7) amplified DNA from all the aerobactin-producing strains (Table 2). The *S. sonnei* and *S. boydii* DNA was also amplified with primers that amplified the region between the aerobactin gene *iucA* and the upstream genes in the *S. flexneri* island (Table 2, primers 5,7). The DNA sequences of the fragments amplified by this primer pair were determined; the sequences shared >90% homology among the three *Shigella* species (data not shown), indicating that the ORFs orf24 and rorf25 upstream of the aerobactin genes are conserved in

Table 4. Sequences present downstream of *selC* in *Shigella* and *E. coli* strains.

Strain	Presence of aerobactin genes[b]	PCR amplification by primer pairs[a]				
		selC–int junction (553 bp)	*selC–yicK* junction (736 bp)	*yicL* (700 bp)	*nlpA* (768 bp)	*yicM* (558 bp)
E. coli W3110	−	−	+	+	+	+
S. flexneri SA100	+	+	−	−	−	+
S. flexneri M90T	+	+	−	−	−	+
S. sonnei PB66	+	+	−	−	+	+
S. boydii 0-1392	+	−	+	ND	ND	ND
S. dysenteriae 0-4576	−	+	−	−	+	+ (≈ 1 kb)
S. dysenteriae Ubon 378	−	+	−	−	ND	ND
E. coli EPEC E2348-69	−	−	−	−	+	+
E. coli EIEC 1107-81	+	−	+	ND	ND	ND
E. coli EIEC 930-78	−	−	+	ND	ND	ND
E. coli EHEC EDL933	−	+ (350 bp)	−	−	+	+ (≈ 1 kb)

a. Approximate locations of primer pairs 1,2 (*selC–int*) and 1,10 (*selC–yicK*) are shown in Fig. 1; *yicL*, *nlpA* and *yicM* were detected by amplification with primer pairs SVO209, SV6; ECS20, ECS17; and ECS21, ECS9 respectively. + indicates amplification of a DNA fragment of the expected size (except as noted); − indicates no amplification; ND, not determined.

b. The presence of aerobactin genes was determined by Southern hybridization and confirmed by bioassays (Lawlor and Payne, 1984; Lawlor *et al*., 1987) to test for aerobactin synthesis and transport.

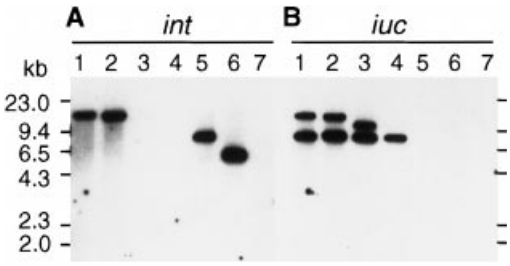

Fig. 3. Presence of sequences homologous to *S. flexneri int* and *iuc* in *Shigella* and *E. coli*. Genomic DNA was digested with *Eco*RI, and the fragments were hybridized to a 866 bp internal fragment of the *int* gene (A) or a 2068 bp probe spanning the *Eco*RI site upstream of the aerobactin promoter (B). Locations of the probes and *EcoRI* sites are shown in Fig. 1. Lanes contain DNA from: lane1, *S. flexneri* SA100; lane 2, *S. sonnei* PB66; lane 3, *S. boydii* 0-1392; lane 4, EIEC strain 1107-81; lane 5, *S. dysenteriae* Ubon 378; lane 6, *S. dysenteriae* 0-4576; lane 7, *E. coli* W3110. Numbers to the left indicate sizes and positions of molecular weight markers.

these species. The third set of primers (primer pair 4,7) failed to amplify either the *S. sonnei* or the *S. boydii* DNA (Table 2). Primer 4 is derived from sequences within the IS*1* upstream of aerobactin. Failure to amplify with this primer pair is consistent with earlier studies of *Shigella* spp. aerobactin genes, showing that the IS*1* upstream of aerobactin in *S. flexneri* was absent in *S. sonnei* and *S. boydii* (Lawlor and Payne, 1984). The downstream primers amplified a fragment in the *S. flexneri* and *S. sonnei* only, indicating that orf35 was present in both these species, but not in the others. DNA from the aerobactin-positive *E. coli* strains could not be amplified with any of the primer pairs other than 6,7. Therefore, the DNA flanking the aerobactin genes in these *E. coli* strains is not the same as that in *S. flexneri* and *S. sonnei* (Table 2).

Additional PCR analyses were used to determine whether SHI-2, or any other pathogenicity island, was located downstream of *selC* in strains other than *S. flexneri*. Primer pair 1,2 (Fig. 1) amplified a 553 bp *selC–int* fragment when the *int* sequence was located immediately downstream of *selC*, thus identifying strains that potentially have SHI-2 at this site; primer pair 1,10 (Fig. 1) amplified the 736 bp *selC–yicK* junction in strains such as *E. coli* K-12 that lack an island or other insertion downstream of *selC*. Among the aerobactin-positive strains tested, both *S. flexneri* and *S. sonnei* had *int* sequences downstream of *selC* (Table 4). The *selC–int* primers amplified a 553 bp fragment in both species, and the *selC–yicK* primers did not amplify these DNA (Table 4). Amplification with the *selC–int* primers indicates that sequences homologous to the *S. flexneri int* are located immediately downstream of *selC* in *S. sonnei* as well. In contrast, the aerobactin island was not found downstream of *selC* in *S. boydii* or in the aerobactin-positive EIEC strain 1107-81. No amplification was detected in *S. boydii* or EIEC using the primer pair that amplified the *selC–int* junction (Table 4), but the *selC–yicK* primers amplified a DNA fragment from these strains that was the same size as that from *E. coli* K-12 strain W3110 (Table 4). These data indicate that there is no insertion downstream of *selC* in these strains and that the aerobactin genes are located at a different site in the chromosome. Interestingly, the *S. dysenteriae* type 1 strains were found to contain an insertion at *selC* that has *int* sequences in common with *S. flexneri*. This strain does not contain aerobactin genes, however, so the insertion is not identical to that found in *S. flexneri* (Table 4).

To obtain additional information about the linkage between the aerobactin genes and the SHI-2 *int* gene, Southern hybridizations were performed using two probes, an internal sequence of the *int* gene and a fragment that spanned the *Eco*RI site upstream of *iucA* (Fig. 1). In *S. flexneri*, both the *int* and *iuc* probes hybridized to the

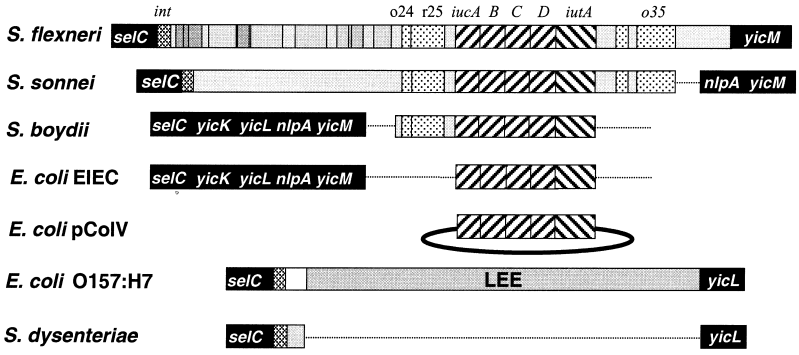

Fig. 4. Comparison of organization of aerobactin genes and their association with the *selC* locus in *Shigella* spp. and pathogenic *E. coli*. The locations of the aerobactin genes and adjacent sequences, *int*, *selC, yicK, yicL, nlpA, yicM*, were determined by Southern hybridization and PCR analysis. Patterned boxes indicate sequences conserved among different strains. Boxes with diagonal lines indicate the aerobactin genes, the hatched box represents *int* and dotted boxes indicate conserved ORFs adjacent to the aerobactin genes. The black boxes represent sequences found in *E. coli* K-12, including *selC*, *yicK, yicL, yicM* and *nlpA*. The dotted lines indicate distances of unknown length separating the genes of interest.

13 kb *Eco*RI fragment of the pathogenicity island DNA in *S. flexneri,* and the *iuc* probe hybridized to a second, 8.5 kb, *Eco*RI fragment that contains the aerobactin genes (Fig. 3, lanes A1 and B1). The same hybridization pattern was observed with *S. sonnei* (Fig. 3, lanes A2 and B2), although the larger of the two *Eco*RI fragments is slightly smaller in *S. sonnei* than in *S. flexneri*. Similarly, the *int* and *iuc* probes hybridized to fragments of the same size when the *S. sonnei* DNA was digested with *Bam*H1, and the fragment was smaller than that observed with *S. flexneri* (data not shown). Because both the *int* and *iuc* probes hybridized to *S. sonnei* DNA restriction fragments of the same size, it is likely that the aerobactin and *int* sequences are physically linked and constitute a pathogenicity island in *S. sonnei*, as they do in *S. flexneri*. However, the islands in these two species are not identical, as indicated by the absences of an IS*1* and *imm* gene upstream of the aerobactin genes in *S. sonnei* (Table 2 and data not shown). Furthermore, the junctions between SHI-2 and the downstream *yic* genes are different in *S. flexneri* and *S. sonnei. nlpA* is present downstream of the island in *S. sonnei*, whereas *nlpA* sequences are deleted in *S. flexneri* (Table 4).

Chromosomal DNA from *S. boydii*, *S. dysenteriae*, EIEC and *E. coli* K-12 was also hybridized with the *S. flexneri int* and *iuc* probes to determine whether these sequences were present and, if so, were the *int* and *iuc* genes linked in any of these strains (Fig. 3). Hybridization with the *int* sequence was found only in *S. dysenteriae* strains, while the *iuc* probe hybridized to sequences in *S. boydii* and the EIEC strain (Fig. 3). The *iuc* probe hybridized to a single *Eco*RI fragment of the EIEC strain, verifying that the DNA upstream of the *Eco*RI site in the aerobactin promoter region is different in *S. flexneri* and in the EIEC strain. Two bands were detected when *S. boydii* DNA was hybridized to the *iuc* probe (Fig. 3, lane B3). The larger band was smaller than that detected either in *S. flexneri* or in *S. sonnei*, and this fragment did not hybridize to the *int* probe. The hybridization and PCR data together suggest that the *S. sonnei* aerobactin genes are found at the same site and are on an island similar to the *S. flexneri* aerobactin island. The *S. boydii* aerobactin genes have sequences homologous to *S. flexneri* upstream, but the genes are located at different sites in the two species. In the pathogenic *E. coli* strains examined, neither the surrounding sequences nor the map locations are the same as in *S. flexneri*. Thus, the aerobactin genes, which are widespread among *Enterobacteriaceae*, are not restricted to a single chromosomal location and are found in a variety of different genetic contexts (Fig. 4). It is likely that these genes are mobile and, in *S. flexneri* and *S. sonnei*, they have become associated with a pathogenicity island that maps at *selC*.

Discussion

Pathogenicity islands are common features of enteric bacterial pathogens (Groisman and Ochman, 1996). These are defined by Hacker *et al*. (1997) as regions of the chromosome that (i) carry virulence genes; (ii) are present in pathogenic strains and absent or sporadically distributed in less pathogenic strains; (iii) have a different G + C content from host bacterial DNA; (iv) occupy large chromosomal regions; (v) represent distinct genetic units; (vi) are associated with tRNA genes or insertion sequences; (vii) contain potential mobility genes such as IS elements or integrases; and viii) are relatively unstable. Studies reported here, along with previous analyses of the *S. flexneri* aerobactin genes, indicate that they are in a chromosomal region that has these characteristics. The aerobactin genes are found in some, but not all, strains of *Shigella* and *E. coli* and are more often associated with highly pathogenic strains (Lawlor and Payne, 1984; Valvano *et al*., 1986). The aerobactin genes are found within a 30 kb region just downstream of a tRNA gene, *selC*, and there are multiple IS elements and an integrase gene in the region. The *int* sequence has homology to *int* genes found in other pathogenicity islands inserted near *selC*. The G + C content of the portion of the SHI-2 that has been sequenced is 46%, slightly lower than the 51% G + C content of the rest of the chromosome. Omitting from this analysis the sequences of the IS elements, which may have transposed onto this region after acquisition of the island, yields a G + C content of 43%. Spontaneous deletions of the aerobactin genes have been observed in *S. flexneri* (Lawlor *et al*., 1987), indicating instability of the region. Thus, the aerobactin region appears to fit within the category of pathogenicity islands. This island is designated SHI-2 (*Shigella* pathogenicity island 2), as it is distinct from a previously described *S. flexneri* pathogenicity island, named *she*, that encodes a homologue of the IgA protease-like family of proteins (Rajakumar *et al*., 1998). Although the precise site of the *she* island has not been reported, it does not map to the same *Not*I fragment as *selC* (Rajakumar *et al*., 1998). SHI-2 contains the aerobactin operon and a colicin immunity gene and also has several novel ORFs. It is possible that one or more of these novel genes is required for pathogenicity, although *S. flexneri* virulence genes other than aerobactin have not been reported to map near *selC*.

Although SHI-2 is similar to other pathogenicity islands, it is not identical to any of the previously described islands found downstream of *selC* or elsewhere in the chromosome. The sequence most closely related to SHI-2 at the *selC* junction is the EPEC LEE pathogenicity island, while the SHI-2 *int* gene is closely related to the EHEC *int* gene. The homology to the EHEC pathogenicity island

appears to be restricted to the *int* gene, with the sequences diverging immediately 3′ of the *int* coding region. The EPEC and EHEC LEEs include genes for attaching and effacing lesions (McDaniel *et al*., 1995), genes that are not found in *S. flexneri* SA100 (data not shown). One of the distinct phenotypes associated with SHI-2, aerobactin synthesis and transport, is absent from the EHEC strains and, when present in other pathogenic *E. coli* strains, it maps at a different location. Similarly, a colicin immunity gene has not been reported within other pathogenicity islands. Thus, while there are common features among these enterobacterial islands, the islands themselves are distinctly different.

Moss *et al*. (1999) have characterized SHI-2 in M90T, a serotype 5a strain of *S. flexneri.* The genetic organization and DNA sequence of the SA100 and M90T islands are almost identical at the left end, i.e. from *selC* through the aerobactin locus. However, the sequences downstream of *iutA* in the two strains are distinct, and the island is ≈30kb in SA100, rather than the 23.8 kb observed in M90T (Moss *et al*., 1999). Downstream of the aerobactin genes, the M90T island contains a copy of IS*600* but no other ORFs, whereas the SA100 island contains a copy of IS*2* and additional sequences of unknown function. Similarly, we found that the island in *S. sonnei* is closely related, but not identical, to the SHI-2 in *S. flexneri* SA100. The *S. sonnei* island lacks some of the sequences found in SA100, and the junctions between the islands and the downstream K-12-like sequences are distinct in the two species. These differences are indicative of the mosaic structure of this island and may indicate that the right-hand end of the island is unstable. Genes or insertion sequences both within and adjacent to the island may have been gained or lost in different isolates.

The aerobactin genes are found in at least three different locations in the *E. coli–Shigella* group: (i) they are plasmid-encoded in ColV strains (Williams, 1979); (ii) they map to the *selC* island in *S. flexneri* and *S. sonnei*; and (iii) they are found in at least one other chromosomal location in the *S. boydii* and EIEC strains. The mobility of the genes may be related to the presence of insertion sequences flanking the genes. Copies of IS*1* are found on either side of the pColV aerobactin genes (McDougall and Neilands, 1984), and the *S. flexneri* aerobactin genes are flanked by IS*2* elements. Movement of these genes does not appear to result from a simple transposition event, however, as the sequences immediately upstream and downstream of the genes and the position and type of associated insertion sequences are different in each case. In contrast to the divergence of sequences flanking the aerobactin operon, the DNA sequences of the aerobactin genes are highly conserved.

The synthesis of a colicin and the presence of a colicin immunity gene upstream of the *S. flexneri* aerobactin genes suggest a common origin of the *S. flexneri* and pColV aerobactin genes. The *S. flexneri* colicin has not been characterized, but it requires the same receptor, Cir, as that used by colicins V and I, and the putative colicin immunity gene renders *E. coli* insensitive to colicins V and I as well as to the *S. flexneri* colicin. Although the putative immunity ORF is approximately the same size as other immunity genes, no significant homology between this sequence and known colicin immunity genes was noted in either the DNA or amino acid sequence. Also, the position of the colicin synthesis and immunity genes relative to the aerobactin genes is different in pColV than in *Shigella*. The immunity genes are tightly linked to the corresponding colicin synthesis genes on pColV but map at a distance from the aerobactin cluster (Ambrozic *et al*., 1998). The lack of similarity in the immunity gene DNA sequences and the different genetic organizations of the regions surrounding the aerobactin genes indicate that the aerobactin and immunity genes were not transferred as a block between the ColV plasmid and the *Shigella* chromosome.

The observation that the aerobactin genes are found in a variety of different locations and are more highly conserved than the flanking sequences suggests that these genes are highly mobile and may be acquired by additional human or animal pathogens. In the case of *S. flexneri*, the genes have become associated with a pathogenicity island and, thus, have effectively created an island within an island. The surrounding island has a number of features in common with, but distinctly different from, islands found at the same site in other pathogens and, at least in *S. dysenteriae*, the island exists independently of the aerobactin genes.

Experimental procedures

Strains and plasmids

Strains and plasmids used in this study are listed in Table 5. Strains were routinely grown in L broth or on L agar. Antibiotics were added at standard concentrations to maintain plasmids. Bacteria were grown in a low-iron, Tris-buffered minimal medium as described previously (Lawlor *et al*., 1987) to assay for the production of siderophores.

DNA sequencing, sequence analysis and amplification of sequences by PCR

DNA sequencing was performed using an ABI Prism 377 automatic sequencer. The GenBank accession number for this sequence is AF097520. Routine DNA sequence analysis was performed using MACVECTOR (Olson, 1994) (Oxford Molecular). Homologies to proteins and genes were analysed using the BLASTX BLOSUM62 and BLASTN programs, respectively, through the National Center for Biotechnology Information (Altschul *et al*., 1990; 1997; Gish and States, 1993). To determine the DNA sequence upstream of the sequence contained

Table 5. Strains and plasmids.

Bacterial strains	Relevant characteristics[a]	Source or reference
Shigella flexneri		
SA100	Serotype 2a; Crb$^+$ Iuc$^+$ Iut$^+$	Payne *et al.* (1983)
M90T	Serotype 5; Crb$^+$ Iuc$^+$ Iut$^+$	P. Sansonetti
Shigella boydii		
O-1392	Iuc$^+$ Iut$^+$, Clinical isolate	TDH[b]
Shigella dysenteriae		
Ubon 378	Serotype 1	A. Hartman
0-4576	Serotype 1	Lawlor and Payne (1984)
Shigella sonei		
PB66	Iuc$^+$ Iut$^+$	D. Winsor
Escherichia coli		
EHEC (EDL933)	0157:H7	J. Kaper
EIEC (930-78)	0124:H$^-$, Iuc$^-$ Iut$^-$	Marolda *et al.* (1987)
EIEC (1107-81)	Iuc$^+$ Iut$^+$	Marolda *et al.* (1987)
EPEC (2348/69)	0127:H6	J. Kaper
LG1315	pColVK-30	Williams (1979)
RM43	Colicin Ib	I. Molineux
JK354	Cir$^+$	J. Konisky
JK458	JK354*cir*	J. Konisky
EPEC (3787-62)	055:H6	T. Whittam
W3110	K-12	I. Molineux
RM1058	K-12	R. Meyer
Plasmids		
Bluescript SK$^-$	Cloning vector	Stratagene
pKLS971	pLAFRI cosmid containing 27 kb fragment encoding aerobactin and flanking sequences from *S. flexneri* SA100; Iuc$^+$ Iut$^+$, colicin I and colicin V immunity	Marolda *et al.* (1987)
pKLS711	8.4 kb *Eco*RI fragment of pKLS971 containing *iuc* cloned into pBR322	Lawlor *et al.* (1987)
pKLS77	6.4 kb *Eco*RI fragment of pKLS971 cloned into pBR322	K. Lawlor
pSAV1	5.5 kb *Hin*dIII fragment of pKLS971 containing *int* cloned into SK$^-$	This work
pSAV2	3 kb *Hin*dIII fragment of pKLS971 cloned into SK$^-$	This work
pSAV3	4 kb *Hin*dIII–*Eco*RI fragment of pKLS971 cloned into SK$^-$, confers colicin immunity	This work
pSAV4	8 kb *Hin*dIII–*Eco*RI fragment of pKLS971 cloned into SK$^-$, contains *iuc*	This work
pJLG1	2.1 kb *Kpn*I–*Sac*I fragment of pSAV3 cloned into SK$^-$	This work
pSAV3NcoΔ	Deletion of the 866 bp *Nco*I fragment from pSAV3	This work

a. Crb, Congo red binding; Iuc, aerobactin biosynthesis; Iut, aerobactin transport.
b. Clinical isolate from the Texas Department of Health.

in pKLS971, inverse PCR was performed on SA100 genomic DNA. The DNA was digested with *Sau*3AI, purified using the Geneclean II Kit (BIO 101) and ligated under conditions favouring circularization. Inverse PCR was performed in a reaction containing 2.5 mM $MgCl_2$, 5 U of *Taq* DNA polymerase (Qiagen) and 1 μM each of primers 2 and 3 (Fig. 1). The PCR reaction consisted of 30 cycles with 1 min at 94°C, 1 min at 65°C and 3 min at 72°C. The PCR product was isolated and sequenced directly.

Genomic PCR was performed under a variety of conditions depending on the length of the product and the melting temperature of the primers.

The sequences of the primers and their exact positions are (numbers indicate locations of the 5′ and 3′ nucleotides, numbering from the start of the island downstream of *selC*): primer 1, 5′-ATCCAGTTGGGGCCGCCAGCGGTCCCGGGCAG-3′ (in *selC*) (Blanc-Potard and Groisman, 1997); primer 2, 5′-CTCGCGAGCATCGGCTAGCGTTACATCGGGGT-3′ (486–455); primer 3, 5′-GTGAAGCCAGGAAACTCCTCGCTGCTGGAGGC-3′ (494–525); primer 4, 5′-TACCACGACCTCAAAGGCCG-3′ (11592–11611); primer 5, 5′-GGCTCGCCAATGCCCTGATA-3′ (12542–12561); primer 6, 5′-CAGCCCTAGCAGGGTAAAG-3′ (13781–13800); primer 7, 5′-GCCGTGACCAGATGAGCAGG-3′ (14402–14421); primer 8,

5′-TTCACCGTGCCATAAGAGCC-3′ (in o35); primer 9, 5′-TATGGAGGTATGCAGGCTGC-3′ (in o35); primer 10, 5′-GTGAGATCAAGTATTTTTGATGGAGTGGTAGC-3′ (in *E. coli yicK*, not present in SA100) (Blanc-Potard and Groisman, 1997).

Primer ECS9, 5′-ACAGAACCTGCTGCAATG-3′ (in *yicN*); primer ECS17, 5′-GAATTCTGCTGGCAGGTT-3′ (in *nlpA*); primer ECS20, 5′-CGACTTCGGGTGATTGAT-3′ (in *nlpA*); primer ECS21, 5′-CGAAATGCCTAAATCCTG-3′ (in *yicM*); primer SVO209, 5′-TGCCATCTTCCTTGGTATTCTCTGTG-GTATCG-3′ (in *yicL*); primer SV6, 5′-CGATAATCGTCGGT-GAGAGGAATTGCAGCA-3′ (in *yicL*).

Colicin and siderophore assays

To detect colicin synthesis or sensitivity, L plates were stabbed with a colony of the colicin-producing strain and incubated overnight. The plates were then inverted over chloroform-saturated Whatman no. 1 disks for 15 min, dried upright for 30 min and overlaid with 3 ml of 0.7% agar containing 100 μl of a fully grown culture of the colicin indicator strain. The plates were incubated at 37°C overnight, and colicin sensitivity was determined by the presence of a clear zone around the site of the stab.

The presence of hydroxamate siderophores was detected by the ferric perchlorate assay (Atkin *et al*., 1970). The synthesis and transport of aerobactin was confirmed by bioassays as described previously (Lawlor *et al*., 1987).

Southern hybridization

Genomic DNA was isolated with DNAzol reagent (Molecular Research) as described by the manufacturer. Southern hybridizations were performed according to the procedure of Maniatis *et al*. (1982). Probe labelling, hybridization and detection with CSPD reagent were performed as described in the Genius II System (Boehringer Mannheim).

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