Iron is essential for the growth of most bacterial pathogens, and the ability to acquire iron is associated with bacterial virulence. To obtain iron, Shigella spp. can use host iron sources such as heme directly, and they have the ability to remove iron from host sources via siderophore-mediated uptake systems (22, 47). Siderophores are low-molecular-weight, high-affinity iron chelators synthesized and secreted into the environment. The iron-siderophore complex is transported back into the cell using specific receptors. Two different siderophore-mediated iron transport systems have been observed in Shigella spp. and clinical Escherichia coli isolates. The catechol siderophore enterobactin is produced by E. coli (39) and some, but not all, Shigella spp. (36, 38), while the hydroxamate siderophore aerobactin is synthesized by Shigella flexneri and Shigella boydii (20) and Shigella sonnei (34), as well as some E. coli clinical isolates (11, 34). The aerobactin operon encodes the IucABCD enzymes for aerobactin synthesis and Iut, the outer membrane receptor for aerobactin. Expression of the aerobactin operon is negatively regulated by the iron-binding repressor protein Fur (3). Under low-iron conditions, expression of the aerobactin operon is derepressed, and the siderophore synthesis proteins and receptor are produced to facilitate iron acquisition.

The aerobactin genes are found on the pColV and F1 plasmids in some strains of E. coli and Salmonella, respectively, and are found chromosomally in Shigella and other E. coli strains (11, 20, 22, 26, 46). While the aerobactin genes were shown to be located in the Shi-2 pathogenicity island downstream of the selC RNA gene in S. flexneri and S. sonnei (30, 43), their location in S. boydii remained unknown. In this report, we show that the aerobactin operon is located in a 21-kb iron transport island between hysU and the pheU tRNA gene in S. boydii. While the sequence of the aerobactin genes is conserved, the sequences flanking the genes are distinct, and the aerobactin island in S. boydii has been designated SHI-3 for Shigella island 3.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Clinical isolates of S. boydii, S. dysenteriae, S. flexneri, and S. sonnei were obtained from the Texas Department of Health. Enteroinvasive E. coli (EIEC) strains were obtained from J. H. Crosa, Oregon Health Science University. E. coli 1017 (HB101 cmlF::Tn5) has been described previously (12). The iron chelator EDDA ([ethylenediaminetetraacetic acid]) was dewatered as described previously and used at a concentration of 300 μg/ml to induce iron starvation (19). Strains were grown in L broth or on L-agar plates with the addition of antibiotics at the following concentrations when necessary: 250 μg of carbenicillin/ml, 30 μg of chloramphenicol/ml, and 200 μg of streptomycin/ml.

Isolation of the S. boydii aerobactin genes. A 5-341 partial library of S. boydii 0-1392 was constructed in the cosmids vector pLAFR3 (13) and screened by colony hybridization using a probe to the S. flexneri iucA gene. Two overlapping cosmids, pGEP1 and pGEP2, were isolated, and their ability to confer aerobactin synthesis to E. coli 1017 was confirmed by the hydroxamate assay and siderophile bioassays (19, 35).

Construction of the iucB mutation. An iucB mutation was constructed in S. boydii 0-1392 by allelic exchange using the suicide plasmid pGP704 (27). In S. boydii 0-1392 iucB, the wild-type allele is replaced with one containing a chloramphenicol resistance cassette inserted into the 5ml site of iucB.

Tissue culture, cell invasion, and plaque assays. The ability of S. boydii 0-1392 and 0-1392 iucB to invade HeLa cells was determined by the procedure of Hale and Formal (16). Plaque assays were performed as described by Oaks et al. (32).

Nucleotide sequence analysis. DNA sequencing was performed using an ABI Prism 377 automatic sequencer. BamHII, HincII, PstI, and EcoRI fragments of pGEP1 and pGEP2 were subcloned into either pBluescript SK− (Stratagene) or pWKS30 (44) plasmid vectors for sequencing. Routine sequence analysis was performed using MacVector software (33) (Oxford Molecular). Sequence homology to known genes and proteins was analyzed using the BlastN and BlastX algorithms, respectively, through the National Center for Biotechnology Information database (1, 2, 14).

PCR. PCRs were performed in a GeneAmp PCR system 2400 (Perkin-Elmer). The primers used to amplify the SHI-3 int3 and int2-selC junction are as follows: primer 1 (5′-GCGCTGAGATGGTGTGCTGAC-3′), primer 2 (5′-GAATCAG GTTGTGATCC-3′), and primer 3 (5′-GGTATTAAGTCCGTCC-3′). The primers used to amplify the SHI-2 int2 and int2-selC junction are as follows: primer 6 (5′-GGCCGCGATATGGTATC-3′) and primers 7 and 8, which correspond to primers 1 and 2, respectively, described by Vokes et al. (43).
and yjdL genes was sequenced, revealing a 21-kb island between (data not shown). The entire region containing the aerobactin shown by positive hydroxamate tests and siderophore bioassays into the cloned constructed by inserting a chloramphenicol resistance cassette responsible for hydroxamate synthesis, an iucB location in any species. Aerobactin genes have not previously been mapped to this location in S. boydii E. coli inferred the ability to synthesize aerobactin upon the 0-1392 aerobactin genes, cosmids were isolated from a library of reversionary iucA-overlapping iucA- strain 0-1392 by DNA hybridization using a probe to containing the aerobactin genes were isolated from a library of primer pairs 2-3 and 6-8, 1 min for primer pair 4-5, and 2 min for primer pairs 1-3 and 7-8. Southern hybridizations. Genomic DNA was isolated using Qiagen Genomic-tip DNA isolation columns according to the manufacturer’s instructions. Probe labeling, hybridization, standard stringency washes, and detection were performed as described in the ECL Direct Nucleic Acid Labeling Kit (Amersham Pharmacia).

Nucleotide sequence accession number. The GenBank accession number for the sequence described here is AF335540.

RESULTS

Isolation of the S. boydii aerobactin genes. Strains of S. boydii, like those of S. flexneri, produce the siderophore aerobactin, but the S. boydii aerobactin synthesis genes do not map to the same location as those in S. flexneri (43). To map the location of the S. boydii 0-1392 aerobactin genes, cosmids containing the aerobactin genes were isolated from a library of strain 0-1392 by DNA hybridization using a probe to iucA. Two overlapping iucA-positive cosmids, pGEP1 and pGEP2, conferred the ability to synthesize aerobactin upon E. coli 1017 as shown by positive hydroxamate tests and siderophore bioassays (data not shown). The entire region containing the aerobactin genes was sequenced, revealing a 21-kb island between pheU and yjdL at min 93 to 94 of the E. coli K-12 map (Fig. 1). Aerobactin genes have not previously been mapped to this location in any species.

To confirm that the aerobactin genes at this locus are responsible for hydroxamate synthesis, an iucB mutation was constructed by inserting a chloramphenicol resistance cassette into the cloned iucB gene and transferring the mutation to 0-1392 by allelic exchange. 0-1392 iucB did not synthesize aerobactin, indicating that there is a single aerobactin operon in 0-1392. 0-1392 iucB invaded Henle cells at wild-type levels and produced plaques in a standard plaque assay (data not shown).

Structure of the S. boydii aerobactin island. The S. boydii aerobactin island, which we have designated SHI-3, contains a functional aerobactin operon. SHI-3 is demarcated by a putative integrase gene inserted 200 bp downstream of the pheU tRNA at min 94 and by an IS600 interruption of yjdL, an uncharacterized open reading frame (ORF) adjacent to yjdL (Fig. 1). Sequence scanning did not reveal direct repeats or any known sequence in the 84 bp between the conserved intergenic sequence downstream of pheU and the start of the integrase gene. The genes, ORFs, and insertion elements present in S. boydii SHI-3 are summarized in Table 1. The second ORF of the S. boydii aerobactin island shares 97% nucleotide identity with the S. flexneri M90T SHI-2 ORF of unknown function, shiB (30). The region between shiB and the aerobactin genes in S. boydii contains ORFs of unknown function with sequence similarity to ORFs upstream of aerobactin in S. flexneri SHI-2 and E. coli pColV-K30 (Table 1). There is 100% nucleotide identity between orf2 of 0-1392 and orf24 of the S. flexneri SA100 SHI-2 aerobactin island. The adjacent region is 99% identical to S. flexneri SA100 SHI-2 rorf25, an ORF transcribed off the minus strand, but the insertion of a thymine codon at base 5333 in the S. boydii SHI-3 island generates a stop codon, creating the defective rorf3 (43). The 398-bp orf4 is 99% homologous at the nucleotide and amino acid levels to SHI-2 orf27 and shares 93% nucleotide identity to sequence upstream of the aerobactin genes in E. coli pColV-K30. The SHI-3 iucA, -B, -C, and -D and intA genes share 99% nucleotide identity with the SHI-2 aerobactin genes (30, 43) and 92 to 95% iden-

FIG. 1. Map of the S. boydii 0-1392 aerobactin island, SHI-3, and the corresponding E. coli K-12 region between pheU and yjdL at min 94. Sequences present in both E. coli K-12 and S. boydii are indicated by the black bars and are designated K-12 on the SHI-3 map. The ORFs, indicated as shiB and o2 to o4, were inferred from sequence analysis. The iucA probe and the int3 probe, as well as the approximate positions of primers used for PCR amplification of the junctions, are positioned below the SHI-3 map.
TABLE 1. ORFs within the S. boydii aerobactin island

<table>
<thead>
<tr>
<th>ORF, gene, or IS</th>
<th>Location</th>
<th>Length (bp)</th>
<th>Similar sequence (reference)</th>
<th>% Nucleotide (protein) identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>int3</td>
<td>84–1345</td>
<td>1,261*</td>
<td>P4 prophage integrase (37)</td>
<td>(63)</td>
</tr>
<tr>
<td>shiB</td>
<td>1800–2249</td>
<td>449</td>
<td>S. flexneri M90T shiB gene in SHI-2 (30)</td>
<td>97 (94 over 143 of 153 aa)</td>
</tr>
<tr>
<td>IS606</td>
<td>3198–4459</td>
<td>1,261</td>
<td>S. sonnet IS600 (10, 24)</td>
<td>98</td>
</tr>
<tr>
<td>orf2</td>
<td>4815–5081</td>
<td>266</td>
<td>S. flexneri SA100 orf24 in SHI-2 (43)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>orf4</td>
<td>5037–6229</td>
<td>1,192</td>
<td>S. flexneri SA100 orf25 in SHI-2 (43)</td>
<td>99 (89 for 297 of 397 aa)</td>
</tr>
<tr>
<td>icrA, -B, -C, -D</td>
<td>6308–12112</td>
<td>5,804</td>
<td>S. flexneri M90T aerobactin synthesis genes in SHI-2 (30)</td>
<td>(99)</td>
</tr>
<tr>
<td>icrA</td>
<td>12115–14313</td>
<td>2,198</td>
<td>S. flexneri M90T icrA in SHI-2 (30)</td>
<td>99 (99)</td>
</tr>
<tr>
<td>IS600</td>
<td>14542–15809</td>
<td>1,267</td>
<td>S. sonnet IS600 (10, 24)</td>
<td>98 (93)</td>
</tr>
<tr>
<td>Partial IS285</td>
<td>15803–16159</td>
<td>356</td>
<td>Y. pestis IS285 (21)</td>
<td>86 (79 for 88 of 270 aa)</td>
</tr>
<tr>
<td>Partial IS200</td>
<td>16374–17106</td>
<td>732</td>
<td>E. coli IS200 (4)</td>
<td>95 (87)</td>
</tr>
<tr>
<td>ileX</td>
<td>17706–17778</td>
<td>72</td>
<td>E. coli ileX tRNA gene (5)</td>
<td>87</td>
</tr>
<tr>
<td>L0006</td>
<td>17928–18590</td>
<td>662</td>
<td>E. coli LEE pathogenicity island prophage gene L0006 (37)</td>
<td>93 (95)</td>
</tr>
<tr>
<td>L0005</td>
<td>18004–18786</td>
<td>182</td>
<td>E. coli LEE pathogenicity island prophage gene L0005 (37)</td>
<td>92 (78)</td>
</tr>
<tr>
<td>Partial L0004</td>
<td>18799–19047</td>
<td>248</td>
<td>E. coli LEE pathogenicity island prophage gene L0004 (37)</td>
<td>90 (95 for 82 of 116 aa)</td>
</tr>
<tr>
<td>IS600</td>
<td>19699–20961</td>
<td>1262</td>
<td>S. sonnet IS600 (10, 24)</td>
<td>98 (93)</td>
</tr>
</tbody>
</table>

* Three ORFs corresponding to the integrase-like sequence were corrected for apparent frameshifts and are presented here as a single ORF.

a Amino acid identity for the partial ORF present in SHI-3.

b orf3 is an ORF transcribed off the minus strand.

c Due to a premature stop codon, orf3 is defective.

d A partial copy of the ileX tRNA gene was the target of a bacteriophage integration event and then was incorporated into SHI-3 via other bacteriophage- or IS element-mediated events.

e Association between SHI-3 and loss of cadA. In S. boydii 0-1392, there is a deletion of more than 6 kb relative to the E. coli K-12 sequence at the site occupied by SHI-3, including the lysine decarboxylase gene, cadA (Fig. 1). Cadavarine, produced by the decarboxylation of lysine, inhibits Shigella enterotoxin activity, and deletion of cadA has been shown to enhance the virulence of Shigella and EIEC (25). Thus, in S. boydii, the acquisition of SHI-3 may have resulted not only in the enhanced ability to scavenge iron from the host using the siderophore aerobactin but also in the loss of a gene whose absence is associated with an increase in virulence.

f Distributions of SHI-3 int3 and SHI-2 int2 among enteric bacteria. PCR was used to determine the distribution and location of the SHI-3 integrase gene, int3, among other Shigella strains, and hydroxamate tests were performed to assess the possible correlation of int3 presence with aerobactin production (Table 2). Primer pair 2-3 (Fig. 1) amplified a 395-bp product internal to int3 in several S. boydii, S. flexneri, S. sonnet, and S. dysenteriae serotypes, as well as EIEC strains, illustrating the distribution of this putative P4-like prophage integrase among Shigella spp. and E. coli. In those strains positive for int3, PCR to detect the yjdc-int3 junction was performed. Using primers 1 and 3, where primer 1 is in yjdc, the uncharacterized ORF upstream of pheU, and primer 3 is internal to int3, a 2.1-kb yjdc-int3 fragment was amplified in S. boydii 0-1392, 0-1393, and 224860, as well as in S. dysenteriae 1-130 (Fig. 1). All four of these strains produced aerobactin, as determined by hydroxamate assays and siderophore bioassays (data not shown). Therefore, it is possible that SHI-3, carrying the aerobactin genes, is located downstream of pheU in each of
these strains. The presence of strains positive for int3 but lacking the yjdC-int3 junction, such as S. boydii 0-1591, suggests that another Int3-mediated bacteriophage integration event occurred at a different map location in these strains.

The presence of int2, the related but distinct integrase gene found in SHI-2, among the Shigella and EIEC clinical isolates was also determined through PCR (Table 2). Although the two integrases are related, no cross-amplification of int3 and int2 genes occurs with primer pair 2-3 and primer pair 6-8. Using primers 6 and 8, a 120-bp fragment internal to int2 was amplified in all but one of the S. flexneri serotypes, in one of five S. dysenteriae strains, and in both S. sonnei strains. int2 was absent from the seven S. boydii strains and from the three EIEC strains tested (Table 2). In S. flexneri serotypes 2a, 2b, and 5, as well as S. sonnei strains, PCR amplification with both the int2 and int3 primer pairs suggested two independent bacterio-

### DISCUSSION

SHI-3 is a 21-kb iron transport island carrying the aerobactin genes and is located downstream of the pheU tRNA gene in S. boydii 0-1392. SHI-3 has many characteristics of a pathogenicity island: it contains mobile elements, including a P4-like prophage integrase and IS elements; it is associated with a tRNA gene; and it may have been acquired via horizontal transfer. However, the role that this island plays in Shigella pathogenicity is unclear. The only potential virulence genes
within this region encode enzymes for aerobactin synthesis and the outer membrane receptor Iut. Aerobactin is known to be important for bacterial survival in low-iron conditions and also may be important in the host. *S. flexneri* aerobactin mutants show reduced fluid accumulation in the rabbit ileal loop model of infection (31), yet *iuc* mutants are capable of wild-type invasion, form plaques in cultured epithelial cells, and are positive in the Serény test. In this study we have shown that *S. boydii* aerobactin mutants are also capable of wild-type invasion and plaque formation, most likely due to the presence of additional iron uptake systems. The presence of multiple functional iron transport systems in *Shigella* suggests that there is selection for the acquisition and maintenance of these genes. These iron acquisition systems may benefit *Shigella* in the different environments it encounters within the host, making the contribution of one iron transport system to virulence difficult to assess. The SHI-3 island also is associated with the absence of the gene encoding lysine decarboxylase activity, the loss of which contributes to *Shigella* pathogenicity (25). Thus, the aerobactin island may contribute to both the survival and the pathogenicity of these bacteria.

The acquisition of SHI-3 may have been bacteriophage mediated, as SHI-3 contains various phage genes, including that for an integrase similar to the LEE P4-like prophage integrase of *E. coli*. *S. boydii* 0-1392 SHI-3 appears to be stable (data not shown), although spontaneous deletions of the aerobactin genes in *S. flexneri* have been observed (19), suggesting the instability of the aerobactin genes in certain strains. The G+C content of the 21-kb SHI-3 island is similar to the observed base composition of the chromosome (51%). Either *S. boydii* acquired this island early in its evolution and the base composition has become similar to that of the chromosome, or SHI-3 was transferred from an organism with a base composition similar to that of *Shigella*.

Many pathogenicity islands are associated with tRNA genes or tRNA-like loci, and the 3’ ends of tRNA genes may act as sites for integration of foreign DNA (15). The *S. boydii* SHI-3 island is the second island to be associated with *pheU* in enteric pathogens. The LEE pathogenicity island is found at *pheU* in clinical isolates of enterohemorrhagic and enteropathogenic *E. coli* strains expressing β-intimin, while it is found immediately downstream of *selC* in strains expressing α- or γ-intimin (41, 45). The *selC* tRNA gene is also the site of the *S. flexneri* and *S. sonnei* SHI-2 aerobactin islands (30, 43).

SHI-3 is distinct from the previously described *S. flexneri* SHI-2, although the aerobactin genes are highly conserved. The G+C content of the 30-kb *S. flexneri* SHI-2 is slightly lower (46%) than that of the *S. boydii* SHI-3 (51%) and contains a colicin immunity gene (43). Additionally, SHI-3 contains three copies of IS600 and incomplete copies of IS200 and IS285, as well as the putative prophage genes L0004 to L0006, which are not present in SHI-2. The presence of different genes and IS elements and the difference between SHI-2 and SHI-3 in G+C content indicate that SHI-2 may have been acquired at a later time, or from another source, than SHI-3.

Horizontal gene transfer involves the introduction of genes into a single lineage via plasmid, bacteriophage, or IS elements, resulting in a scattered phylogenetic distribution among closely related species. Mapping of several iron transport loci among the *Enterobacteriaceae* suggests horizontal transfer of these genes. The various locations of the aerobactin genes, the distribution of the SHI-2 and SHI-3 aerobactin islands among enteric bacteria, and the association of aerobactin genes with bacteriophage or mobile elements suggest that genes for aer-

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**FIG. 3.** Map locations of the aerobactin genes among the *Enterobacteriaceae*. The islands containing aerobactin genes are shown as insertions relative to the *E. coli* K-12 chromosome. Hatched boxes indicate the aerobactin operon, white boxes indicate P4-like prophage integrase genes, and dark grey boxes indicate ISs. (a) The SHI-2 aerobactin pathogenicity island at *selC* in some *S. flexneri* strains and in *S. sonnei*. (b) The SHI-3 aerobactin island at *pheU* in *S. boydii*. (c) The aerobactin genes are encoded on the pColV and F1me plasmids in some strains of *E. coli* and *Salmonella enterica*, respectively. On pColV, the aerobactin genes are flanked by IS1 elements.
obactin synthesis and transport have been acquired through horizontal transfer (Fig. 3). Similarly, the presence of the shu gene transfer locus in S. dysenteriae type I and various E. coli strains, but not in other S. dysenteriae serotypes or Shigella spp., suggests that the shu genes also spread via one of these transfer mechanisms (28, 29, 42, 47). Finally, the genes encoding the siderophore versiniabactin and its receptor are present in the high-pathogenicity islands of Yersinia enterocolitica, Y. pestis, and P. pseudotuberculosis (6, 7, 9). High-pathogenicity islands also have been found in the chromosomes of some pathogenic E. coli strains, suggesting that the high-pathogenicity islands of S. dysenteriae strains, but not in other S. dysenteriae. Finally, the genes encoding the Shigella dysenteriae

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