Flanking and Internal Regions of Chromosomal Genes Mediating Aerobactin Iron Uptake Systems in Enteroinvasive Escherichia coli and Shigella flexneri

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We have investigated the presence of the aerobactin system and the location of the aerobactin genes in enteroinvasive strains of Escherichia coli. Also, we cloned the aerobactin region and its flanking sequences from the chromosome of a strain of Shigella flexneri and compared the molecular organization of the aerobactin genes in the two genera. Of the 11 enteroinvasive E. coli strains studied, 5 possessed the aerobactin genes, which were located on the chromosome in each case. These strains produced and utilized aerobactin and also were susceptible to the bacteriocin cloacin-DF13. Restriction endonuclease mapping and hybridization experiments showed that the regions corresponding to the aerobactin-specific sequences were very similar in both enteroinvasive E. coli and S. flexneri. However, differences were found in the region corresponding to the aerobactin receptor gene. The regions flanking the aerobactin system in enteroinvasive E. coli and S. flexneri exhibited some similarities but were different from those in pColV-K30. Under iron-limiting conditions, aerobactin-producing enteroinvasive E. coli and S. flexneri synthesized outer-membrane proteins of 76 and 77 kDa, respectively, which cross-reacted immunologically with rabbit antiserum raised against the 74 kDa pColV-K30-encoded ferric aerobactin receptor.

INTRODUCTION

The aerobactin-mediated iron transport system is found in several members of the Enterobacteriaceae (Crosa, 1984; Payne, 1980; Payne et al., 1983) and is encoded by ColV-type plasmids in some Escherichia coli strains (Williams, 1979; Braun, 1981; Warner et al., 1981). However, in the majority of E. coli K1 strains associated with human neonatal infections the aerobactin system is located on the bacterial chromosome (Valvano & Crosa, 1984; Valvano et al., 1986). Some species of Shigella such as S. flexneri and S. boydii also possess a chromosome-mediated aerobactin system (Lawlor & Payne, 1984). Recently, the presence of the aerobactin system in enteroinvasive E. coli has been reported (Griffiths et al., 1985), although the location and the molecular organization of the aerobactin genes in these strains was not investigated. In this study, we demonstrate that certain enteroinvasive strains of E. coli possess a chromosome-determined aerobactin system. Also we compare the physical maps of the aerobactin region and its adjacent sequences in one of these E. coli isolates and the cloned aerobactin genes from S. flexneri.

METHODS

Bacterial strains and plasmids. Laboratory strains and plasmids used in this study are described in Table 1. Enteroinvasive E. coli were human diarrhoeal isolates referred to the Center for Disease Control, Atlanta, Georgia, USA.
### Strain or plasmid

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and relevant characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td>ara azi fepA lac leu mtl proC rpsL supE tonA thi</td>
<td>Carbonetti &amp; Williams (1984)</td>
</tr>
<tr>
<td>LG1522</td>
<td>(pColV-K30, iuc)</td>
<td></td>
</tr>
<tr>
<td>HB101 BC3</td>
<td>hsdS recA ara proA lacY galK rpsL xyI mtl supE cir rpsL</td>
<td>Maniatis et al. (1982)</td>
</tr>
<tr>
<td><strong>Shigella</strong></td>
<td>Prototrophic, Iuaer+, Tc'</td>
<td>J. Konisky</td>
</tr>
<tr>
<td><strong>S. boydii 1392</strong></td>
<td>Prototrophic, Iuaer+, Tc'</td>
<td>Lawlor &amp; Payne (1984)</td>
</tr>
<tr>
<td><strong>S. flexneri SA100</strong></td>
<td>8.6 kb and 16.3 kb HindIII fragments from pColV-K30 harbouring the aerobactin region and flanking sequences cloned in pVK102; Iu&lt;sup&gt;ser&lt;/sup&gt;, Tc&lt;sup&gt;c&lt;/sup&gt;; source of control DNA for the hybridization experiments</td>
<td>Valvano &amp; Cosa (1984)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>16.3 kb HindIII fragment from pColV-K30 harbouring the aerobactin genes cloned in pVK102; Iu&lt;sup&gt;ser&lt;/sup&gt;, Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Valvano et al. (1986)</td>
</tr>
<tr>
<td>pJHCV8</td>
<td>6.7 kb BamHI fragment from pJHCV8 carrying the aerobactin receptor gene cloned in pBR322; Ap'; source for the KPNI probe (Fig. 1c)</td>
<td>Bindereif &amp; Neilands (1983)</td>
</tr>
<tr>
<td>pJHCPl</td>
<td>6.8 kb HindIII-EcoRI fragment carrying the siderophore genes; Ap'; source for the HAM and AVAL probes (Fig. 1c)</td>
<td>This study</td>
</tr>
<tr>
<td>pKLS10</td>
<td>4.8 kb HindIII-BglII fragment carrying part of the pColV-K30 aerobactin siderophore genes cloned in pBR322; Ap'; source for the HIND-BGL probe (Fig. 1c)</td>
<td>Lawlor &amp; Payne (1984)</td>
</tr>
<tr>
<td>pLAFR1</td>
<td>Cosmid cloning vector; Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Friedmann et al. (1982)</td>
</tr>
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<td>pAT153</td>
<td>High-copy-number cloning vector; Tc&lt;sup&gt;c&lt;/sup&gt;, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Maniatis et al. (1982)</td>
</tr>
<tr>
<td>pKLS971</td>
<td>Recombinant plasmid in pLAFR1 carrying the aerobactin region and flanking sequences from S. flexneri SA100; Iu&lt;sup&gt;ser&lt;/sup&gt;, Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pKLS719</td>
<td>13 kb EcoRI fragment from pKLS971 cloned in pLAFR1; Iu&lt;sup&gt;ser&lt;/sup&gt;, Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJHCP107</td>
<td>2 kb EcoRI-SalI fragment from pJHCP1 (Perez-Casal &amp; Crosa, 1984) containing the pColV-K30 aerobactin promoter region; source for the ECO-SAL probe (Fig. 1c)</td>
<td>This study</td>
</tr>
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*Auc, inability to synthesize aerobactin (Carbonetti & Williams, 1984); Iu<sup>ser</sup>, possession of a functional aerobactin system; Ap, ampicillin; Tc, tetracycline.

### Aerobactin production and utilization.

Hydroxamates in supernates of strains grown under iron-limiting conditions were detected by the method of Csaky (1948). The production of aerobactin was determined by a cross-feeding bioassay (Valvano & Crosa, 1984). An aerobactin-utilization test was also performed as described elsewhere (Valvano et al., 1986), using aerobactin purified from Enterobacter aerogenes 62-1 as described by Gibson & Magrath (1969). In the case of the E. coli harbouring recombinant cosmids from the S. flexneri SA100 gene library, the production of aerobactin was assessed by plate bioassays using both E. coli LG1522 and S. boydii 1392 as indicator strains (Lawlor & Payne, 1984). The susceptibility to the bacteriocin cloacin DF-13 (Van Tiel-Menkveld et al., 1982) was used as an indication of the presence of the ferric-aerobactin receptor protein. A crude cloacin preparation (25 μl volumes) was dropped onto Luria broth agar plates seeded with the strains to be tested (Bindereif et al., 1982).

### Isolation of plasmid and chromosomal DNA.

Plasmids from clinical strains were obtained by the method of Kado & Liu (1978), and recombinant plasmids were obtained by an alkaline procedure (Maniatis et al., 1982). Further purification of plasmid DNA was achieved as previously described (Valvano & Crosa, 1984). Total DNA was isolated either by the procedure of Marmur (1961) or as described by Hull et al. (1981).

**Restriction enzymes, cosmid cloning and DNA hybridizations.** Restriction endonucleases and T4 ligase were used under the conditions recommended by the supplier (Bethesda Research Laboratories). The size fractionation of the EcoRI-digested chromosomal DNA from S. flexneri, the preparation of λ-packaging extracts, and the transduction of in vitro-packaged ligated DNA were performed as described by Maniatis et al. (1982). Southern
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Aerobactin genes were identified in various DNA probes using the conditions previously described (Valvano & Cosa, 1984). Colony hybridizations were performed as described by Maniatis et al. (1982). pJHCMV666, a recombinant cosmid harbouring the 8-6 kb and 16-3 kb HindIII fragments from pColV-K30 (Table 1 and Fig. 1c) was used as a control in the hybridization experiments. The probes, (Fig. 1c) consisted of restriction endonuclease fragments that were eluted from agarose gels and labelled with [32P]dCTP by nick-translation (Maniatis et al., 1982). These fragments were as follows: a 3-4 kb HindIII-BamHI fragment from pABN5 (HAM probe); the mixture of two consecutive AvaI fragments of 1-6 kb and 2-7 kb, respectively, from pABN5 (AVAI probe); a 2-9 kb KpnI fragment from pJHCV15 (KPN1 probe); a 8-3 kb HindIII-BglII fragment from pKLS10 (HIND-BGL probe); and a 2 kb SalI-EcoRI fragment from pJHCP107 (ECO-SAL probe, Table 1). The detection of IS1 insertion sequences was achieved using as a probe a labelled DNA fragment from the bacteriophage VA3(1S1) (Lawlor & Payne, 1984). The precise location of the S. flexneri IS1 was determined by hybridizations to Bal1 digests (Maniatis et al., 1982) of the EcoRI insert of pKLS719 (Fig. 1b and Table 1).

**RESULTS**

**Presence of the aerobactin system in enteroinvasive strains of E. coli**

Five of the eleven enteroinvasive E. coli strains examined produced hydroxamates and also cross-fed the E. coli indicator strain LG1522 (Table 2). Only the aerobactin-producing strains were able to grow in M9 minimal medium with 200 μM-α,α'-dipyridyl or 50 μM-FeCl3, were prepared and analysed as described previously (Crosa & Hodges, 1981; Actis et al., 1985). Rabbit antiserum against the 74 kDa outer-membrane protein expressed by pColV-K30 was raised in 6-month-old New Zealand White rabbits by subcutaneous injections of crushed polyacrylamide gel fragments containing the aerobactin receptor protein band from outer-membrane proteins obtained from the E. coli cir strain BC3 harbouring pJHCV8 (Table 1). Outer-membrane proteins separated by SDS-PAGE were transferred to nitrocellulose paper and treated as described by Actis et al. (1985).

**Restriction endonuclease mapping of the aerobactin genes in the E. coli 1107-81**

A preliminary hybridization experiment using the HAM probe (Fig. 1c) on HindIII digests of total DNA from the five aerobactin-producing E. coli strains resulted in the detection of a single HindIII fragment of about 18 kb in the case of strains 1107-81, 1885-77, 978-77, and SSU-5076, and of about 12 kb in the case of strain SSU-5144 (Fig. 2a and data not shown). By contrast, in pColV-K30 the aerobactin genes are found on a 16-3 kb HindIII fragment (Figs 1c and 2a; Bindereif & Neilands, 1983).

To study in more detail the flanking and internal regions of the chromosome-determined aerobactin system in enteroinvasive E. coli, strain 1107-81 was chosen for further analysis. Restriction-endonuclease-cleaved total DNA from this strain was hybridized with different probes containing subsets of the aerobactin region from the plasmid system in pColV-K30 (Fig. 1c). As a result of these experiments we constructed a restriction map of the aerobactin region and its flanking sequences in strain 1107-81 (Fig. 1a). Hybridizations with the HAM probe resulted in the detection of the following fragments: 3-4 kb HindIII-BamHI, 5-5 kb HindIII-BsrEII, 0-7 kb HindIII-SalI and 7 kb HindIII-EcoRI (Fig. 2a, and data not shown), which had the same molecular masses as those of the corresponding fragments in the pColV-K30 aerobactin region (Figs 1a, c and 2a). When the AVAI probe was used, we found homologies with EcoRI and SalI fragments of about 8-3 kb and 12 kb, respectively, and also with two BsrEII fragments of about 7-5 kb and 4 kb (Fig. 2b). The EcoRI chromosome fragment was slightly smaller (8-3 kb) than the corresponding 9 kb EcoRI fragment in pColV-K30 (Figs 1c and 2a, b).
(a) *E. coli* 1107-81

(b) *S. flexneri* SA100

(c) pColV-K30

Fig. 1. Genetic and physical map of the aerobactin region and flanking sequences present in enteroinvasive *E. coli* 1107-81 (a), *S. flexneri* SA100 (b) and pColV-K30 (c). ---, Location of the aerobactin regions in *E. coli* 1107-81 and *S. flexneri* SA100. REPI and REPII, replication regions. AEROBACTIN and 74K indicate the regions containing siderophore and receptor genes, respectively. IS1 insertion sequences. KPNI, AVAI, HIND–BGL, HAM and ECO–SAL are the boundaries of the fragments used as radiolabelled probes (Table 1). Restriction endonucleases: B, BamHI; E, EcoRI; Bs, BstEII; S, SalI; H, HindIII; A, Ad; Bg, BglII; K, KpnI. The restriction sites for BstEII, Ad, BglII and KpnI in (c) are not completely mapped.

### Table 2. Properties of the enteroinvasive *E. coli* strains

| Strain* | O-type† | Hydroxamate‡ | Aerobactin bioassay | Aerobactin utilization | Sensitivity to cloacin DF-13§ | Aerobactin probe||
|---------|---------|--------------|---------------------|------------------------|-------------------------------|------------------------|
| 497-78  | O28ac : NM | –            | –                   | –                      | R                             | –                      | Hybridization with: Colonies Plasmids |
| SSU-5144 | O28ac : NM | +           | +                   | +                      | S                             | +                      |                                      |
| 978-77  | O29 : NM  | +           | +                   | +                      | S                             | +                      |                                      |
| 1885-77 | O29 : NM  | +           | +                   | +                      | S                             | +                      |                                      |
| 929-78  | O124 : NM | –           | –                   | –                      | R                             | –                      |                                      |
| 930-78  | O124 : NM | –           | –                   | –                      | R                             | –                      |                                      |
| 931-78  | O124 : NM | –           | –                   | –                      | R                             | –                      |                                      |
| 3189-75 | O124 : Hnt| –           | –                   | –                      | R                             | –                      |                                      |
| 5898-71 | O124 : H30| –           | –                   | –                      | R                             | –                      |                                      |
| SSU-5076 | O136 : NM | +           | +                   | +                      | S                             | +                      |                                      |
| 1107-81 | Ont : NM  | +           | +                   | +                      | S                             | +                      |                                      |

* Other characteristics of these strains are described by Harris *et al.* (1982).
† NM, non-motile; nt, non-typable.
‡ Hydroxamates were detected by the method of Csaky (1948).
§ R, resistant; S, sensitive.
|| Hybridization experiments were performed using HAM and KpnI probes (Fig. 1c).
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These results demonstrate that in the chromosome of strain 1107-81 there is a region of homology with the pColV-K30-encoded aerobactin biosynthesis genes.

Also, both KPNI and AVAI probes (Fig. 1c) were used to map the region in strain 1107-81 containing sequences corresponding to the pColV-K30 aerobactin receptor region. Three BamHI fragments, of about 8 kb, 3.9 kb and 0.3 kb hybridized with the AVAI probe (Fig. 2b). Of these fragments, only the 3.9 kb fragment was detected with the KPNI probe (Fig. 2c). Total DNA was successively cleaved with EcoRI and BamHI, and also hybridized with the KPNI probe, resulting in the detection of two fragments, one of about 3.2 kb showing a strong homology with the probe and the other of about 0.7 kb (Fig. 2c). Therefore, we concluded that the 3.9 kb BamHI fragment must correspond to the aerobactin receptor region in strain 1107-81 (Fig. 1a). In the case of the pColV-K30 aerobactin system the receptor region lies on a 6.7 kb BamHI fragment (Fig. 2b, c; Krone et al., 1983). These experiments demonstrated that the physical map of the regions corresponding to the aerobactin receptor gene in strain 1107-81 and in pColV-K30 are different (Figs 1a, c and 2c).

Restriction endonuclease mapping of the sequences flanking the aerobactin region of *E. coli* 1107-81

We first determined the restriction map of the sequences flanking the receptor region in strain 1107-81. Total DNA cleaved with both HindIII–BstEII and BstEII was hybridized to the KPNI probe. This experiment resulted in the detection of two hybridizing fragments of 4 kb and 5.5 kb in the case of the HindIII–BstEII-cleaved DNA (Fig. 2c), or 4 kb and 7.5 kb in the case of the BstEII-cleaved DNA (Fig. 2c). The larger HindIII–BstEII and BstEII fragments shown in Fig. 2(c) must contain sequences of the siderophore biosynthesis genes because they strongly hybridized with the HAM and AVAI probes (Fig. 2a, b). In contrast, the 4 kb BstEII fragment showing homology with the KPNI probe must contain sequences lying within the receptor region and its immediate vicinity (Fig. 1a). In pColV-K30, the corresponding BstEII fragment has a size of 3 kb (Figs 1c and 2c). Another hybridization experiment with the KPNI probe performed on EcoRI-cleaved chromosomal DNA from strain 1107-81 showed two EcoRI fragments of 8.3 kb and 7.8 kb, respectively (data not shown). Since the 8.3 kb EcoRI fragment harbours all the siderophore and part of the receptor region (Figs 1a and 2a, b), the 7.8 kb EcoRI fragment must contain the other portion of the receptor region and its flanking sequences. Fig. 1(a, c) shows that a 10.5 kb EcoRI fragment in pColV-K30 corresponds to that of the 7.8 kb EcoRI fragment in strain 1107-81. Therefore, the restriction pattern of the sequences adjacent to the aerobactin receptor region in strain 1107-81 differs from the corresponding region in pColV-K30.

Also differences were noticed in the region of the 1107-81 chromosome flanking the left-hand side of the aerobactin genes as shown in Fig. 1(a, c). Hybridization experiments using the ECO–SAL probe (Table 1 and Fig. 1c) showed homology with the following fragments: 5 kb HindIII, 4.1 kb SalI and 3.4 kb HindIII–SalI (Fig. 2d). In addition, the EcoRI–SalI fragment hybridizing to the ECO–SAL probe had a size of about 1.6 kb whereas the corresponding fragment in pColV-K30 is 2 kb in length (Figs 1c and 2d). Also, in pColV-K30 the next SalI site lies at 23 kb from the left-hand side of the aerobactin region (Fig. 2d; Lawlor & Payne, 1984).

Cloning of the aerobactin region from *S. flexneri* and comparison with the corresponding region in *E. coli* 1107-81

A *S. flexneri* SA100 gene library was generated by cosmid cloning using the pLAFR1 cosmid vector as described in Methods. *E. coli* HB101 cells containing recombinant cosmids were screened by DNA colony hybridization using a 4-8 kb HindIII–BglII fragment from pKLS10 specific for the aerobactin siderophore genes (Fig. 1c). Of 1200 transductants screened, three colonies cross-fed the indicator strain *S. boydii* and were also sensitive to cloacin DF-13 (Van Tiel-Menkveld et al., 1982). One of these clones, harbouring a plasmid designated pKLS971, was chosen for further analysis. EcoRI-cleaved pKLS971 DNA afforded, in addition to vector DNA, fragments of 13 kb, 8-4 kb and 6-4 kb. A hybridization experiment using the HIND–BGL probe (Fig. 1c) revealed homology with a single 8-4 kb EcoRI fragment both in pKLS971
Fig. 2. Southern blot hybridization analysis of cleaved total DNA from *E. coli* 1107-81. (a)–(d) are autoradiographs of Southern blots containing either total DNA or pJHCMV66 DNA (identified in the figure as chromosome or pColV-K30, respectively) cleaved with various restriction enzymes: H, *HindIII*; B, *BamHI*; Bs, *BsrEII*; E, *EcoRI*; S, *SalI*. The *32*P-labelled probes were HAM, AVAI, KPNI and ECO-SAL (see Fig. 1c). To visualize the low-intensity bands in the case of total DNA it was necessary to expose the film longer, resulting in overexposure of the DNA in lanes corresponding to pColV-K30. Thus, those lanes in panel A were obtained from a shorter exposure of the same Southern-blotted gel. The molecular mass markers used were the following: 16.3 kb *HindIII*, 12 kb *SalI*, 9 kb *EcoRI*, 5.5 kb *HindIII*–*BsrEII*, 3.4 kb *HindIII*–*BamHI*, 0.7 kb *HindIII*–*SalI*, 0.4 kb *BamHI* fragments from pJHCV8 (Table 1). The asterisks in (b) indicate restriction endonuclease fragments barely visible in the photograph.
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and in the EcoRI-cleaved total DNA from the S. flexneri chromosome (data not shown). The EcoRI fragments in pKLS791 were subcloned into the high-copy-number vector pAT153. Further restriction endonuclease mapping analysis followed by hybridization experiments using the siderophore probe allowed us to construct a physical map of the aerobactin region in S. flexneri (Fig. 1b).

The comparison of the restriction endonuclease maps of the siderophore and receptor region both in the cloned DNA from S. flexneri SA100 and in the chromosome of E. coli 1107-81 revealed common features (Fig. 1a, b). However, the HindIII fragments carrying the aerobactin genes in the two strains have different sizes: 18 kb in the case of E. coli 1107-81 (Fig. 1a), and 12 kb in S. flexneri (Fig. 1b). The EcoRI fragment containing sequences flanking the aerobactin receptor region in E. coli 1107-81 has a size of about 7.8 kb (Fig. 1a) whereas the corresponding fragment in S. flexneri is 6.4 kb. The sequences flanking the siderophore region in S. flexneri and in E. coli 1107-81 were conserved up to approximately 3 kb to the left-hand side of the HindIII site (Fig. 1a, b).

In pColV-K30 the aerobactin genes are flanked by two copies of the IS1 insertion sequences (Fig. 1c). To determine whether the cloned S. flexneri aerobactin determinants were also flanked by IS1 elements, EcoRI-cleaved pKLS971 DNA was hybridized with a probe specific for the IS1 insertion sequences. Hybridization was detected with the 13 kb EcoRI fragment only in the left flanking region of the S. flexneri aerobactin genes (Fig. 1b and data not shown). Hybridization of the IS1 probe with a subclone containing this 13 kb EcoRI fragment, pKLS719 (shown in Fig. 1b), digested with EcoRI, EcoRI–HindIII, HindIII, HindIII–SalI and SalI (Fig. 3b), allowed us to position the IS1 insertion sequences in the 4.2 kb HindIII–EcoRI fragment (Fig. 1b). A more precise localization of the IS1 within this fragment was achieved by Bal31 digestion of the 13 kb EcoRI fragment after cleavage with HindIII, and hybridization of the IS1 probe to Southern blots of these fragments. A hybridization experiment using the IS1 probe was also performed on total DNA from the enteroinvasive E. coli strain 1107-81 cleaved with various restriction endonucleases. About 10 to 20 DNA fragments, depending on the restriction endonuclease used, showed homology with the IS1 probe (data not shown). Therefore, we could not determine the presence of IS1 insertion sequences in the proximity of the 1107-81 aerobactin genes.

Analysis of iron-regulated outer-membrane proteins expressed by enteroinvasive strains of E. coli and S. flexneri

Several outer-membrane proteins with molecular masses ranging from approximately 74 kDa to 85 kDa could be detected in the cells grown under iron limitation (Fig. 4). These proteins were not apparent when the culture was grown at high iron concentration (data not shown). To identify the aerobactin receptor in these strains we did an immunoblotting experiment using rabbit antiserum raised against the purified pColV-K30 aerobactin receptor expressed by an E. coli cir strain carrying pJHCV8 (Table 1). This antiserum specifically reacted with outer-
Fig. 4. Iron-regulated outer-membrane proteins in enteroinvasive E. coli and S. flexneri. SDS-PAGE profiles of outer-membrane proteins from E. coli K12 cir carrying pJHCV8 (lane 1), aerobactin-producing enteroinvasive E. coli (lanes 3-7), aerobactin-producing S. flexneri SA100 (lane 8), and aerobactin-nonproducing enteroinvasive E. coli (lanes 2 and 9-13). The enteroinvasive E. coli strains were: (2) 929-78, (3) 978-77; (4) 1107-81, (5) 1885-77, (6) SSU-5076, (7) SSU-5144, (9) 497-78, (10) 930-78, (11) 931-78, (12) 3189-75 and (13) 5898-71.

Fig. 5. Immunoblot analysis of iron-regulated outer-membrane proteins from enteroinvasive E. coli and S. flexneri SA100. Outer-membrane proteins from an SDS-PAGE similar to that in Fig. 4 were transferred to nitrocellulose paper and incubated with antiserum raised against the pColV-K30-74 kDa aerobactin receptor outer-membrane protein. Lanes: (1) E. coli HB101 (cir'), (2) E. coli BC3 (cir) carrying pJHCV8, (3) 978-77, (4) 1107-81, (5) 1885-77, (6) SSU-5076, (7) SSU-5144, (8) S. flexneri SA100, (9) 497-78, (10) 929-78, (11) 930-78, (12) 931-78, (13) 3189-75 and (14) 5898-71. Only the region of the blot containing the iron-regulated outer-membrane proteins is shown.

membrane proteins of an apparent molecular mass of 76 kDa in the case of the enteroinvasive E. coli strains (Fig. 5, lanes 3-7), and 77 kDa in the case of S. flexneri SA100 (Fig. 5, lane 8). These proteins are larger than the pColV-K30 aerobactin receptor, which has an apparent molecular mass of 74 kDa. Immunological reactions to outer-membrane proteins from either the aerobactin nonproducers or the Cir+ control strain were not detected (Fig. 5).

DISCUSSION

The present study shows that the genes for the aerobactin system are located on the chromosome of aerobactin-producing enteroinvasive E. coli strains. Since in the case of S. flexneri the aerobactin system is also located on the chromosome (Lawlor & Payne, 1984), we compared the aerobactin regions in these organisms. This analysis was facilitated by the cloning of the aerobactin genes from the chromosome of S. flexneri SA100. Restriction endonuclease mapping and hybridization experiments showed that the regions encompassing the cloned S. flexneri aerobactin biosynthesis genes were identical to the corresponding regions in both the
enteroinvasive *E. coli* 1107-81 and the pColV-K30 plasmid system. However, differences were found in the receptor regions of the chromosome-mediated aerobactin systems with respect to pColV-K30. In the case of enteroinvasive *E. coli* and *S. flexneri* the regions adjacent to the left-hand side of the aerobactin system exhibited some similarities, but were different from those in pColV-K30. The regions adjacent to the right-hand side in *E. coli*, *S. flexneri* SA100 and pColV-K30 were all different (Fig. 1).

The aerobactin system in pColV-K30 is flanked by two replication regions, REP1 and REP1I (Perez-Casal & CroSA, 1984), and by two IS1 elements (Lawlor & Payne, 1984; McDougall & Neilands, 1984; Perez-Casal & CroSA, 1984). In *S. flexneri*, one copy of IS1 was found on the left flanking region of the cloned aerobactin genes (Fig. 1b), but none was detected within 5 kb to the right end of the aerobactin genes. The structure of the flanking *S. flexneri* IS1 also differs from the pColV-K30 IS1 insertion sequences. The *S. flexneri* sequence hybridizes to IS1 under stringent conditions but lacks at least one restriction site, *PstI*, which is present in the pColV IS1/s. The presence of IS1 in the vicinity of the aerobactin genes of *E. coli* 1107-81 could not be determined since various chromosome DNA fragments from this strain showed homology with the IS1 probe.

The plasmid ColV-K30 encodes an iron-regulated outer-membrane protein with an apparent molecular mass of 74 kDa (Krone et al., 1983), which is the receptor for ferric-aerobactin (Van Tiel-Menkveld et al., 1982). In the aerobactin-producing enteroinvasive *E. coli* and *S. flexneri*, we found several iron-regulated outer-membrane proteins. Rabbit antiserum raised against the purified pColV-K30 74 kDa aerobactin receptor reacted with iron-regulated outer membrane proteins of about 76 kDa in enteroinvasive *E. coli*, and of 77 kDa in *S. flexneri* cell envelopes. Griffiths et al. (1985) also reported that *S. flexneri* strains and *S. flexneri*–*E. coli* K12 hybrids carrying the aerobactin system express an iron-regulated outer-membrane protein of about 76 kDa. Our results demonstrate that the iron-regulated outer-membrane proteins with molecular masses larger than 74 kDa cross-react immunologically with the aerobactin receptor protein encoded by pColV-K30. The fact that the cross-reacting outer-membrane proteins in these isolates have a higher molecular mass than the pColV-K30 aerobactin receptor may be due to some heterogeneity at the level of the DNA nucleotide sequence at the downstream end of the receptor genes.

The role of the aerobactin system as a virulence determinant in enteroinvasive *E. coli* and *S. flexneri* has not been defined. In the case of *E. coli* strains invading extraintestinal tissues, the aerobactin system may play a role in the infectious process by allowing bacterial growth under conditions in which iron is not readily available (Williams, 1979). Similarly, the aerobactin system could contribute to overcoming the iron limitation in the intestinal lumen due to the presence of lactoferrin, a high-affinity iron-binding protein which is commonly present in mucosal surfaces (Bullen et al., 1978). Experiments to test this hypothesis are currently being carried out.

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**REFERENCES**


Role of iron in bacterial infections. Current Topics in Microbiology and Immunology 80, 1-35.