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# Aerobactin Iron Transport Genes Commonly Encoded by Certain ColV Plasmids Occur in the Chromosome of a Human Invasive Strain of *Escherichia coli* K1

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**The aerobactin-mediated iron uptake system encoded by pColV-K30 and other ColV plasmids has been associated with the ability of *Escherichia coli* strains to cause disease. We investigated whether the pColV-K30 aerobactin system is present in *E. coli* K1 VW187 isolated from a human neonate with meningitis. This strain exhibited a functional aerobactin-mediated iron uptake system, as assessed by a cross-feeding bioassay and by its sensitivity to cloacin, a bacteriocin that recognizes the outer membrane receptor for iron-aerobactin complexes. By using a variety of techniques, we could not find any plasmid harboring the aerobactin genes. Hybridization of restriction endonuclease-cleaved chromosomal DNA from strain VW187 with various clones containing subsets of the pColV-K30 aerobactin region showed that the aerobactin genes were located on a 10.5-kilobase-pair chromosomal *Hin*dIII restriction fragment which also contained IS*1*-like insertion sequences. The chromosomal aerobactin region showed a high degree of conservation when compared with the homologous region in plasmid pColV-K30, although it was located on a different restriction endonuclease site environment.**

The ability to obtain iron, an essential element in bacterial metabolism, has been correlated with virulence for a variety of microorganisms pathogenic to humans and animals (7a, 24, 41). Many species of bacteria have developed high-affinity mechanisms to take up iron from the environment. In most cases, these processes involve soluble iron-carrying molecules, siderophores, and their corresponding membrane-associated transport systems (24).

Recently, plasmid-mediated iron transport mechanisms were demonstrated in invasive strains of *Escherichia coli* and *Vibrio anguillarum*. The plasmids pColV-K30 in *E. coli* and pJM1 in *V. anguillarum* endowed these bacteria with the capacity to grow under iron-limiting conditions (10, 11, 42). This property was correlated with an enhanced ability to cause septicemic infections in the respective animal hosts (10, 11, 42). The pColV-K30-specified siderophore has been identified as aerobactin (39), a hydroxamate compound originally found in culture supernatants of *Enterobacter aerogenes* (14). The genes specifying aerobactin and its membrane receptor complex in plasmid pColV-K30 are located on a 16.3-kilobase-pair (kb) *Hin*dIII restriction endonuclease fragment (3).

To study the spread of the aerobactin-mediated iron uptake system in *E. coli* K1 strains associated with human neonatal infections, a number of clinical isolates were examined for the presence of DNA sequences related to the pColV-K30 aerobactin genes, as well as for the existence of functional components of this system. In this report, we present evidence indicating that one of these strains, *E. coli* K1 VW187, possesses genetic determinants for the aerobactin-mediated iron uptake system and that these genes are located on the bacterial chromosome.

(These results were presented, in part, at the 84th Annual Meeting of the American Society for Microbiology, 4–9 March 1984, St. Louis, Mo. [Y. Mitoma, J. Perez-Casal, M. A. Walter, and J. H. Crosa, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B175, p. 158], and at the Conference on Plasmids in Bacteria, 14–18 May 1984, Urbana, Ill. [M. A. Valvano, M. K. Wolf, L. M. Crosa, and J. H. Crosa, Abstract W-29].)

## MATERIALS AND METHODS

**Bacterial strains.** The *E. coli* strains used in this study, their relevant characteristics, and sources are listed in Tables 1 and 2. Strain VW187 is an isolate obtained from the cerebrospinal fluid of a human neonate with meningitis. The presence of K1 capsular polysaccharide was determined by the agar-antiserum test and by the sensitivity to K1-specific bacteriophages, as previously described (15, 33). This strain was sensitive to all of the antibiotics tested, which included ampicillin (Ap), tetracycline (Tc), kanamycin (Km), gentamicin (Gm), and chloramphenicol (Cm). *E. coli* HB101 and C600 were used as a recipient in transformation experiments and as a source of chromosomal DNA, respectively.

**Biological detection of aerobactin siderophore and its outer membrane receptor.** Aerobactin production was determined by the ability of sterile supernatants to support the growth of strain LG1522. This iron uptake-deficient derivative carries the plasmid pColV-K30 with a mutation in genes specifying aerobactin synthesis, which does not affect the biosynthesis of the aerobactin receptor. The bioassay test was carried out on bacteria grown in a Tris minimal medium (31) with 1% sodium succinate as a carbon source (TMS medium) and 50 μM α,α′-dipyridyl as an iron chelator, as previously described (43). Positive and negative controls were strains LG1315 and HB101, respectively. Strain RWB18 was used as a control lawn for cross-feeding specificity, since this plasmidless bacterium does not have the aerobactin receptor.

Since the outer membrane receptor for ferric aerobactin complexes is also the receptor for the bacteriocin cloacin (2, 38), its presence was biologically determined by the cloacin

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TABLE 1. Properties of *E. coli* strains

| Strain | Genotype | Relevant phenotype[a] | Plasmid | Reference or source |
|---|---|---|---|---|
| LG1315 | *ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl* (pColV-K30) | $Iu^+$ $C^s$ | pColV-K30 | 43 |
| LG1522 | *ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi* (pColV-K30 *iuc*) | $Iu^-$ $C^s$ | pColV-K30 (*iuc*) | 43 |
| RW193 | *entA proC leu trp tsx thi galK ara entA mtl xyl azi supE44* | $Iu^-C^r$ | Plasmidless | 40 |
| RWB18 | Same as strain RW193 but *fepA* | $Iu^-$ $C^r$ | Plasmidless | 40 |
| C600 | *thi thr leu lacY tonA supE* | $Iu^+$ $C^r$ | Plasmidless | 1 |
| HB101 | *hsdS recA ara proA lacY galK rpsL xyl mtl supE* | $Iu^+$ $C^r$ | Plasmidless | 7 |
| C600-1 | 600(pBRG29) | $Cm^r$ $Tc^r$ $Ap^r$ | pBR322 with Tn*9* insertion | D. Berg, personal communication |
| HB101-1 | HB101(pBR325) | $Cm^r$ $Tc^r$ $Ap^r$ | pBR325 | 5 |

[a] Iu, Iron uptake ability. In the case of strains LG1315 and LG1522, this phenotype includes both the enterobactin iron transport system as well as the aerobactin-mediated iron uptake system present in pColV-K30. *iuc* is a mutation on this plasmid which affects the genes for aerobactin synthesis but leaves intact the receptor genes. In the rest of the strains, Iu refers only to the enterobactin system. The symbols $C^s$ and $C^r$ stand for cloacin sensitivity and resistance, respectively.

sensitivity test (2, 38). Cloacin-sensitive strain LG1315 and cloacin-resistant strain RWB18 were included as controls.

**Plasmid transfer and curing experiments.** The possible existence of transmissible plasmids harboring the genes for the aerobactin system in strain VW187 was investigated by a plate-mating procedure (8). A nalidixic acid- (Nx) resistant derivative of *E. coli* RWB18 was used as a recipient in the conjugation experiments. Exconjugant selection was carried out in TMS agar plates containing 120 μM α,α'-dypyridyl and 20 μg of nalidixic acid per ml. Under these conditions, only exconjugants receiving the aerobactin-mediated iron uptake system could grow, as demonstrated in control experiments by using as a donor strain LG1315 which harbors plasmid pColV-K30. Transformation and cotransformation experiments were performed by previously described methods (9, 18), with strain HB101 used as a recipient. In the case of cotransformation experiments with plasmid pBR322, indirect selection of the cotransformed cryptic plasmids from strain VW187 was carried out by plating on L-agar plates containing 20 μg of tetracycline per ml and 200 μg ampicillin per ml.

Plasmid curing experiments were carried out by using the nonmutagenic sodium dodecyl sulfate method described by Tomoeda et al. (37).

**Isolation of plasmid and chromosomal DNA.** Large-scale purification of plasmid DNA was performed by the methods of Hansen and Olsen (16) and Birnboim and Doly (4). Further purification of plasmid DNA was achieved by centrifugation in cesium chloride-ethidium bromide density gradients at 50,000 rpm for 16 h at 15°C in a VTi65 rotor in a Beckman ultracentrifuge L8-70. Plasmid screening in transformation and recombinant DNA experiments was carried out by using a rapid alkaline lysis procedure (4). A mild in situ lysis procedure for screening of large plasmids was performed as described by Rosenberg et al. (29).

Chromosomal DNA was isolated after bacterial lysis with lysozyme and Sarkosyl as described previously (23) but modified by adding a subsequent treatment with proteinase K (30 μg/ml) at 65°C for 2 h.

**Restriction endonuclease analysis and molecular cloning experiments.** Restriction enzymes were used under the conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Electrophoresis of restriction endonuclease-cleaved DNA was performed in horizontal agarose slab gels (0.5% for chromosomal DNA and 0.7% for plasmid DNA) in a Tris-borate buffer system (21) at 20 mA during 16 h. Analysis of covalently closed circular DNA was performed in a vertical 0.7% agarose electrophoresis gel as described previously (21).

Molecular cloning of the aerobactin and receptor regions from the plasmid pColV-K30 was carried out by ligating pColV-K30 DNA partially digested with *Hin*dIII into the *Hin*dIII site of the cosmid vector pVK102 (17), a derivative of pRK290 (13). Cosmid clones were packaged in vitro following the conditions recommended by the supplier (Promega Biotec, Madison, Wis.), and transductants were examined for cloacin sensitivity. To obtain DNA probes specific for internal and flanking regions of aerobactin and receptor genes, we subcloned the 16.3-kb *Hin*dIII fragment by digestion with various restriction endonucleases, ligation with the appropriate cloning vectors, and transformation in strain HB101. Thus, the recombinant plasmids pJHC-V4, pJHC-V8, pJHC-V10, pJHC-V11, and pJHC-V12 were obtained by cloning subsets of the 16.3-kb *Hin*dIII fragment into various cloning vectors. The details of the plasmid constructions and

TABLE 2. Aerobactin and aerobactin receptor production by clinical isolates and strains harboring recombinant plasmids

| Strain | Plasmids[a] | Aerobactin production[b] | Aerobactin receptor[c] |
|---|---|---|---|
| VW187 | Five cryptic (25.4, 4.6, 3.3, 2, and 1.2 Md) | + | + |
| VW187-19[d] | Four cryptic (4.6, 3.3, 2, and 1.2 Md) | + | + |
| HB101-V8 | pJHC-V8 | + | + |
| HB101-V12 | pJHC-V12 | − | − |
| HB101-V4 | pJHC-V4 | − | + |
| HB101-V10 | pJHC-V10 | − | − |
| HB101-V11 | pJHC-V11 | − | − |

[a] Values in parentheses are the molecular masses of the plasmids present in the strain expressed in Md.

[b] Aerobactin production was determined by a cross-feeding bioassay, as described in the text.

[c] Presence of the aerobactin receptor was assessed by the cloacin sensitivity test, as described in the text.

[d] VW187-19 was obtained from VW187 by curing the 25.4-Md plasmid, as described in the text.

phenotype conferred by the recombinant plasmids are given in the legend to Fig. 3 and Table 2.

$^{32}$P-labeled plasmid DNA from the recombinant clones labeled by nick translation (27) was used as a probe in Southern blot hybridization experiments (32) as described previously (21). When needed, radioactive probes were prepared from purified fragments. Restriction endonuclease fragments were isolated from agarose gels by electroelution, and the DNA was concentrated by ethanol precipitation (21).

## RESULTS

**Presence of components of the aerobactin-mediated iron uptake system in the *E. coli* K1 strain VW187.** Table 2 shows that culture supernatants of strain VW187 obtained under iron-limiting conditions cross-fed the indicator strain LG1522. In addition, strain VW187 was sensitive to cloacin. Thus, both components, the siderophore aerobactin as well as its cognate receptor, must be synthesized by VW187.

Since it has been shown in previous studies that in many strains of *E. coli*, the aerobactin-mediated iron uptake system is specified by certain ColV plasmids (2, 35, 42), strain VW187 was first screened for a plasmid-carried aerobactin system. After alkaline lysis, followed by agarose gel electrophoresis, five plasmid classes of 25.4, 4.6, 3.3, 2, and 1.2 megadaltons (Md) were detected (Fig. 1a, lane A). We could not demonstrate the existence of plasmid DNA molecules larger than 25.4 Md, even when using methods specifically designed for the isolation of large plasmids (see above).

To investigate whether aerobactin sequences were present in any of the five plasmid classes identified in strain VW187, we performed Southern blot hybridizations of plasmid DNA with various probes containing the aerobactin genes from pColV-K30. These probes were prepared by using as cloning vectors derivatives of the plasmid pRK290 (13), which did not show any background hybridization with plasmid or chromosomal DNA from the strain VW187 (Fig. 2, lanes A through C). The recombinant plasmid pJHC-V8, containing the 16.3-kb *Hin*dIII fragment from pColV-K30, showed homology with the 25.4-Md plasmid (Fig. 1b, lane A), whereas the smaller plasmids did not hybridize (Fig. 1b, lanes A and B). However, a derivative of VW187 in which the 25.4-Md plasmid had been cured (Fig. 1a, lane B) still exhibited a biologically detectable aerobactin system (Table 2). The smaller plasmids did not hybridize with the pJHC-V8 probe, and the 25.4-Md plasmid is not necessary for aerobactin production. Therefore, the results of this part of the study indicate that the aerobactin genes must not be encoded by any of the VW187-identified plasmids. In that case, the homology shown by the 25.4-Md plasmid with the pJHC-V8 probe must be due to a region other than the sequences coding for aerobactin.

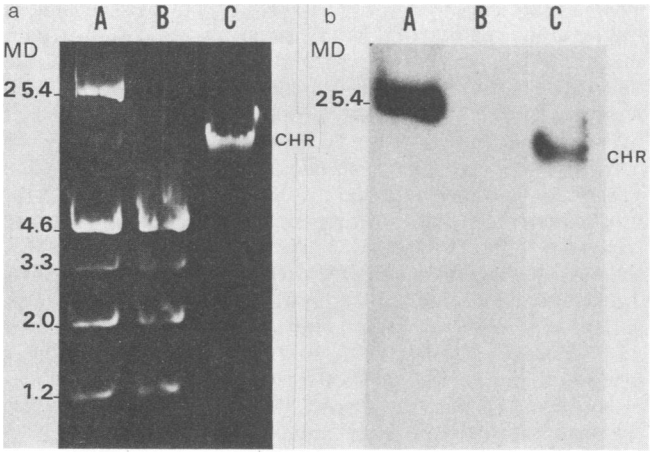


FIG. 1. Southern blot hybridization analysis of plasmid DNA from the *E. coli* K1 strain VW187. (a) A 0.7% agarose gel stained with ethidium bromide. The plasmid DNA complement of strains VW187 and VW187-19 are shown in lanes A and B, respectively; lane C, uncleaved VW187-19 chromosomal DNA (CHR). MD, Megadaltons. (b) Autoradiograph of a Southern blot hybridization of the gel in (a), using pJHC-V8 plasmid DNA as a $^{32}$P-labeled probe.

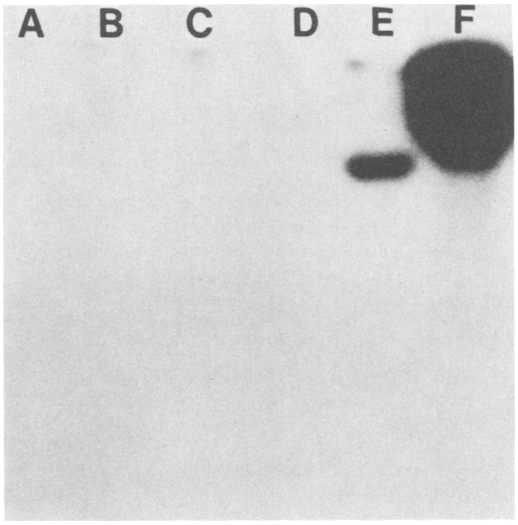


FIG. 2. Southern blot hybridization of restriction endonuclease-cleaved chromosomal and plasmid DNA from VW187, with $^{32}$P-labeled pVK102 DNA used as a probe. Southern blot of a 0.6% agarose gel. Lane A, covalently closed circular plasmid DNA from VW187; lanes B and C, total DNA from strain VW187 cleaved with restriction endonucleases (lane B, *Eco*RI; lane C, *Hin*dIII); lane D, *Hin*dIII-cleaved plasmid DNA from pABN1, a ColE1 derivative containing the pColV-K30 16.3-kb *Hin*dIII fragment (3); lane E, *Hin*dIII-cleaved lambda DNA; lane F, pVK102 covalently closed circular DNA. The homology in lane E is due to the *cos* sites present in both pVK102 and lambda DNA.

To investigate the region of the 16.3-kb *Hin*dIII fragment responsible for the hybridization with this plasmid, purified plasmid DNA preparations were electrophoresed in agarose gels and Southern blots hybridized with probes pJHC-V4, pJHC-V10, pJHC-V11, and pJHC-V12. The internal segments of the 16.3-kb *Hin*dIII fragment contained in each one of these recombinant derivatives are identified in Fig. 3. The results (Fig. 4, lane B) indicate that there is homology between the pJHC-V11 probe and the 25.4-Md plasmid. Probes pJHC-V12, pJHC-V4, and pJHC-V10 did not hybridize with this plasmid (data not shown). Therefore, the 25.4-Md plasmid hybridizes with the right end of the 16.3-kb *Hin*dIII fragment of pColV-K30 and hence does not contain the aerobactin sequences (Fig. 3).

Further characterization of the location of aerobactin genes in strain VW187 was carried out by attempting to transfer by conjugation the aerobactin determinants of VW187 to a Nx$^r$ derivative of RWB18, an iron uptake-deficient *E. coli* strain (Table 1). Any exconjugants obtained from this mating could be selected by their ability to grow under iron-limiting conditions after transfer of the aerobactin determinants. In control experiments, using as a donor strain

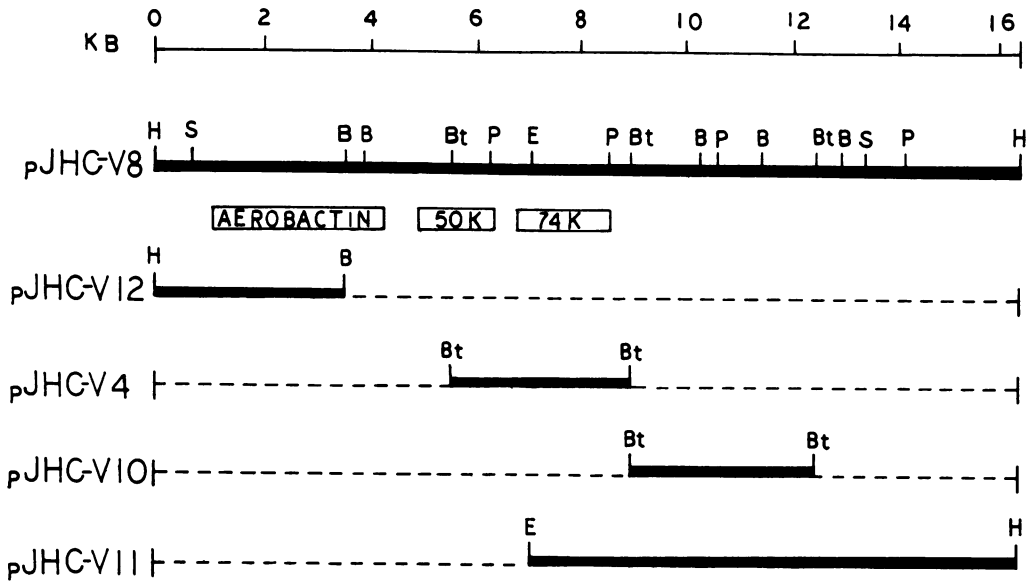


FIG. 3. Genetic and physical map of cloned pColV-K30 DNA containing components of the aerobactin region. The map of the pColV-K30 16.3-kb *Hin*dIII fragment cloned in the vector pVK102 (17) is shown with the appropriate map coordinates expressed in kilobase pairs (KB). Restriction endonuclease mapping of this fragment in the recombinant clone, designated pJHC-V8, showed no differences with respect to data previously reported (3, 19, 20), except that we found an extra *Bst*EII cleavage site on the right end of the fragment. The regions labeled aerobactin, 50 K, and 74 K are those regions identified as encoding, respectively, siderophore and receptor complex components of the aerobactin iron uptake system (3, 19, 20). Thicker lines represent the regions from the 16.3-kb *Hin*dIII fragment that were subcloned in various cloning vehicles. Dashed lines correspond to the regions of this fragment that were not included in the clones. The cleavage sites for various restriction endonucleases are shown: H, *Hin*dIII; S, *Sal*I; B, *Bam*HI; Bt, *Bst*EII; P, *Pvu*II; E, *Eco*RI. The recombinant plasmid pJHC-V12 consists of the 3.4-kb *Hin*dIII-*Bam*HI fragment located at coordinates 0 to 3.4 cloned in the *Hin*dIII-*Bgl*II ends of pVK101 (17). Plasmid pJHC-V4 contains the 3.3-kb *Bst*EII fragment located at coordinates 5.5 to 8.8 cloned in the vector pJBK45, which was constructed and kindly provided by J. Kaper, Center for Vaccine Development, University of Maryland, Baltimore. Plasmid pJHC-V10 contains a 3.5-kb *Bst*EII fragment, located at coordinates 8.8 to 12.3, which was also cloned in pJBK45. Plasmid pJHC-V11 consists of a 9.6-kb *Eco*RI-*Hin*dIII fragment located at coordinates 6.7 to 16.3 cloned in pVK101.

LG1315 harboring plasmid pColV-K30, we observed a frequency of $10^{-6}$ iron uptake-proficient, $Nx^r$ exconjugants. In contrast, no exconjugants with such a phenotype were detected when the donor was VW187 (conjugation frequency, less than $10^{-8}$). Consequently, a transferable plasmid does not appear to harbor aerobactin genes in VW187. We also purified total plasmid DNA from strain VW187 for transformation experiments. Since plasmids of strain VW187 do not code for resistance to any of the antibiotics tested (see above), a small amount of pBR322 DNA was used to cotransform strain HB101 with these plasmids, as described above. The small plasmid classes of VW187 were detected in about 50% of the transformants, whereas the 25.4-Md plasmid was found in only 4% of the colonies. Bacteria harboring either the small plasmids or the 25.4-Md plasmid did not produce aerobactin, and they were not sensitive to cloacin.

Therefore, the genetic and molecular experiments described in this part of the study suggest that the aerobactin genes in strain VW187 are not encoded by any plasmid, and hence, they must be encoded in the chromosome.

**Localization of internal and adjacent regions of aerobactin genes in the chromosome of *E. coli* K1 VW187.** If the aerobactin sequences are present in the chromosome of strain VW187, chromosomal DNA from the cured derivative of this strain, designated VW187-19, lacking the 25.4-Md plasmid, should hybridize to the 16.3-kb *Hin*dIII fragment of pColV-K30. Evidence shown in Fig. 1b, lane C indicates that this was the case. To localize the aerobactin system genes in the VW187 chromosome, Southern blots of restriction endonuclease-digested total DNA were hybridized with probes containing subsets of the pColV-K30 aerobactin region (Fig. 3). The presence of siderophore and receptor genes was investigated by using as probes recombinant plasmids pJHC-V12, which contains a 3.4-kb *Hin*dIII-*Bam*HI fragment carrying sequences specific for siderophore genes, and pJHC-V4, which contains a 3.3-kb *Bst*EII fragment carrying sequences for the aerobactin receptor genes, as well as about 400 base pairs of DNA sequences adjacent and to the right of the receptor region (Fig. 3). The hybridization results with the pJHC-V12 probe (Fig. 5a and c, lanes B) demonstrate that there is homology with a single chromosomal *Hin*dIII fragment of 10.5 kb. Further characterization of the nature of these sequences was achieved by successively digesting the VW187-19 chromosome with two restriction endonucleases. Treatment with *Hin*dIII and *Eco*RI changed the hybridization pattern so that a fragment of about 7 kb showed homology with the pJHC-V12 probe (Fig. 5a and c, lanes C). Successive digestions with *Hin*dIII and *Bst*EII resulted in a 5.5-kb fragment which also hybridized with that probe (Fig. 5a and c, lanes D). The sizes of these fragments coincided with the *Hin*dIII-*Eco*RI and *Hin*dIII-*Bst*EII distances mapped along the left half of the 16.3-kb pColV-K30 fragment (Fig. 3). Thus, these results indicate that not only are the chromosomal aerobactin genes located on a single 10.5-kb *Hin*dIII fragment of the VW187-19 chromosome, but the *Eco*RI and *Bst*EII sites must also be located at essentially the same positions as those found in the pColV-K30 16.3-kb *Hin*dIII fragment.

The hybridization results with the pJHC-V4 probe (Fig. 5b and d) demonstrate that fragments obtained by treatment with either *Hin*dIII alone (10.5, 8, and 3.3 kb; Fig. 5b and d, lanes B) or *Hin*dIII followed by *Eco*RI (7, 4.4, 3.4, and 2.3

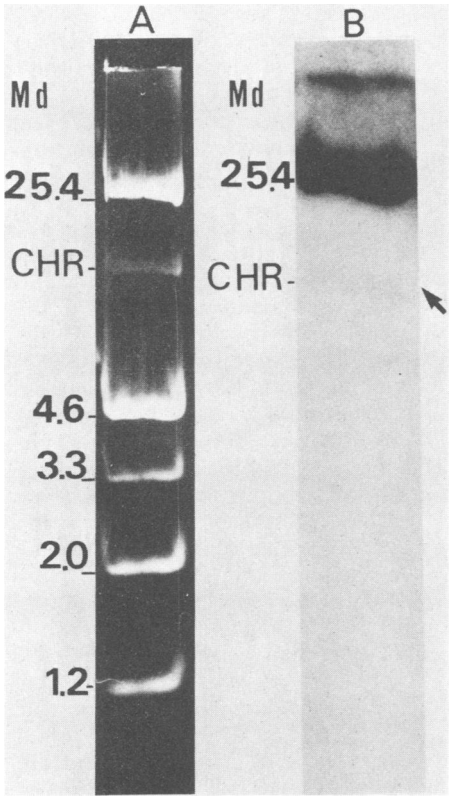


FIG. 4. Southern blot hybridization of plasmid DNA complement from VW187 with the pJHC-V11 probe. Lane A, Plasmid DNA complement of strain VW187 in an ethidium bromide-stained 0.7% agarose gel. Molecular weights are indicated in Md. CHR, residual chromosomal DNA. Lane B, Southern blot hybridization of the gel shown in lane A, with $^{32}$P-labeled pJHC-V11 used as a probe. Arrow shows the region in which some homology could be observed due to residual VW187 chromosomal DNA still present after plasmid purification.

kb; Fig. 5b and d, lanes C) hybridized with the pJHC-V4 probe. The homologies were particularly high with the 10.5-kb *Hin*dIII fragment (Fig. 5b and d, lanes B) as well as with both the 7- and 3.4-kb fragments obtained by successive digestions with both *Hin*dIII and *Eco*RI restriction endonucleases (Fig. 5b and d, lanes C). In the case of the sample treated with both *Hin*dIII and *Bst*EII (Fig. 5b and d, lanes D), a 4-kb fragment exhibited the highest homology with that probe, whereas there were two other fragments of 2.8 and 1 kb which showed very low homology (not visible in the photograph). Since the pJHC-V4 probe consisted mostly of the regions of pColV-K30 DNA containing the cloacin-aerobactin receptor genes, our results demonstrate that these genes are included in the 10.5-kb *Hin*dIII fragment of VW187-19 chromosomal DNA, which also contained the aerobactin siderophore genetic determinants. In addition, since the pJHC-V4 probe showed hybridization with more chromosomal fragments than did the pJHC-V12 probe, other sequences present in the 3.3-kb *Bst*EII fragment in pJHC-V4 but absent from the 3.4-kb *Hin*dIII-*Bam*HI fragment in pJHC-V12 must be repeated in several locations of the VW187-19 chromosome.

When cleaved chromosomal DNA from C600 was hybridized with the pJHC-V12 probe, we did not detect homologies (Fig. 5a and c, lanes A). In contrast, the pJHC-V4 probe showed homology with four C600 chromosomal *Hin*dIII restriction fragments of 13, 6.8, 5, and 1.3 kb (Fig. 5b and d, lanes A). The homology at a location corresponding to fragments of a molecular length larger than 23 kb could be due to a partial digestion of the C600 chromosomal DNA. Therefore, the cloned DNA in the pJHC-V4 probe, but not in

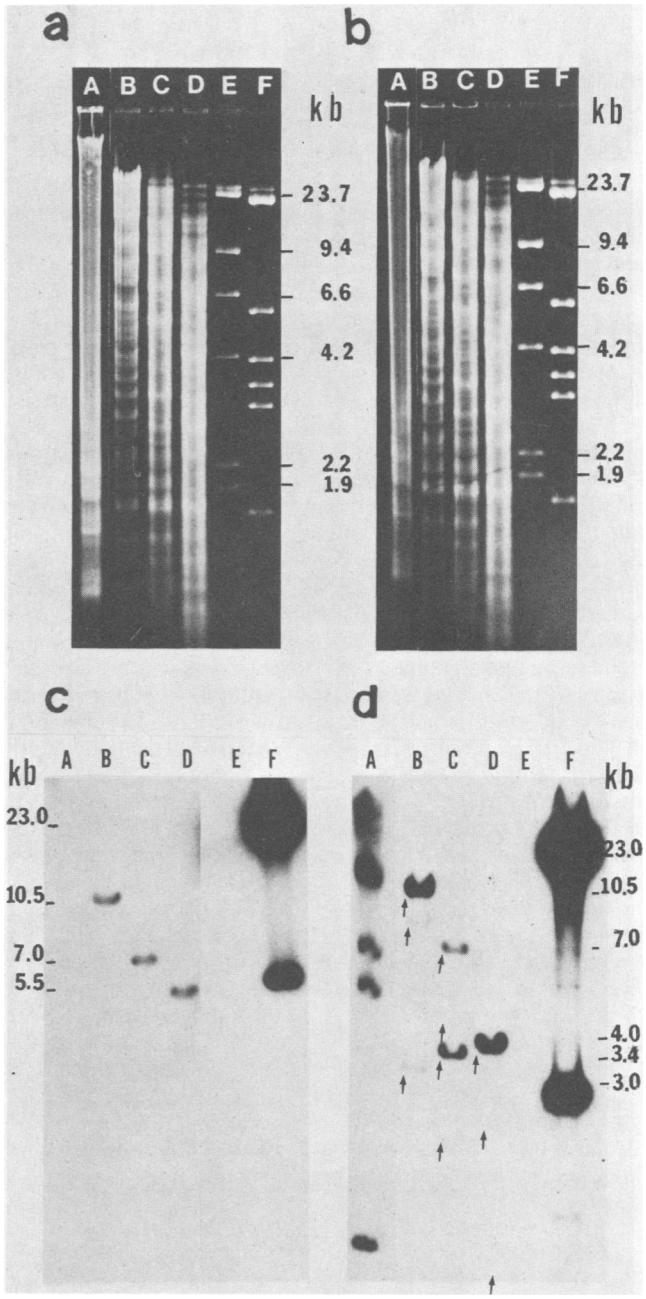


FIG. 5. Southern blot hybridization of restriction endonuclease-cleaved DNA with specific probes for the aerobactin siderophore and receptor regions. (a and b) Duplicate ethidium bromide-stained 0.5% agarose gels of restriction endonuclease-cleaved DNA. Lanes A, C600 DNA; lanes B through D, VW187-19 DNA; lanes E, lambda DNA; lanes F, pJHC-V8 DNA. The restriction endonucleases used were: lanes A, B and E, *Hin*dIII; lanes C, successive digestions with *Eco*RI and *Hin*dIII; lanes D and F, successive digestions with *Hin*dIII and *Bst*EII. (c and d) Autoradiographs of Southern blot hybridizations of the gels described in (a and b) with $^{32}$P-labeled probes; (c) was obtained from (a), and (d) was obtained from (b). (c) pJHC-V12 probe. (d) pJHC-V4 probe; arrows show VW187-19 chromosomal restriction fragments hybridizing with the pJHC-V4 probe.

the pJHC-V12 recombinant plasmid, contained sequences repeated several times in the *E. coli* K-12 C600 chromosome, as was previously found with the chromosome of strain VW187-19.

Since IS*1*-like insertion sequences were recently found to flank the aerobactin region in pColV-K30 (25a), we suspected that the repeated sequences present in the chromosomal DNA of these strains which hybridized with the pJHC-V4 probe were IS*1*-like elements. Therefore, we performed Southern blot hybridizations of restriction endonuclease-cleaved chromosomal DNA from strains VW187-19 and C600, using as a probe a 1.5-kb *Eco*RI fragment containing one of the IS*1* elements and part of the $Cm^r$ gene of the transposon Tn*9* transposition sequence. This fragment was obtained from plasmid pBRG29 (constructed and kindly provided by D. Berg, Washington University, St. Louis, Mo.), which carries a complete copy of Tn*9*. The results of this experiment (Fig. 6a and c, lanes A through C) indicate that this probe hybridized with several chromosomal fragments in both C600 (Fig. 6a and c, lane A) and VW187-19 (Fig. 6a and c, lanes B and C). The homology pattern obtained with the C600 chromosome in the hybridization with the IS*1* probe (Fig. 6a and c, lanes A) is identical to that obtained with this same chromosomal DNA hybridized with the pJHC-V4 probe (Fig. 5b and d, lanes A) which confirms that the 3.3-kb *Bst*EII fragment from pColV-K30 contains IS*1*-like elements. Several fragments, including a 10.5-kb *Hin*dIII fragment of VW187-19 chromosome, hybridized with the IS*1* probe (Fig. 6a and c, lanes B). The 10.5-kb *Hin*dIII fragment must be the same as that which contains the aerobactin sequences, since this fragment is not present in the VW187 chromosomal DNA successively digested with *Eco*RI and *Hin*dIII and hybridized with the IS*1* probe (Fig. 6a and c). In addition to other fragments, the IS*1* probe showed homology with a fragment of about 3.4 kb, as was the case with pJHC-V4, whereas there was no homology at the location corresponding to a 7-kb fragment (Fig. 6a and c, lanes C). This was an expected result, since the 7-kb chromosomal *Eco*RI-*Hin*dIII fragment contains a portion of the aerobactin region but lacks the IS*1*-like sequences (Fig. 5a and c, lanes C). In the control experiment, C600 and VW187-19 chromosomal DNAs did not hybridize when the probe was a 1.2-kb *Eco*RI-*Hin*dIII fragment from pBR325 (5). This fragment contains part of the $Cm^r$ gene also present in Tn*9*, but does not have IS*1* sequences (Fig. 6b and d, lanes A through C).

Thus, the results of this part of the study indicate not only that the aerobactin region is present in the 10.5-kb *Hin*dIII chromosomal fragment from strain VW187-19, but also that this fragment contains IS*1*-like sequences.

## DISCUSSION

*E. coli* is one of the most important agents responsible for neonatal sepsis and meningitis (28). It is now well recognized that most *E. coli* strains isolated from these infections possess the K1 capsular polysaccharide antigen (28), which is associated with resistance of these bacteria to both phagocytosis by polymorphonuclear cells and lysis by serum complement (6, 25, 26, 34, 36). However, to cause sepsis and meningitis, microorganisms must also grow and reach a relatively high concentration in body fluids and tissues (12), which depends on their capacity to obtain iron, an essential nutrient factor (7a, 41). In vertebrates, iron is bound to high-affinity iron-binding proteins, such as transferrin in serum and lactoferrin in secretions, and is not free to be used by the invading pathogen (41). Thus, *E. coli* K1 strains attempting to establish an infection must possess specific iron transport systems to help them in overriding the iron starvation imposed by the host.

In recent years, there has been a careful examination of those properties that enable *E. coli* K1 strains to resist host killing mechanisms (6, 25, 26, 30, 34, 36); however, the analysis of iron transport systems as a potential virulence attribute in these pathogens has received less attention.

In the case of other invasive *E. coli* strains causing septicemia in humans and animals, the aerobactin-mediated iron uptake system, commonly encoded by pColV-K30 and other ColV plasmids, has been associated with their ability to cause disease (42). Consequently, we studied the occurrence of aerobactin genes in *E. coli* K1 strains isolated from cerebrospinal fluid of human neonates with meningitis.

*E. coli* K1 VW187 exhibited a functional aerobactin-mediated iron uptake system, as assessed by a cross-feeding bioassay and by its sensitivity to cloacin, a bacteriocin that recognizes the outer membrane receptor for iron-aerobactin complexes (2, 38). Plasmid analysis of strain VW187 revealed the presence of five plasmid classes. The aerobactin genes were not carried by any of these plasmids, although one of them (25.4 Md) showed homology with pColV-K30 DNA sequences other than the aerobactin region. Moreover, strain VW187-19, a derivative of VW187 cured of the 25.4-Md plasmid, retained a functional aerobactin-mediated system.

By using a variety of genetic and molecular techniques, we could not find an additional, still undetected plasmid harboring the aerobactin genes in VW187, suggesting that these genes must be located on the chromosome.

Hybridization of restriction endonuclease-cleaved chromosomal DNA from strain VW187-19 with various clones containing subsets of the pColV-K30 aerobactin region demonstrated that the genetic determinants for aerobactin production and the cloacin receptor are part of a chromosomal *Hin*dIII fragment which is about 6 kb smaller than the *Hin*dIII fragment in which they are found in plasmid pColV-K30. Nonetheless, the chromosomal aerobactin and receptor regions appeared to be highly conserved when compared with the homologous regions in plasmid pColV-K30. Thus, the lengths that fragments *Hin*dIII-*Eco*RI and *Hin*dIII-*Bst*EII mapped along the left end of the 16.3-kb pColV-K30 *Hin*dIII fragment were identical to those found with the VW187 aerobactin regions (Fig. 3). However, the 3-kb *Bst*EII fragment containing part of the receptor genes and part of flanking sequences in pColV-K30 was smaller than the chromosomal *Bst*EII digest (4 kb as compared with 3 kb in pColV-K30). Hence, although the internal sequences of the aerobactin region appeared to be conserved, there were a considerable number of rearrangements in the right adjacent areas.

The aerobactin genes in pColV-K30 are flanked by IS*1*-like sequences (25a). One of these IS*1* elements occurs at the right end of the pColV-K30 aerobactin region, which is the area in which we detected rearrangements in the case of the chromosomal aerobactin sequences. IS*1* elements also occur within the 10.5-kb *Hin*dIII chromosomal fragment which carries the conserved aerobactin region in VW187-19, indicating that possibly the combination of aerobactin genes and IS*1* sequences may have resulted in their conservation as a unit in these two cases through recombination or transposition events mediated by the IS*1*-like elements. This hypothesis is strengthened by the fact that aerobactin sequences are also found in chromosomes and plasmids of other enteric

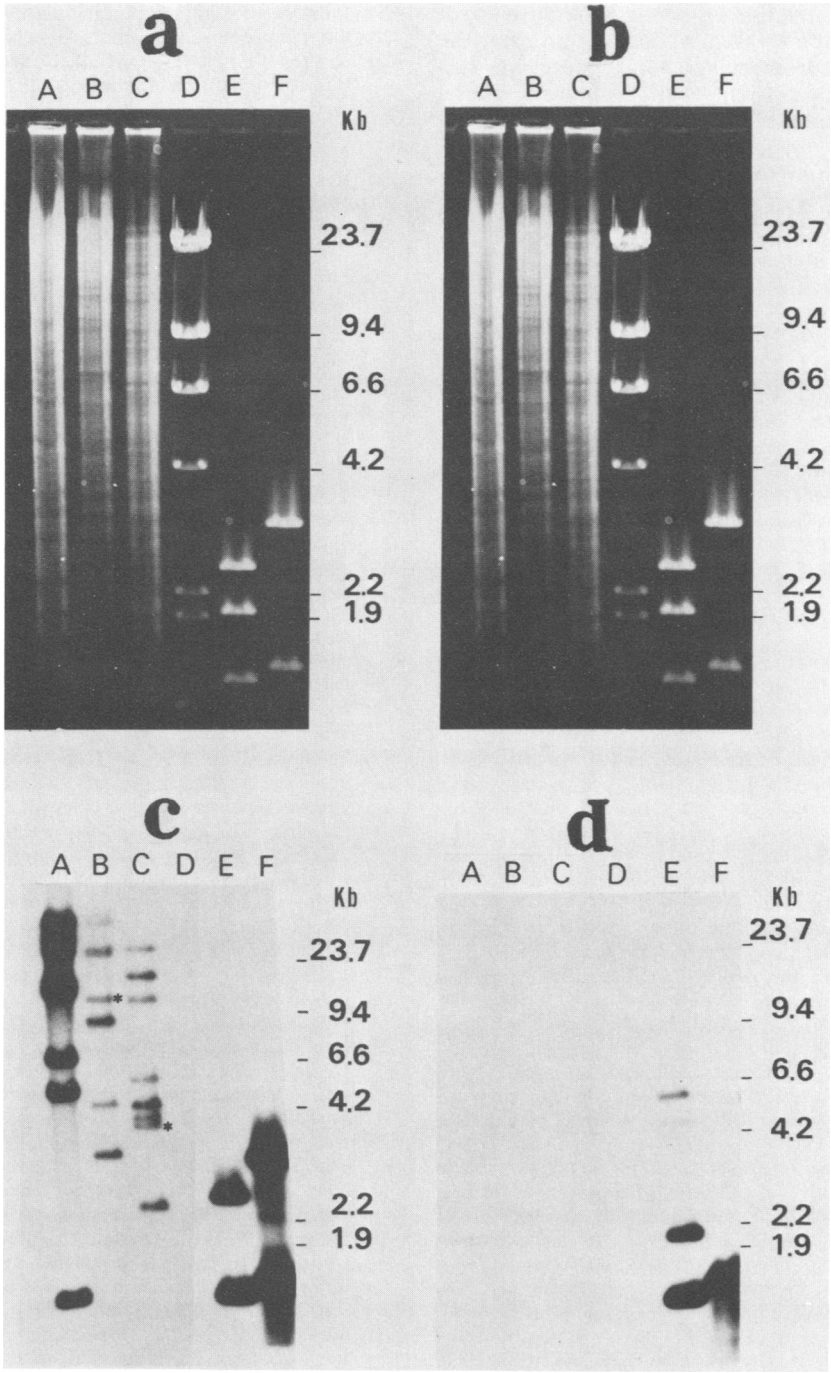


FIG. 6. Hybridization of an IS*1* probe with restriction endonuclease-cleaved DNA. (a and b) Duplicate ethidium bromide-stained 0.5% agarose gels of restriction endonuclease-cleaved DNA. Lanes A, C600; lanes B and C, VW187-19; lanes D, lambda phage; lanes E, pBR325; lanes F, pBRG29 (a pBR322 derivative containing Tn*9*). The restriction endonucleases are: lanes A, B, and D, *Hin*dIII; lanes C, successive digestion with *Eco*RI-*Hin*dIII; lanes E, successive digestion with *Pvu*II-*Hin*dIII; lanes F, *Eco*RI. (c and d) Autoradiographs of Southern blot hybridizations of the gels described in (a and b) with $^{32}$P-labeled probes: (c) a 1.5-kb *Eco*RI fragment from pBRG29 (IS*1* probe). (d) A 1.2-kb *Hin*dIII-*Eco*RI fragment of pBR325; (c) was obtained from (a), and (d) was obtained from (b). The restriction endonuclease fragments used as labeled probes were obtained by electroelution as described in the text. Asterisks in (c) indicate fragments suspected to correspond to the chromosomal aerobactin region: lane B, 10.5 kb; lane C, 3.4 kb.

bacteria which also possess IS*1* elements (20a, 22; M. A. Valvano, M. K. Wolf, L. M. Crosa, and J. H. Crosa, Abstract W-29, Conference on Plasmids in Bacteria, May 14–18, 1984, Urbana, Ill.).

The conservation of aerobactin sequences as a genetic unit in *E. coli* K1 isolates and other enteric pathogens suggests a recombinational mobility of these genes which may have played a role in the spread of this virulence factor in nature.

Another implication of our findings is that the absence of ColV plasmids from *E. coli* K1 isolates does not preclude the existence of an aerobactin-mediated iron-sequestering system, which may still be an important component of the virulence repertoire of invasive *E. coli* K1 strains.

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