JOURNAL OF BACTERIOLOGY, Feb. 1985, p. 727–735
0021-9193/85/020727-09$02.00/0

Vol. 161, No. 2

Aerobactin Genes in Clinical Isolates of *Escherichia coli*

ALBRECHT BINDEREIF AND J. B. NEILANDS*

Department of Biochemistry, University of California, Berkeley, California 94720

Received 20 September 1984/Accepted 26 November 1984

The location of the aerobactin gene complex on either the chromosome or plasmid was determined in eight aerobactin-positive clinical isolates of *Escherichia coli* by Southern hybridization analysis, using as probes the cloned aerobactin genes from the ColV-K30 plasmid. The aerobactin genes were in two cases detected on large plasmids, whereas in the other strains the aerobactin genes are most likely located on the chromosome. Restriction mapping revealed only slight variations in the structural genes and an at least 3.4-kilobase-long upstream region conserved in all three plasmid-coded systems. A 7.7-kilobase *Hin*dIII fragment upstream and adjacent to the 16.3-kilobase *Hin*dIII fragment carrying the complete aerobactin system was cloned from the ColV-K30 plasmid. Fine-structure restriction mapping identified the left insertion sequence in the upstream region as IS*1*, in inverted orientation to the IS*1* element downstream from the aerobactin operon. The upstream and downstream sequences of IS*1* appear to have perfect homology, as indicated by S1 nuclease resistance of a 760-base-pair DNA duplex formed by both IS*1* elements.

A wide range of plasmid-determined phenotypes are associated with virulence of pathogenic strains of *Escherichia coli* (10, 15). The factors include multiple drug resistances, enterotoxin formation, and production of colonization antigen and hemolysin.

Colicin V was described some six decades ago by Gratia (17) as "principle V" in a cell-free filtrate of *E. coli* V which killed the sensitive *E. coli* strain φ. Large, conjugative, F-like ColV plasmids were observed by Smith (43) and Smith and Huggins (44) to be present in a high proportion of invasive *E. coli* strains. In one animal model, Quackenbush and Falkow (39) showed that virulence is not dependent on the presence of colicin V activity.

The role of iron as a virulence factor has long been recognized (51), but an understanding of the molecular basis of this phenomenon has lagged behind that of other virulence phenotypes. Iron, although an indispensable nutrient for microbial growth, is not freely available in mammals (9). Therefore, the ability of microbial pathogens to compete successfully with the host for this vital element is considered to be a prerequisite to infection (51).

The discovery (52, 53) of a novel iron assimilation system coded on *E. coli* ColV plasmids combined these two lines of research and provided for the first time an experimental basis to define the role of iron as a virulence factor in molecular biological terms. A mutational analysis proved that not the ColV phenotype but, rather, the plasmid-coded iron assimilation system is responsible for the virulence character (54). Subsequently, the siderophore component of this system was identified as aerobactin (50), a hydroxamate-type siderophore originally isolated from *Aerobacter aerogenes* 62-I (16). A specific component of the uptake system was determined to be the outer-membrane receptor protein, a 74-kilodalton polypeptide (3). It became clear around that time that aerobactin is a siderophore widespread among enteric bacteria. Aerobactin was isolated from *Shigella flexneri* (35, 36) and from *Salmonella* and *Arizona* isolates (28). Iron-suppressible hydroxamate material was also found to be widely distributed among natural *E. coli* isolates (48).

* Corresponding author.

Recently we reported a survey of a large number of *E. coli* clinical isolates for aerobactin synthesis (J. Z. Montgomerie, A. Bindereif, J. B. Neilands, G. Kalmanson, and L. B. Guze, submitted for publication). A high incidence of aerobactin-positive strains (39%) was found. In this paper we determined the genetic location, viz., whether plasmid coded or chromosomal, of the aerobactin biosynthesis and transport determinants in eight aerobactin-positive clinical isolates of *E. coli* and compare the particular organization of the aerobactin gene complex in these strains.

MATERIALS AND METHODS

Clinical isolates of *E. coli*. A collection of 54 clinical isolates of *E. coli* was obtained from J. Z. Montgomerie, Rancho Los Amigos Hospital, Downey, Calif. In addition, the survey included *E. coli* 0111, a clinical isolate from the Centers for Disease Control, Atlanta, Ga.

Recombinant DNA. Restriction enzymes, S1 nuclease, DNA polymerase I, and T4 DNA ligase were purchased from Bethesda Research Laboratories (Bethesda, Md.). The large fragment of DNA polymerase I was from New England Biolabs (Beverly, Mass.) The conditions recommended by the supplier were used for restriction enzyme digestions and ligations. The protocols given by Maniatis et al. (26) were followed for most standard recombinant DNA procedures.

The cloning vector for the construction of pABN20 was pUC8, 2.7 kilobases (kb) (49). *E. coli* JM83 [*ara* Δ(*lac-pro*) *thi rpsL* φ80d*lacZ*ΔM15] was the host for pUC8-derived recombinant plasmids. *E. coli* JM83 was screened for plasmids by a quick minilysate procedure, which was also used for large-scale isolations of small plasmids (20). For large-scale isolations of large plasmids (pColV-K30 and total plasmid DNA from *E. coli* isolates) the Portnoy procedure was employed (38). Cells from 11 cultures were harvested after 2 to 3 days of growth in Tris-buffered medium (41) with sodium succinate (30 mM). For total genomic DNA isolations from *E. coli* strains, a combination of two procedures (2, 14) was used: 100 ml of L-broth cultures were grown to stationary phase (8 h), cells were harvested and resuspended in 4 ml of TE buffer (50 mM Tris-hydrochloride [pH 8.0], 25 mM EDTA), lysozyme was added to 1 mg/ml, and the cell suspension was left on ice for 20 min. RNase was added to 100 μg/ml, *N*-laurylsarcosine (Na salt) to 1%, and proteinase

K to 0.5 mg/ml, and incubation was continued for 2 h at 37°C and then overnight at room temperature. The viscous lysate was next passed 10 times through a 23-gauge needle to shear chromosomal DNA, extracted with 3% NaCl-saturated phenol-chloroform, and dialyzed against TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA). This procedure routinely gave yields of 3 to 10 mg of DNA per 100 ml of culture.

Recombinant plasmids were mapped by single and double digestions with restriction enzymes, followed by agarose gel electrophoresis. Fine-structure mapping was done by the method of Smith and Birnstiel (42) with DNA fragments which had been end labeled by the large fragment of DNA polymerase I and [α-^{32}P]dinucleosidetriphosphate (Amersham Corp., Arlington Heights, Ill., and ICN Radiochemicals, Irvine, Calif.) and gel purified by the DEAE-cellulose paper method of Dretzen et al. (13). DNA elution was achieved by two incubations of the paper at 65°C for 10 min each. Purified DNA fragments were nicktranslated to specific activities greater than 10^7 cpm/μg (26), and at least 10^6 cpm was used per Southern hybridization. Washing of the blots was carried out under high stringency conditions. Marker fragments were a *Sal*I digest of pABN1 DNA (4) (12.4 and 5.9 kb), a *Bgl*II digest (7.7 and 1.0 kb), an *Ava*I digest (1.8, 2.9, and 4.0 kb), and an *Eco*RI digest (8.7 kb) of pABN5 DNA (4) and were run on both sides of the gel. As supercoiled size markers, the following plasmid DNAs were used: pABN1 (18.3 kb) (4), pABN5 (8.7 kb) (4), pABN15 (4.0 kb) (A. Bindereif and J. B. Neilands, submitted for publication), and pPlac (2.0 kb) (4).

Isolation of insertion sequence. The procedure for isolating the inverted repeat sequence basically followed the protocol of Ohtsubo and Ohtsubo (33). An amount of 10 μg of pABN20-6 DNA, linearized by *Hin*dIII, was denatured by the addition of 200 μl of 0.15 M NaOH and kept for 20 min at room temperature; after neutralization with 0.15 M CHl the solution was chilled on ice, and the NaCl concentration was adjusted to 0.24 M in a total volume of 600 μl. Renaturation of inverted repeat sequences was performed at 68°C for 45 s. S1 nuclease buffer (to a final concentration of 30 mM NaAc [pH 4.6], 1 mM $ZnSO_4$) was added. The solution was then divided into aliquots and exposed to various amounts of S1 nuclease (0.13 U/μg in a 200-μl reaction with 2.5 μg of DNA; 1 U as defined by the manufacturer solubilizes 1 μg of denatured DNA in 1 min at 37°C) for 30 min at 37°C. The DNA fragments were ethanol precipitated and analyzed on a 1.5% agarose gel.

RESULTS AND DISCUSSION

Plasmid versus chromosome location of aerobactin genes. To determine the location of the aerobactin determinants, a group of 15 isolates found positive for the synthesis of the siderophore, comprising 7 isolates from blood, 5 from ascites, and 3 from urine, were screened for their plasmid profile. The survey also included *E. coli* 0111, a clinical isolate from the Centers for Disease Control, Atlanta, Ga., which we reported to form aerobactin (32). In four isolates (JM1195, JM1264, JM1389, and JM1559) no plasmid DNA could be detected, whereas four other strains (JM605, JM2309, JM2310, and JM1304) gave insufficient amounts of large plasmid DNA for mapping experiments. The remaining eight isolates (five from blood [JM616, JM1363, JM1358, JM1372, and JM1403], one from ascites [JM1205], one from urine [JM901], and *E. coli* 0111) yielded sufficient total plasmid DNA and were chosen for further characterization. Purified ColV-K30 DNA was included as a well-characterized reference DNA in all the following screening and mapping experiments. Plasmid DNA was analyzed on 0.7% agarose gels by ethidium bromide staining and Southern hybridization by using cloned aerobactin genes from plasmid ColV-K30 as a DNA probe. Fig. 1A shows that the aerobactin-positive isolates contained a variety of small and large plasmids: JM1205 contained only one large plasmid; *E. coli* 0111, JM616, and JM901 contained all small plasmids (2.8, 9.0, 5.4, and ca. 27 kb, respectively) in addition to a large plasmid; JM1372 and JM1403 had two large plasmids; JM1358 had three large plasmids in addition to a small plasmid (5.8 kb); JM1304 contained three small plasmids (5.6, 6.7, and ca. 17 kb) and traces of a large plasmid; and JM1363 contained only a ca. 30-kb plasmid. The different staining intensities probably do not reflect copy numbers, since the recoveries of small and large plasmids may differ. Several bands in a particular lane may represent different forms of the same plasmids. All large plasmids, like pColV-K30, were ca. 100 kb in size.

To detect plasmid-coded aerobactin genes Southern hybridizations were performed with purified plasmid DNA and the 6.8-kb *Hin*dIII-*Eco*RI probe (see Fig. 6). Only large plasmid DNA from JM616 (blood) and JM1205 (ascites) showed stringent homology to the cloned aerobactin sequences (Fig. 1B), indicating that at least in these two isolates the aerobactin genes, and most likely the entire gene complex, are located on large plasmids. Southern hybridizations were also done with *Ava*I-digested plasmid DNA and the 1.7-kb *Hin*dIII-*Hpa*I probe, which covers most of the first biosynthetic gene (see Fig. 6). The result confirmed that JM616 and JM1205 plasmid DNA have the first biosynthetic gene in common with the cloned aerobactin system (Fig. 1C). Furthermore, the size of this *Ava*I fragment (5.6 kb) is the same in all three plasmid systems (ColV-K30, JM616, and JM1205), indicating that the *Ava*I site (3.5 kb upstream from the left *Hin*dIII site) is conserved in these three cases (see Fig. 6).

Because of the fragility of large plasmids, it is impossible to isolate chromosomal DNA without contaminating nicked circular or linear DNA. Therefore, total genomic DNA was prepared from the same eight clinical isolates, digested with restriction enzymes, separated on agarose gels, and subjected to Southern hybridization by using as probes aerobactin sequences cloned from the ColV-K30 plasmid. Digested ColV-K30 plasmid DNA was always used for comparison. Positive hybridization to total DNA, but negative hybridization to purified plasmid DNA, was considered strongly suggestive of a chromosomal location for the genes.

We cannot rule out the possibility that the lack of detectable high-molecular-weight plasmid DNA reflects a technical problem of the isolation procedure, rather than the absence of plasmid DNA, but considering the successful isolation of large plasmids in most cases (Fig. 1), this possibility seems unlikely.

Positive hybridization of the cloned aerobactin genes to total DNA from all eight clinical isolates (Fig. 2, 3, and 4) and positive hybridization to two plasmid DNAs (Fig. 1) strongly indicate that the aerobactin genes map either on large plasmids (ColV-K30, JM616, and JM1205) or on the chromosome (JM1363, JM1358, JM1372, JM1403, JM901, and *E. coli* 0111).

Restriction mapping of aerobactin genes and flanking regions in chromosomal and plasmid-coded systems. To characterize and compare chromosomal and plasmid-coded aerobactin systems, restriction sites for *Bam*HI, *Sal*I, *Eco*RI, and *Ava*I were mapped on the ColV-K30 plasmid upstream

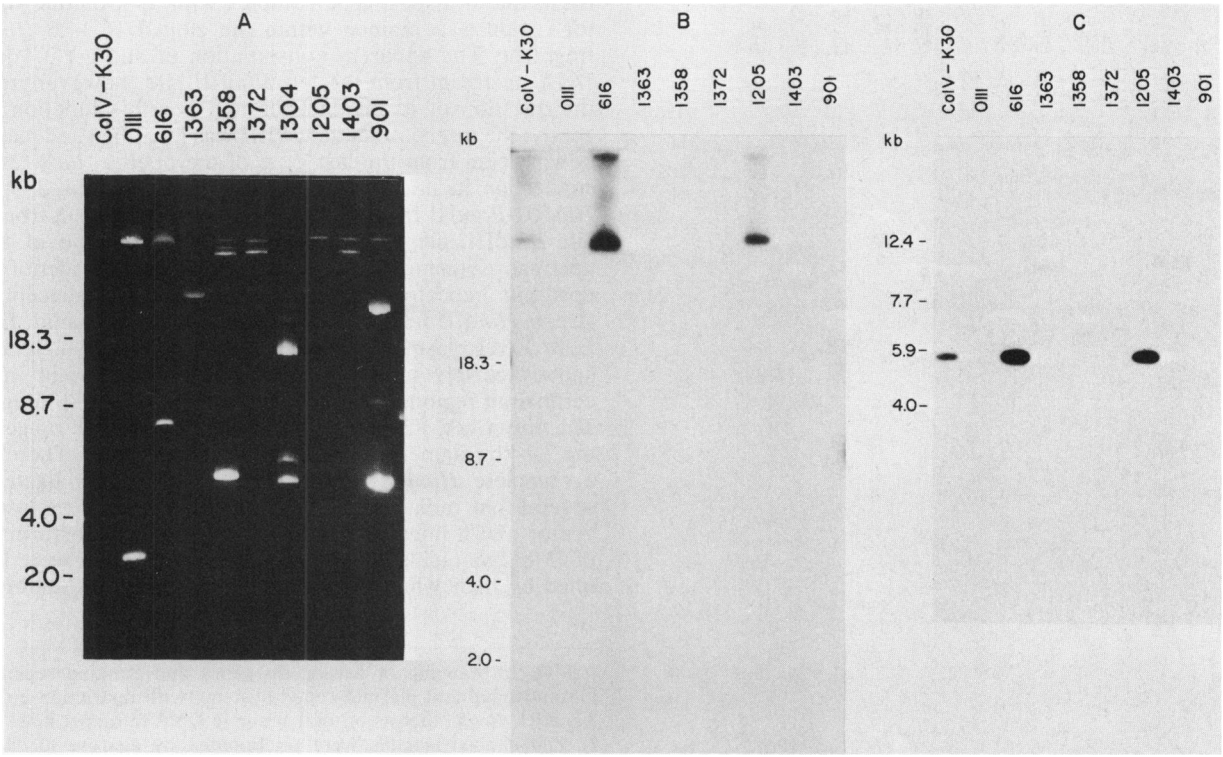
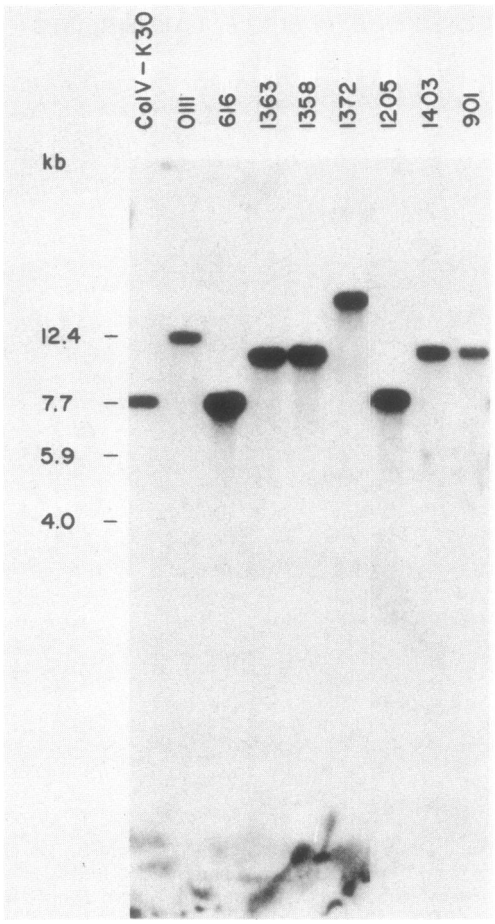


FIG. 1. Plasmid-coded aerobactin gene complexes in *E. coli*. (A) ColV-K30 plasmid DNA and total plasmid DNA from *E. coli* clinical strains as isolated and analyzed by agarose (0.7%) gel electrophoresis in Loen buffer (36 mM Tris base, 30 mM NaH_2PO_4, 1 mM EDTA). (B) After transfer to nitrocellulose (46), Southern hybridization analysis was performed by using as DNA probe the 6.8-kb *Hin*dIII-*Eco*RI fragment after it had been gel purified ^{32}P-nick translated (26). (C) ColV-K30 plasmid DNA and total plasmid DNA from *E. coli* clinical isolates were cut with *Ava*I, fractionated by agarose (1%) gel electrophoresis, transferred to nitrocellulose (46), and analyzed by Southern hybridization (26) by using the gel-purified and ^{32}P-nick translated 1.7-kb *Hin*dIII-*Hpa*I fragment as DNA probe.

from the cloned 16.3-kb *Hin*dIII fragment by Southern hybridization, with the 6.8-kb *Hin*dIII-*Eco*RI DNA fragment as a probe. Plasmid pABN1 digests and the known restriction map of pABN1 (4) allowed the identification of overlapping fragments and mapping of the upstream sites. The results are shown in Fig. 5. The large fragments in the *Ava*I A and B lanes are not identical; their apparent size similarity is purely fortuitous. A comparative analysis of chromosomal and plasmid-coded systems was then carried out by mapping restriction sites for *Eco*RI, *Ava*I, and *Bam*HI in the aerobactin structural genes and flanking regions by using Southern hybridization and three different DNA probes (Fig. 6): the 6.8-kb *Hin*dIII-*Eco*RI probe represents the entire biosynthetic gene region and part of the transport genes, the 1.7-kb *Hin*dIII-*Hpa*I probe covers most of the first biosynthetic gene, and the 2.1-kb *Kpn*I-*Eco*RI probe includes part of the transport genes. This analysis enabled us to answer these questions. (i) Are the various aerobactin systems all organized in the same way? (ii) Are there characteristic differences between chromosome and plasmid-coded systems? (iii) Is the map position of the aerobactin gene complex on the plasmid or chromosome in different isolates identical? The Southern blots used for mapping are shown on Fig. 2 through 4 and are summarized in Fig. 6. Three assumptions were made for the interpretation of the Southern hybridizations. (i) The map position of the 1.8-kb *Ava*I fragment covering the *iucB* gene and part of the *iucC* gene is the same in the ColV plasmid and in the other systems, except in JM1403. This is very likely the case, since the 1.8-kb *Ava*I fragment is conserved in all systems except JM1403 (Fig. 3A) and hybridizes only to the 6.8-kb *Hin*dIII-*Eco*RI probe, but not to probes to the left (Fig. 3B) or to the right (Fig. 3C) of the 1.8-kb *Ava*I fragment. (ii) The 0.3-kb *Bam*HI fragment in the *iucC* gene is conserved. (iii) The map position of the central *Eco*RI site in the *iutB* gene is conserved.

The mapping results for the plasmid-coded systems (pColV-K30, large plasmids from JM616 and JM1205) may be summarized as follows. The structural genes appear to be conserved based on common *Ava*I fragments (0.75, 1.8, and 2.9 kb). There is a conserved region in all three systems at least 3.4 kb long and upstream from the aerobactin gene complex, based on identical *Eco*RI, *Ava*I, and *Bam*HI restriction sites in this region. The three plasmids, however, are different from each other, as demonstrated by different positions of the downstream *Bam*HI site (Fig. 6).

In the chromosomal systems, in contrast, the organization of the aerobactin genes and the upstream region is more varigated. Generally the conserved upstream region of the plasmid systems was absent. A group of three isolates (JM1363, JM1358, and JM901) were identical as far as restriction sites were mapped, suggesting that the aerobactin system may be integrated at the same chromosomal location in these three cases. Furthermore, in this group in *E. coli* 0111, there is an additional *Ava*I site 0.8 kb to the left of the *Eco*RI site. The *Ava*I site 0.9 kb to the left of the *Hin*dIII site is common to JM1303, JM1358, JM901, JM1372, and *E. coli* 0111. JM1403 turned out to be unusual in that no *Ava*I sites could be mapped within an 8.9-kb *Ava*I fragment. Both flanking *Bam*HI sites, as well as the upstream *Eco*RI site, however, mapped as in JM1363, JM1358, and JM901.



Cloning of upstream sequences and identification of the upstream insertion sequence. The entire aerobactin biosynthesis and transport gene complex with its promoter-operator region is contained within the cloned 16.3-kb *Hin*dIII fragment. The apparent conservation of at least 3.4 kb of DNA upstream from the left *Hin*dIII site, however, raised the question of its significance and possible function. Recently, the insertion sequence IS*1* was identified and mapped downstream from the last gene of the operon, *iutB* (28). A similar or identical element was found within a 3.2-kb *Hin*dIII-*Bam*HI fragment, at least 4.9 kb upstream from the left *Hin*dIII site (28). To search for a function of these upstream sequences, to map accurately, and to identify the upstream repeated element, we cloned the adjacent upstream *Hin*dIII fragment from the ColV-K30 plasmid. In the ColV-K311 plasmid, the restriction pattern of which corresponds closely to that of the ColV-K30 plasmid in the aerobactin region, this *Hin*dIII fragment is 8.4 kb long (8). The only ColV-K30 *Hin*dIII fragment in this size range was the 7.7-kb fragment *Hin*dIII-E (data not shown). Therefore, a complete *Hin*dIII digest of pColV-K30 was ligated in several-fold molar excess into the insertional inactivation vector pUC8 linearized with *Hin*dIII. After transformation of JM83, ampicillin-resistant clones with inserts were identified by their white color on 5-bromo-4-chloro-3-indolyl-β-

FIG. 2. Mapping of aerobactin genes and flanking regions in chromosomal and plasmid-coded systems with restriction enzyme *Eco*KRI, *Eco*RI digests of total genomic DNA (5 αg per lane) from *E. coli* clincial isolates were fractionated by agarose (0.7%) gel electrophoresis, transferred to nitrocellulose (46), and analyzed by Southern hybridization (26). The Dna probe was the gel-purified and ^{32}P-nick-translated 1.7-kb *Hin*dIII-*Hpa*I gragment. Size markers are described in the text.

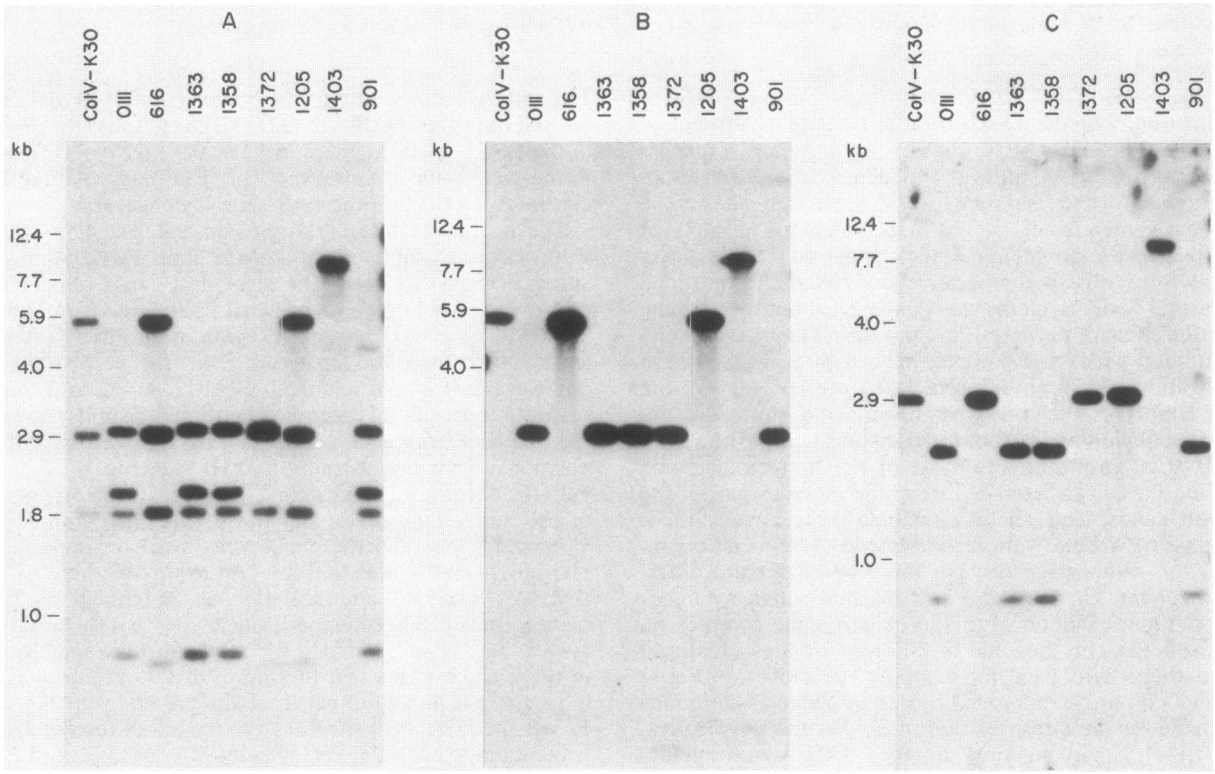


FIG. 3. Mapping of aerobactin genes and flanking regions in chromosomal and plasmid-coded systems with restriction enzyme *Ava*I. *Ava*I digests of total genomic DNA (5 μg per lane) from *E. coli* clinical isolates were fractionated by agarose (1%) gel electrophoresis, transferred to nitrocellulose (46), and analyzed by Southern hybridization (26). The DNA probes used were the 6.8-kb *Hin*dIII-*Eco*RI fragment (A), the 1.7-kb *Hin*dIII-*Hpa*I fragment (B), and the 2.1-kb *Kpn*I-*Eco*RI fragment (C), all gel purified and ^{32}P-nick translated.

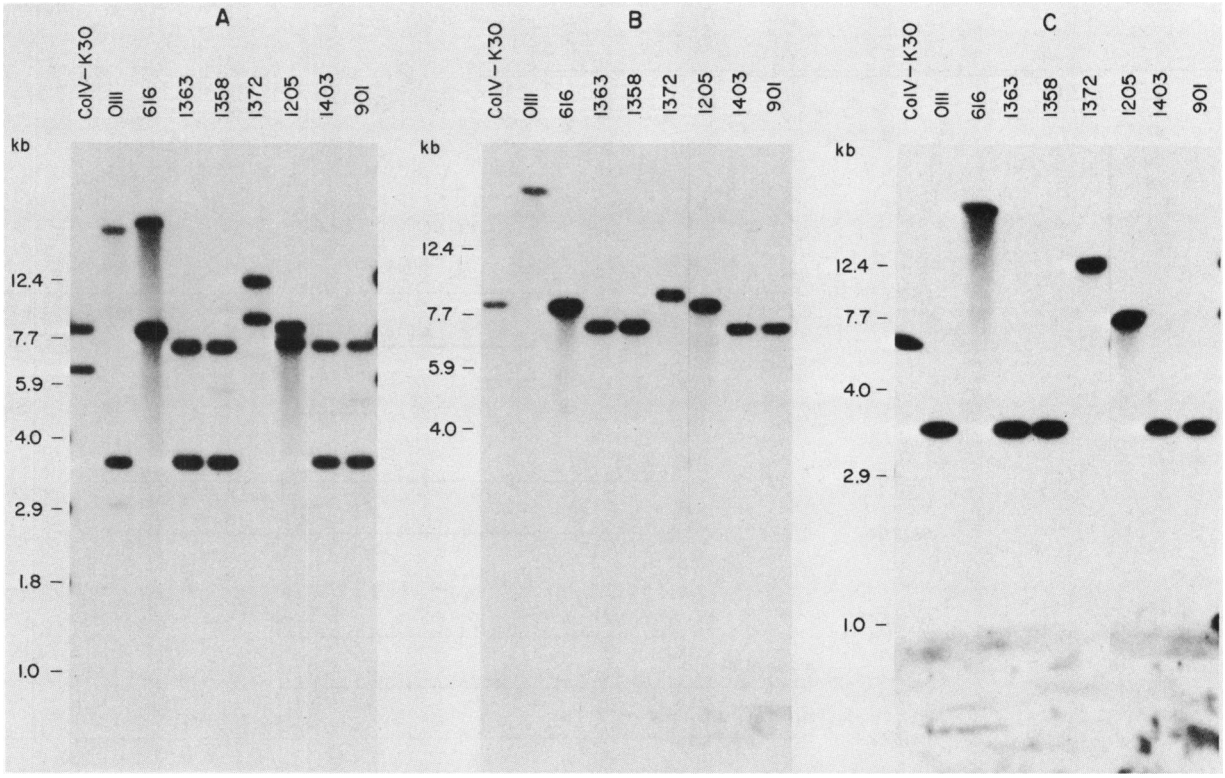


FIG. 4. Mapping of aerobactin genes and flanking regions in chromosomal and plasmid-coded systems with restriction enzyme *Bam*HI. *Bam*HI digests of total genomic DNA (5 μg per lane) from *E. coli* clinical isolates were fractionated by agarose (0.7%) gel electrophoresis, transferred to nitrocellulose (46), and analyzed by Southern hybridization (26). The DNA probes used were the 6.8-kb *Hin*dIII-*Eco*RI fragment (A), the 1.7-kb *Hin*dIII-*Hpa*I fragment (B), and the 2.1-kb *Kpn-Eco*RI fragment (C), all gel purified and ^{32}P-nick translated.

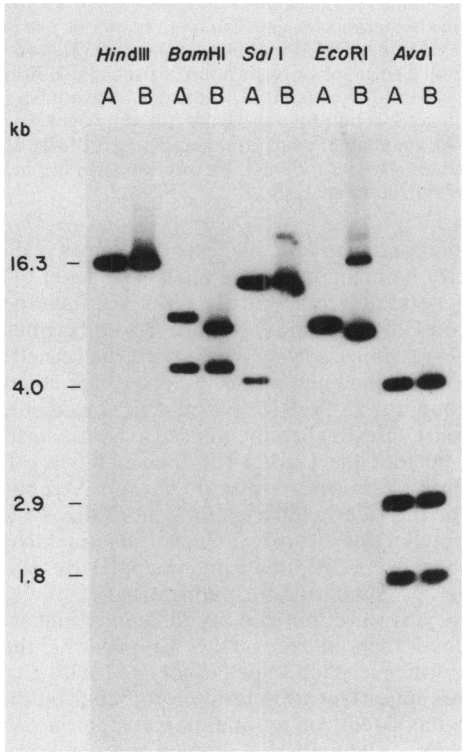


FIG. 5. Restriction mapping of the upstream flanking region in the ColV-K30 plasmid. *Bam*HI, *Sal*I, *Eco*RI, and *Ava*I digests of purified pABN1 (A) and pColV-K30 (B) DNA were fractionated by agarose (0.7%) gel electrophoresis, transferred to nitrocellulose (46), and analyzed by Southern hybridization by using the gel-purified and ^{32}P-nick-translated 6.8-kb *Hin*dIII-*Eco*RI fragment as DNA probe.

D-galactoside indicator plates (39%) and screened for a 7.7-kb *Hin*dIII insert. Among 30 clones, 1 was found which, upon *Hin*dIII digestion, gave the expected fragment of 7.7 kb and the 2.7-kb vector fragment. This recombinant plasmid of 10.4 kb was designated pABN20; its restriction map is shown in Fig. 7. The agreement of the upstream *Bam*HI, *Ava*I, and *Eco*RI sites with the corresponding sites mapped on the intact ColV-K30 plasmid by Southern hybridization further confirmed the location of the cloned fragment upstream and adjacent to the 16.3-kb fragment *Hin*dIII-C.

To map precisely and to identify the upstream insertion element, the 3.2-kb *Hin*dIII-*Bam*HI fragment where it previously had been localized was purified and end labeled at the *Hin*dIII site. Partial digests by *Pvu*II, *Pst*I, *Bal*I, *Bst*EII, *Dde*I, *Alu*I, *Hpa*II, *Hae*II, and *Hae*III were obtained and resolved by agarose or polyacrylamide gel electrophoresis (data not shown). These mapping results agreed with the published DNA sequence of the insertion element IS*1* (34) and are included in Fig. 6. The two IS*1* copies are oriented as inverted repeats. The upstream IS*1* copy maps between 0.4 and 1.2 kb to the right of the upstream *Hin*dIII site (Fig. 6).

The identity of the two insertion sequences as IS*1* was further corroborated by their isolation. For this purpose the two IS*1* copies which enclose in the ColV-K30 plasmid almost 15 kb of DNA were moved closer together by excising the 4.5-kb *Bam*HI fragment of pABN20 to give

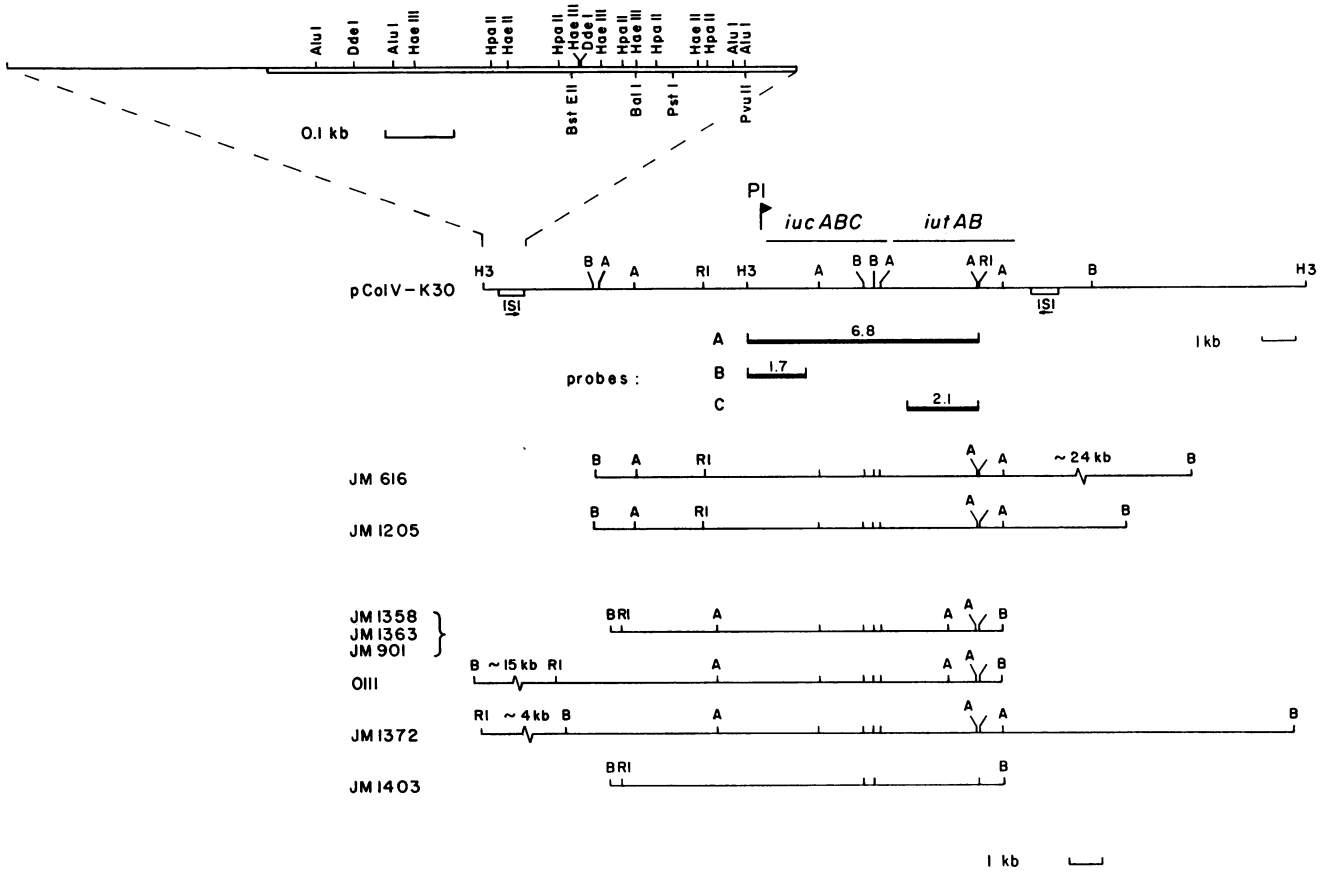


FIG. 6. Restriction mapping of aerobactin genes and flanking regions in chromosomal and plasmid-coded systems. Data from Fig. 2 through 5 are summarized in maps for *Bam*HI, *Ava*I, and *Eco*RI restriction sites in the aerobactin and flanking regions of plasmid-coded systems (pColV-K30, plasmids from JM616 and JM1205) and chromosomal systems (JM1363, JM1358, JM901, 0111, JM1372, and JM1403). Heavy lines indicate the three DNA fragments used as probes in Southern hybridization. The genetic organization of the ColV-K30 aerobactin system is outlined above the map of the ColV-K30 region. Restriction sites to the right of the downstream IS*1* sequence have not been mapped completely. The position and orientation of the two IS*1* copies flanking the ColV-K30 aerobactin genes are represented by open boxes under the ColV-K30 map. A fine-structure restriction map of the left IS*1* sequence is shown on a larger scale in the upper part of the figure. The locations of the restriction sites are abbreviated as follows: H3, *Hin*dIII; B, *Bam*HI; A, *Ava*I; RI, *Eco*RI. Restriction sites are indicated in the JM plasmids and in the 0111 plasmid only if they were mapped by Southern hybridization analysis.

pABN20Δ*Bam*HI and inserting into the resulting single *Bam*HI site the 6.4-kb *Bam*HI fragment from pABN6 (A. Bindereif, B. H. Paw, and J. B. Neilands, submitted for publication) carrying the transport genes *iutA* and *iutB* and the downstream IS*1* element. Screening for the original inverted-repeat orientation of the two IS*1* copies yielded pABN20-6, 12.3 kb (Fig. 7). The procedure of isolating the inverted repeat DNA involved denaturation of *Hin*dIII-linearized pABN20-6 DNA, followed by rapid renaturation of the inverted-repeat sequences which resulted, following first-order kinetics, in the formation of an intramolecular fold-back structure. After S1 nuclease treatment, S1-resistant duplex DNA was recovered and analyzed by gel electrophoresis. Figure 8 shows that a ca. 760-base-pair (bp) fragment was the product and was dependent on S1 nuclease treatment. The larger fragments are probably products of the competing intermolecular renaturation. The 760-bp hybrid DNA fragment was cleaved by *Bal*I into a 230- and a 520-bp fragment. These results are as predicted by the published IS*1* sequence (34). The absence of any discrete smaller products of S1 nuclease treatment, even at 400-fold excess of S1 nuclease (Fig. 8, lane D) and at S1 nuclease concentrations sufficient to cause degradation of the duplex DNA fragment (data not shown), suggests that the two IS*1* copies are perfectly homologous at the nucleotide level (40).

Three genetic determinants for virulence have now been identified on ColV plasmids. First, a 5.3-kb fragment of the ColV,I-K94 plasmid carrying the *iss* gene (increased survival in serum) was cloned and mapped close to the colicin genes (5, 6). Second, the ColV-B188 plasmid enhanced adhesion in vitro to mouse intestinal epithelium (11). Third, certain ColV plasmids, but not the ColV,I-K94 plasmid, were found to code for an iron transport system (47, 52). Aerobactin was shown to be the siderophore component of this system (50), and the polypeptides involved in aerobactin biosynthesis and transport were identified and mapped by cloning (4; Bindereif et al., submitted for publication).

ColV$^+$ strains were found in a significantly higher proportion in blood than in feces (12). Considering the newly identified virulence phenotypes associated with ColV plasmids, it was important to determine the distribution of the aerobactin-mediated iron assimilation system in pathogenic *E. coli* isolates. The finding of an unusually high proportion of aerobactin-positive strains among blood isolates (Montgomerie et al., submitted for publication) led to speculations about what the selective advantage may be in having aerobac-

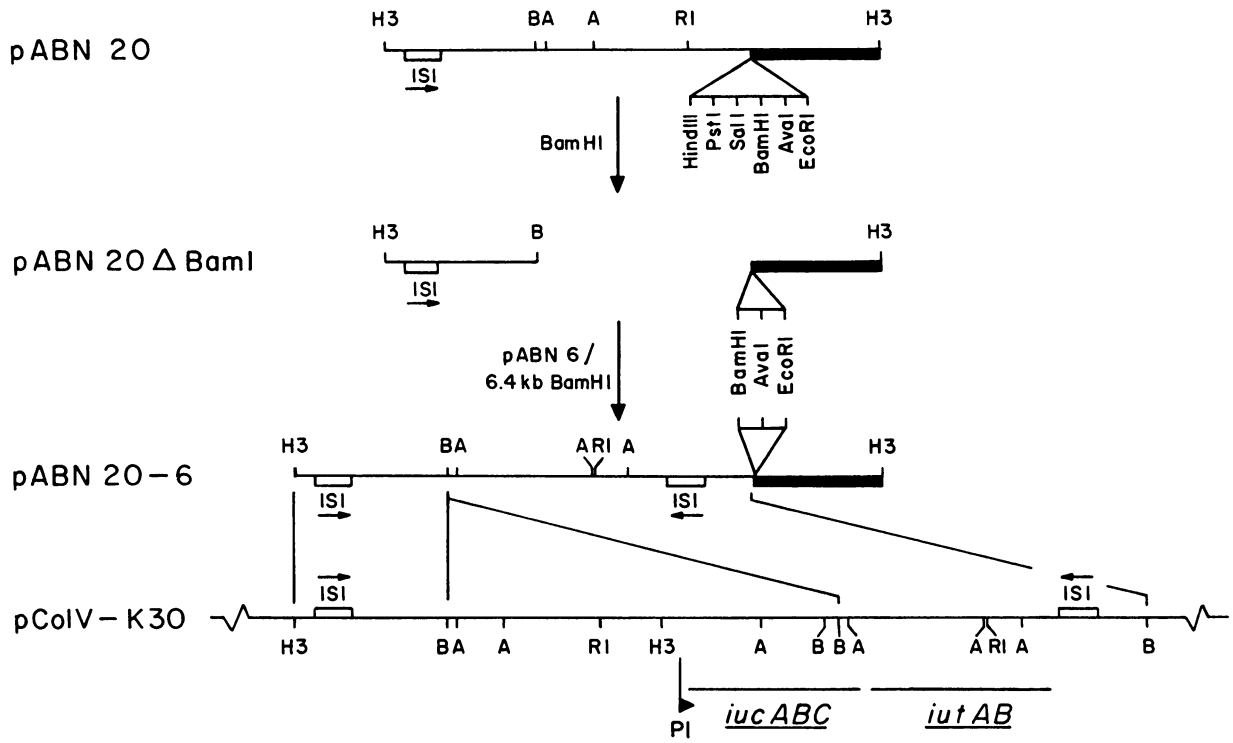


FIG. 7. Construction of plasmid pABN20-6. Plasmid maps are shown linearized at the *Hin*dIII site of the vector DNA, pUC8 (49), which is represented by heavy lines. The construction of plasmid pABN20-6 is described in the text. The figure shows the location of the two IS*1*-containing fragments in the aerobactin region of pColV-K30. The location and orientation of the IS*1* copies are denoted by open boxes. The genetic organization of the ColV-K30 aerobactin system is outlined below the map of the ColV-K30 region. Restriction sites are abbreviated as follows: H3, *Hin*dIII; B, *Bam*HI; A, *Ava*I; RI, *Eco*RI.

tin as a second siderophore system in addition to the intrinsic enterobactin system, particularly in serum (23, 32). Konopka et al. (23) found that in serum aerobactin, despite its inferior thermodynamic affinity for iron (19), proved more efficient than enterobactin in the transfer of transferrin-bound iron to *E. coli* cells. One reason for this is that enterobactin, but not aerobactin, forms a stable complex with serum albumin (24). In addition, enterobactin has a relatively low water solubility (37) and is chemically unstable, and its highly aromatic character may cause it to adhere to proteins as a haptene, explaining the presence of specific antibodies in human serum (30, 31). Aerobactin, however, is a much more water-soluble, stable, and even recyclable molecule (1, 7). In summary, the properties and the distribution of aerobactin support our present view (24, 32) that enterobactin, although having the largest known stability constant of any iron chelate (18), is inferior to other siderophores, such as aerobactin, which evolved structural and functional qualities adapted for iron acquisition in serum. Nonetheless, it is clear that the aerobactin-mediated iron assimilation system is only one factor in the interaction between host and pathogen. Bacterial virulence undoubtedly depends on a multitude of factors, but in certain circumstances, such as in the strictly iron-limited environment of serum, iron assimilation may be crucial for the proliferation of *E. coli*.

The occurrence of the aerobactin genes on either large plasmids or on the chromosome, the wide distribution of the genes in enteric bacterial species other than *E. coli*, and the two IS*1* sequences flanking the operon in inverted orientation make it tempting to speculate about the mobility of the entire gene complex as a transposable element. It has been suggested that any sequence flanked by identical insertion elements can form a transposon (25). So et al. (45) demonstrated for the heat-stable toxin of *E. coli* that flanking IS*1* elements can mediate cointegrate formation in a *recA*-host. Furthermore the *argF* gene region of the *E. coli* K-12

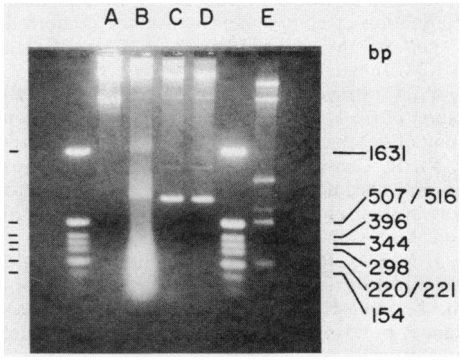


FIG. 8. Isolation of the insertion sequence IS*1*. The isolation of the insertion sequence IS*1* is described in the text. S1 nuclease-resistant duplex DNA, after rapid renaturation of denatured pABN20-DNA and S1 nuclease treatment, was analyzed by agarose (1.5%) gel electrophoresis. Each reaction started with 2.25 μg of pABN20-6 DNA. Shown are the products without S1 nuclease treatment (lane A) and with S1 nuclease at 0.4 U/μg (lane B), 2.2 U/μg (lane C), and 8.9 U /μg (lane D) (1-min units; 30-min reaction at 37°C). Lane E displays a *Ball* digest of S1 nuclease-resistant duplex DNA. Size markers are *Hin*fI fragments of pBR322 DNA.

chromosome (21, 56) and the plasmid-coded *raf* operon in *E. coli* (27) is IS*1* flanked. Several other genes involved in virulence or substrate utilization have in the last few years been shown to be coded within transposon-like structures, such as the *E. coli* genes for citrate utilization (22), raffinose utilization (27), heat-stable enterotoxin (45), heat-labile enterotoxin (55), and the *Vibrio cholerae* toxin genes (29). A 5-kb-long conserved region upstream from the cholera toxin genes was proposed to function in gene amplification and transposition (29).

Whether one or both of the IS*1* sequences flanking the aerobactin operon are actually functional in promoting cointegrate formation or transposition and the significance of the upstream conserved region in the plasmid systems will be the subjects of further investigation. Mobility of the aerobactin gene complex between plasmids and chromosome and between bacterial species via conjugative plasmids would explain the wide distribution of this iron assimilation system.

ACKNOWLEDGMENTS

We are indebted to J. Z. Montgomerie for providing the clinical isolates used in this work.

Financial support was provided by Public Health Service grants A104156 and ES07075 from the National Institutes of Health and by National Science Foundation grant PCM78-12198.

LITERATURE CITED

1. **Arceneaux, J. E. L., W. B. Davis, D. N. Downer, A. H. Haydon, and B. R. Byers.** 1973. Fate of labeled hydroxamates during iron transport from hydroxamate iron chelates. J. Bacteriol. **115**:919–927.
2. **Better, M., B. Lewis, D. Corbin, G. Ditta, and D. R. Helinski.** 1983. Structural relationships among *Rhizobium meliloti* symbiotic promoters. Cell **35**:479–485.
3. **Bindereif, A., V. Braun, and K. Hantke.** 1982. The cloacin receptor of ColV-bearing *Escherichia coli* is part of the Fe^{3+}-aerobactin transport system. J. Bacteriol. **150**:1472–1475.
4. **Bindereif, A., and J. B. Neilands.** 1983. Cloning of the aerobactin-mediated iron assimilation system of plasmid ColV. J. Bacteriol. **153**:1111–1113.
5. **Binns, M. M., D. L. Davies, and K. G. Hardy.** 1979. Cloned fragments of the plasmid ColV,I-K94 specifying virulence and serum resistance. Nature (London) **279**:778–781.
6. **Binns, M. M., J. Mayden, and R. P. Levine.** 1982. Further characterization of complement resistance conferred on *Escherichia coli* by the plasmid genes *traT* or R100 and *iss* of ColV,I-K94. Infect. Immun. **35**:654–659.
7. **Braun, V., C. Brazel-Faisat, and R. Schneider.** 1984. Growth stimulation of *Escherichia coli* in serum by iron(III) aerobactin. Recycling of aerobactin. FEMS Microbiol. Lett. **21**:99–103.
8. **Braun, V., R. Gross, W. Koester, and L. Zimmermann.** 1983. Plasmid and chromosomal mutants in the iron(III)-aerobactin transport system of *Escherichia coli*. Use of Streptonigrin for selection. Mol. Gen. Genet. **192**:131–139.
9. **Bullen, J. J., H. J. Rogers, and E. Griffith.** 1978. Role of iron in bacterial infections. Curr. Top. Microbiol. Immunol. **80**:1–35.
10. **Cabello, F., and K. N. Timmis.** 1979. Plasmids of medical importance, p. 55–69. *In* K. N. Timmis and A. Puehler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.
11. **Clancy, J., and D. C. Savage.** 1981. Another colicin V phenotype: in vitro adhesion of *Escherichia coli* to mouse intestinal epithelium. Infect. Immun. **32**:343–352.
12. **Davies, D. L., F. R. Falkiner, and K. G. Hardy.** 1981. Colicin V production by clinical isolates of *Escherichia coli*. Infect. Immun. **31**:574–579.
13. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112**:295–298.
14. **Elledge, S. J., and G. C. Walker.** 1983. Proteins required for ultra-violet light and chemical mutagenesis. J. Mol. Biol. **164**:175–192.
15. **Elwell, L. P., and P. L. Shipley.** 1980. Plasmid-mediated factors associated with virulence of bacteria to animals. Annu. Rev. Microbiol. **34**:465–496.
16. **Gibson, F., and D. I. Magrath.** 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. Biochim. Biophys. Acta **192**:175–184.
17. **Gratia, A.** 1925. Sur un remarquable exemple d'antagonisme entre deux souches de Colibacille. C. R. Soc. Biol. (Paris) **93**:1040–1041.
18. **Harris, W. R., C. J. Carrano, S. R. Cooper, S. R. Sofen, A. E. Avdeef, J. V. McArdle, and K. N. Raymond.** 1979b. Coordination chemistry of microbial iron transport compounds. 19. Stability constants and electrochemical behaviour of ferric enterobactin and model complexes. J. Am. Chem. Soc. **101**:6097–6104.
19. **Harris, W. R., C. J. Carrano, and K. N. Raymond.** 1979a. Coordination chemistry of microbial iron transport compounds. 16. Isolation, characterization, and formation constants of ferric aerobactin. J. Am. Chem. Soc. **101**:2722–2727.
20. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
21. **Hu, M., and R. C. Deonier.** 1981. Mapping of IS*1* elements flanking the *argF* gene region on the *Escherichia coli* K12 chromosome. Mol. Gen. Genet. **181**:222–229.
22. **Ishiguro, N., G. Sato, C. Sasakawa, H. Danbara, and M. Yoshikawa.** 1982. Identification of citrate utilization transposon Tn*3411* from a naturally occurring citrate utilization plasmid. J. Bacteriol. **149**:961–968.
23. **Konopka, K., A. Bindereif, and J. B. Neilands.** 1982. Aerobactin-mediated utilization of transferrin iron. Biochemistry **21**:6503–6508.
24. **Konopka, K., and J. B. Neilands.** 1984. Effect of serum albumin on siderophore-mediated utilization of transferrin iron. Biochemistry **23**:2122–2127.
25. **Kopecko, D. J.** 1980. Specialized genetic recombination systems in bacteria: their involvement in gene expression and evolution, p. 135–142. *In* F. E. Hahn (ed.), Progress in molecular and subcellular biology. Springer Verlag, Berlin.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Mattes, R., J. Altenbuchner, and R. Schmitt.** 1978. The translocation of the raffinose operon occurs by IS*1*-mediated, site specific recombination. Hoppe-Seyler's Z. Physiol. Chem. **359**:1118–1119.
28. **McDougall, S., and J. B. Neilands.** 1984. Plasmid- and chromosome-coded aerobactin synthesis in enteric bacteria: insertion sequences flank operon in plasmid-mediated systems. J. Bacteriol. **159**:300–305.
29. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35**:253–263.
30. **Moore, D. G., and C. F. Earhart.** 1981. Specific inhibition of *Escherichia coli* ferrienterochelin uptake by a normal human serum immunoglobulin. Infect. Immun. **31**:631–635.
31. **Moore, D. G., R. J. Yancey, C. E. Lankford, and C. F. Earhart.** 1980. Bacteriostatic enterochelin-specific immunoglobulin from the normal human serum. Infect. Immun. **27**:418–423.
32. **Neilands, J. B.** 1983. Significance of aerobactin and enterobactin in siderophore-mediated iron assimilation in enteric bacteria, p. 284–287. *In* D. Schlessinger. (ed.), Microbiology—1983. American Society for Microbiology, Washington, D.C.
33. **Ohtsubo, H., and E. Ohtsubo.** 1976. Isolation of inverted repeat sequences, including IS*1*, IS*2*, and IS*3*, in *Escherichia coli* plasmids. Proc. Natl. Acad. Sci. U.S.A. **73**:2316–2320.
34. **Ohtsubo, H., and E. Ohtsubo.** 1978. Nucleotide sequence of an insertion element, IS*1*. Proc. Natl. Acad. Sci. U.S.A. **75**:615–619.
35. **Payne, S. M.** 1980. Synthesis and utilization of siderophores by *Shigella flexneri*. J. Bacteriol. **143**:1420–1424.

36. **Payne, S. M., D. W. Niesel, S. S. Peixotto, and K. M. Lawlor.** 1983. Expression of hydroxamate and phenolate siderophores by *Shigella flexneri*. J. Bacteriol. **155**:949–955.
37. **Pollack, J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **38**:989–992.
38. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
39. **Quackenbush, R. L., and S. Falkow.** 1979. Relationship between colicin V ativity and virulence in *Escherichia coli*. Infect. Immun. **24**:562–564.
40. **Shenk, T. E., C. Rhodes, P. W. J. Rigby, and P. Berg.** 1975. Biochemical method for mapping mutational alterations in the DNA with S1 nuclease: The location of deletions and temperature-sensitive mutations in SV40. Proc. Natl. Acad. Sci. U.S.A. **72**:989–993.
41. **Simon, E. H., and I. Tessman.** 1963. Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. U.S.A. **50**:526–532.
42. **Smith, H. O., and M. L. Birnstiel.** 1976. A simple method for DNA restriction site mapping. Nucleic Acids Res. **3**:2387–2398.
43. **Smith, H. W.** 1974. A search for transmissible pathogenic characters in invasive strains of *Escherichia coli*: the discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character closely associated, or identical, with Colicine V. J. Gen. Microbiol. **83**:95–111.
44. **Smith, H. W., and M. B. Huggins.** 1976. Further observations on the association of the Colicine V plasmid of *Escherichia coli* with pathogenicity and with survival in the alimentary tract. J. Gen. Microbiol. **92**:335–350.
45. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS*1*. Nature (London) **277**:453–456.
46. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
47. **Stuart, S. J., K. T. Greenwood, and R. K. J. Luke.** 1980. Hydroxamate-mediated transport of iron controlled by ColV plasmids. J. Bacteriol. **143**:35–42.
48. **Stuart, S. J., K. T. Greenwood, and R. K. J. Luke.** 1982. Iron-suppressible production of hydroxamate by *Escherichia coli* isolates. Infect. Immun. **36**:870–875.
49. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with universal primers. Gene **19**:259–268.
50. **Warner, P. H., P. H. Williams, A. Bindereif, and J. B. Neilands.** 1981. ColV plasmid-specified aerobactin synthesis by invasive strains of *Escherichia coli*. Infect. Immun. **33**:540–545.
51. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection and neoplasia. Physiol. Rev. **64**:65–102.
52. **Williams, P. H.** 1979. Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. Infect. Imun. **26**:925–932.
53. **Williams, P. H., and George H. F.** 1979. ColV plasmid-mediated iron uptake and the enhanced virulence of invasive strains of *Escherichia coli*, p. 161–172. *In* K. N. Timmis and A. Puehler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.
54. **Williams, P. H., and P. J. Warner.** 1980. ColV plasmid-mediated, Colicin V-independent iron uptake system of invasive strains of *Escherichia coli*. Infect. Immun. **29**:411–416.
55. **Yamamoto, T., and T. Yokota.** 1981. *Escherichia coli* heat-labile enterotoxin genes are flanked by repeated deoxyribonucleic acid sequences. J. Bacteriol. **145**:850–860.
56. **York, M. K., and M. Stodolsky.** 1981. Characterization of P1*argF* derivatives from *Escherichia coli* K12 transduction. I. IS*1* elements flank the *argF* gene segment. Mol. Gen. Genet. **181**:230–240.