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Aerobactin Iron Uptake Sequences in Plasmid ColV-K30 Are Flanked by Inverted IS*1*-Like Elements and Replication Regions

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By using Southern blot hybridization procedures, we found that a specific sequence within a 16.3-kilobase *Hin*dIII restriction fragment of pColV-K30 was also present in at least three other pColV-K30 *Hin*dIII fragments. Restriction endonuclease mapping of these *Hin*dIII fragments indicated that two of these repeated sequences, identified as IS*1*-like, occur in reverse orientation adjacent to the ends of the aerobactin iron uptake region, also included in the 16.3-kilobase *Hin*dIII fragment. There are two distinct replication regions enclosing the IS*1*-flanked aerobactin genes. A pColV-K30 *Bam*HI restriction endonuclease fragment, carrying one of these replication regions, showed homology with the F plasmid *Eco*RI fragment f5, which carries the F replication sequences.

Plasmid ColV-K30 specifies an iron uptake system which is associated with the ability of invasive strains of *Escherichia coli* to cause disease (32). Recently, aerobactin sequences related to the iron uptake region present in the ColV-K30 plasmid, have been found in the chromosomes and plasmids of enteric bacteria in which production of aerobactin and its outer membrane receptor could be demonstrated (19, 21, 30). Thus the pColV-K30 aerobactin sequences were found to be present in the chromosome of an *E. coli* K1 strain isolated from a case of human neonatal meningitis (30) and in the chromosome of strains of *Shigella flexneri* (19). Plasmids other than ColV-K30 were also reported to possess aerobactin sequences, i.e., pRJ100 (28) and ColV-K311 (8) in *E. coli*, pSMN1 in *Enterobacter aerogenes* (21), and pSMN2 and pSMN3 in *Salmonella arizona* (21). The aerobactin region appeared to be highly conserved in these systems. Therefore, study of the sequences flanking the iron uptake region of plasmid ColV-K30 will help in understanding the high degree of conservation and ubiquity of these sequences. We report in this paper that the pColV-K30 iron uptake region is flanked by repeated sequences homologous to IS*1* and that, in addition, two replication regions are also found at both ends of the aerobactin iron uptake region. These features may have played an important role in the recombinational mobility and high degree of conservation of this iron uptake region.

(These results were presented in part at the 84th Annual Meeting of the American Society for Microbiology, St. Louis, Mo., 4 to 9 March 1984 [Y. Mitoma, J. Perez-Casal, M. A. Walter, and J. H. Crosa, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B175, p. 46].) Related independent reports of the presence of IS*1* elements adjacent to the aerobactin region in pColV-K30 were recently communicated (19, 21).

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains and plasmids used in this investigation and their relevant characteristics are listed in Table 1. *E. coli* 3478 was used as a recipient in transformation experiments, whereas *E. coli* LE392 was used as a recipient in in vitro transductions.

Detection of aerobactin siderophore. Aerobactin production was determined by the ability of sterile supernatants to support the growth of strain LG1522. This iron uptake-deficient derivative possesses a mutation in the ColV-K30 plasmid genes specifying aerobactin synthesis, which does not affect the biosynthesis of the aerobactin receptor (33). The bioassay test was carried out essentially as previously described (33), with some modifications. Bacteria were grown in a Tris minimal medium with 1% sodium succinate as a carbon source (TMS medium) and 50 μM dipyridyl as an iron chelator. After centrifugation, cell-free supernatant fluid was sterilized by filtration through a membrane filter (0.22 μm; Millipore Corp., Bedford, Mass.). Sterile supernatant fluid (10 μl) was applied onto sterile filter disks that had been placed on TMS agar plates (containing 120 μM dipyridyl) that had been seeded with 0.1 ml of an overnight culture of 10^8 cells of LG1522, the indicator strain, per ml. Aerobactin-producing activity was assessed by growth halos of the indicator strain around the appropriate disks. Positive and negative controls were the aerobactin-producing LG1315 and HB101 strains, respectively. Strain RW193 was used as a control lawn for cross-feeding specificity since this plasmidless bacterium does not have a receptor for aerobactin.

Detection of the aerobactin outer membrane receptor. Presence of the aerobactin outer membrane receptor was determined by the cloacin sensitivity test. Partially purified cloacin (10) was streaked across an L-agar plate and dried at 37°C for 10 min. Liquid cultures of the strains to be tested were streaked across the plates at a right angle with respect to the original cloacin streak. After incubation at 37°C for 4 to 6 h, a zone of inhibition in the growth of any of the culture streaks indicated that the strain was sensitive to cloacin.

Isolation of plasmid DNA. Large-scale purification of plasmid DNA was performed by the methods of Hansen and Olsen (13), Birnboim and Doly (4), and Maniatis et al. (20). Further purification was achieved by centrifugation in cesium chloride-ethidium bromide density gradients at 50,000 rpm for 16 h at 15°C in the VTi65 rotor in a Beckman L8-70 ultracentrifuge. Plasmid screening in transformation and recombinant DNA experiments was carried out by a rapid alkaline lysis procedure (4).

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TABLE 1. Properties and sources of *E. coli* strains and plasmids

Strain	Genotype	Relevant phenotype conferred by plasmid[a]	Source
LE392	F^- *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	None	Bethesda Research Laboratories (20)
HB101	F^- *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	None	H. W. Boyer (7)
C600	F^- *thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	None	Laboratory stock (1)
3478	F^- *polA thy*	None	Laboratory stock (11)
LG1315	F^-, *ara entA lac leu mtl proC rpsL supE tonA thi trpE xyl* (pColV-K30)	Iu^+ C^s	P. H. Williams (32)
LG1522	*ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi* (pColV-K30 *iuc*)	Iu^- C^s	P. H. Williams (33)
RW193	*entA proC leu trp tsx thi lacY galK ara mtl xyl azi supE44*	None	J. B. Neilands (31)
294(pABN1)	*endA hsdR thi pro* (pABN1)	Iu^+ C^s Ap^r (16.3-kb *Hin*dIII fragment from ColV-K30 cloned in a pBR322 derivative [3])	J. B. Neilands (3)
3478(pJHC-P1)		Iu^+ C^s Ap^r	This work
3478(pJHC-P2)		Tc^r C^r	This work
3478(pJHC-P3)		Cm^r C^r	This work
HB101(pJHC-P4)		Cm^r Tc^r Ap^s (obtained from pBR325 by *Bal*31 deletion of Ap^r determinants)	This work
HB101(pJHC-P5)		Ap^r Tc^s (2.3-kb *Hin*dIII fragment from ColV-K30 cloned into pBR322)	This work
C600(pBRG29)		Cm^r Tc^r Ap^r (pBR322 with Tn*9* insertion)	D. Berg (S. Biel and D. Berg, personal communication); (pBRG29 supplied in strain 3510 and transformed into C600)
MV12	C600 *trpE5*, *recA56* (pDF11)	Km^r (*Eco*RI f5 fragment from F plasmid cloned in a ColE1-Km^r vector)	D. Figurski (15)
HB101(pBR325)		Cm^r Tc^r Ap^r	F. Bolivar (5)
HB101(pBR322)		Tc^r Ap^r	Laboratory stock (6)
HB101(pVK102)		Km^r Tc^r (pRK290 with λ *cos* sites)	E. Nester (16)
HB101(pKY2662)		Ap^r cosmid vector	Laboratory stock (12)
HB101(pJHC-V9)		Km^r Tc^s (8.6-kb ColV-K30 *Hin*dIII fragment cloned in pVK102)	This work

[a] Iu, Iron uptake ability; aerobactin-mediated iron uptake system present in ColV-K30. C^s or C^r, Cloacin sensitivity or resistance, respectively. *iuc* is a mutation on this plasmid which results in defective aerobactin synthesis but in an intact receptor. Plasmid-mediated resistance to 20 μg of various antibiotics per ml: ampicillin (Ap^r), tetracycline (Tc^r), chloramphenicol (Cm^r), and kanamycin (Km^r).

Restriction endonuclease analysis and molecular cloning of the pColV-K30 aerobactin regions. Restriction enzymes were used as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Electrophoresis of restriction endonuclease-cleaved DNA was performed in 0.8 to 1% horizontal agarose slab gels in a Tris-borate buffer system (9) at 30 mA for 12 h. To obtain partial restriction endonuclease digests of plasmid DNA, 10 μg samples of ColV-K30 plasmid DNA were treated with 5 U of the restriction endonuclease *Eco*RI for 40 min at 37°C. Partial digests of pColV-K30 DNA were ligated with DNA from cosmid vector pKY2662 (12), which had been completely digested with *Eco*RI, by using T4 DNA ligase prepared by the method of Tait et al. (29). Reactions were carried out at 15°C for 12 h at an approximate ratio of picomoles of ends of vector to target of 1:1. The reaction mixture consisted of 20 mM Tris-hydrochloride (pH 7.4), 10 mM $MgSO_4$, 10 mM dithiothreitol, 0.6 mM ATP, and 1 U of ligase in a final volume of 20 μl. Successful ligation was tested by agarose gel electrophoresis. Ligated DNA was precipitated with ethanol after phenol extraction. DNA was next packaged in vitro using a commercially available lambda in vitro packaging system, under the conditions recommended by the suppliers (Bethesda Research Laboratories; or Promega Biotech, Madison, Wisc.). Exponential phase *E. coli* LE392 cells from an L-broth culture containing 0.2% maltose and 10 mM $MgSO_4$ grown at 37°C were infected with the phage particles at 30°C in the same medium. After 20 min, 1 volume of L-broth was added, the temperature was raised to 37°C, and incubation was continued for an additional 2 h. The cells were then spread onto L-agar plates containing 20 μg of ampicillin (Ap) per ml, and plates were incubated at 37°C for 16 h. Ap^r colonies were then examined for cloacin sensitivity. One of these clones was selected for further examination. The derivative contained a plasmid, pJHC-P1, which was characterized by restriction endonuclease analysis.

Molecular cloning of the pColV-K30 replication regions. pJHC-P1 was cleaved with *Eco*RI and *Bam*HI in separate experiments. Each of these preparations of cleaved pJHC-P1 DNA was ligated with vector DNA cleaved with the appropriate restriction endonuclease by using T4 DNA ligase. The vector used, pJHC-P4, is a plasmid derived from pBR325 (5), by deletion of part of the Ap^r gene with *Bal*31 nuclease (20). The ligation mixture was used to transform *E. coli* 3478,

which is deficient in the synthesis of polymerase I (11). In this strain, ColE1-type plasmids cannot replicate, permitting the selection of clones in which replication regions from pColV-K30 had been cloned. Recombinant plasmids obtained by the cloning of *Eco*RI-cleaved pJHC-P1 DNA in pJHC-P4 should confer resistance to tetracycline, whereas derivatives resulting from the cloning of *Bam*HI-cleaved DNA into pJHC-P4 should harbor the Cm^r gene. Clones containing regions of the original cloning vector pKY2662 should confer resistance to ampicillin. Thus, by selection of Tc^r Ap^s (for *Eco*RI) or Cm^r Ap^s (for *Bam*HI) clones, it was possible to obtain the recombinant derivatives pJHC-P2 (from the *Eco*RI digestions) and pJHC-P3 (from the *Bam*HI digestions). Since both pJHC-P2 and pJHC-P3 replicate in the *polA* derivative of *E. coli*, they must carry pColV-K30 replication regions. The original clone, pJHC-P1, from which these two plasmids were derived, also replicated in the *polA* background. ^{32}P-plasmid DNA from the recombinant clones labeled by nick translation (24) was used as a probe in Southern blot hybridization experiments (27) essentially as described previously (20). Briefly, nitrocellulose filters containing the DNA transferred by the Southern blotting technique (27) were next placed in plastic bags containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. After 3 h at 37°C, the appropriate heat-denatured, ^{32}P-labeled plasmid DNA (10^6 cpm) was added, and hybridization was carried out by incubation in the same preincubation solution with carrier salmon testes DNA at 100 μg/ml for 16 h at 37°C. Washing of the filters was carried out at 65°C in 6× SSC–0.1% sodium dodecyl sulfate.

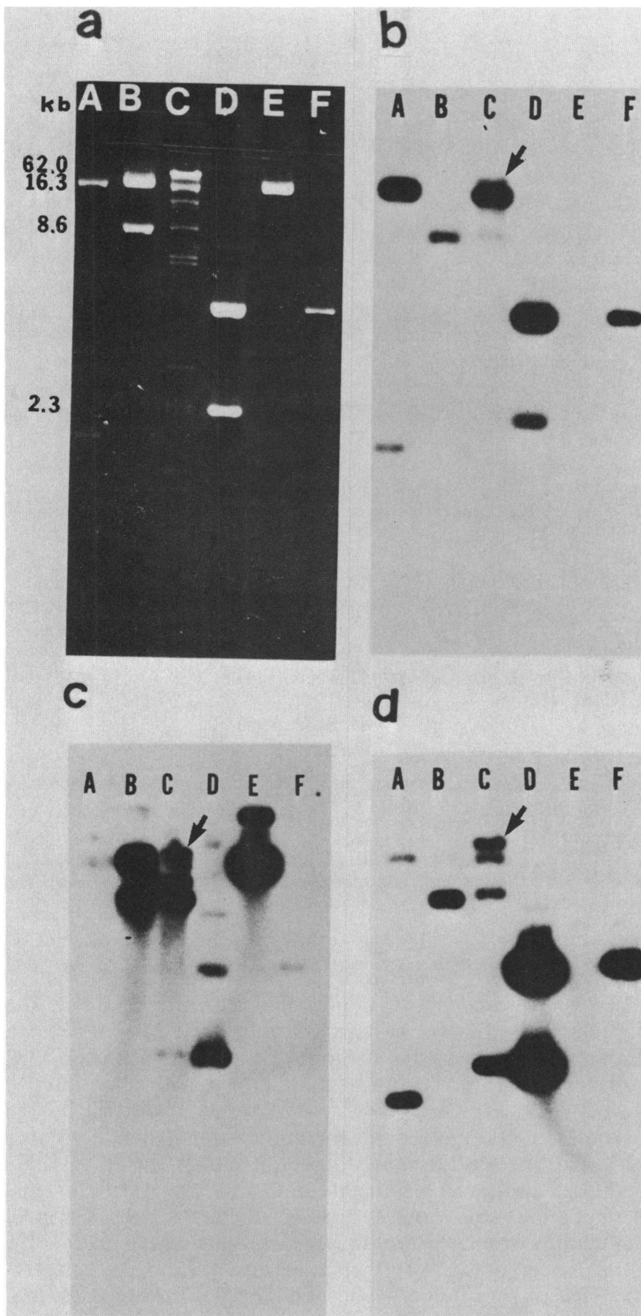

FIG. 1. Presence of repeated sequences in the pColV-K30 genome. (a) Ethidium bromide-stained 0.8% agarose gel of *Hin*dIII restriction endonuclease patterns: lanes A, pABN1 (16.3-kb *Hin*dIII fragment of pColV-K30 cloned in pPlac (a pBR322 derivative [3]); B, pJHC-V9 (8.6-kb *Hin*dIII fragment of pColV-K30 cloned in pVK102); C, pColV-K30; D, pJHC-P5 (2.3-kb *Hin*dIII fragment of pColV-K30 cloned in pBR322); E, pVK102; F, pBR322. (b, c, and d) Autoradiographs of Southern blot hybridizations of gels identical to that in (a), using the following ^{32}P-labeled probes: (b) pABN1, (c) pJHC-V9, and (d) pJHC-P5. The arrows in lanes C of (b), (c), and (d) show the location of the 62-kb *Hin*dIII fragment containing repeated sequences. The molecular weights (in kb) of the pColV-K30 *Hin*dIII fragments shown in lane C are 62, 16.3, 16.3, 12.5, 8.6, 6.4, 6, 3, 2.3, 2.1, 1.75, 1.7, 1.6, 1.15, 0.88, 0.76, and 0.6 (the last three bands are not visible in this photograph).

RESULTS

Presence of repeated sequences in pColV-K30. pColV-K30 is ca. 144 kilobase pairs (kb) in size and is cleaved by the *Hin*dIII restriction endonuclease into 17 fragments which range from 62 to 0.6 kb (Fig. 1a, lane C). One of these, a 16.3-kb fragment, was recently shown to carry the pColV-K30 iron uptake regions (3). We used plasmid pABN1 (3), containing the 16.3-kb *Hin*dIII fragment of pColV-K30, as a probe in Southern blot hybridization experiments with *Hin*dIII-cleaved ColV-K30 plasmid DNA and clones containing pColV-K30 *Hin*dIII fragments. Results of such a hybridization (Fig. 1b, lanes B, C, and D) indicated that in addition to hybridization with the homologous fragment of 16.3-kb, the probe showed homology with at least three other *Hin*dIII fragments of 62, 8.6, and 2.3 kb, respectively. These results indicated that sequences present on the 16.3-kb *Hin*dIII fragment were repeated in the other fragments. Radioactive probes prepared with clones carrying *Hin*dIII fragments of either 8.6 kb cloned in pVK102, i.e., pJHC-V9, or 2.3kb cloned in pBR322, i.e., pJHC-P5, were also hybridized with *Hin*dIII-cleaved plasmid DNA from ColV-K30 and the recombinant derivatives (Fig. 1c and d, lanes B, C, and D). These two probes hybridized with each other and with the *Hin*dIII fragments of 62 and 16.3 kb, in addition to the homologous fragment. The pJHC-V9 probe also hybridized with a 12.5-kb *Hin*dIII fragment. Thus, the results are consistent with the presence of at least one class of repeated sequences in the *Hin*dIII fragments of 62, 16.3, 8.6, and 2.3 kb. Since only the 8.6-kb *Hin*dIII fragment showed homolo-

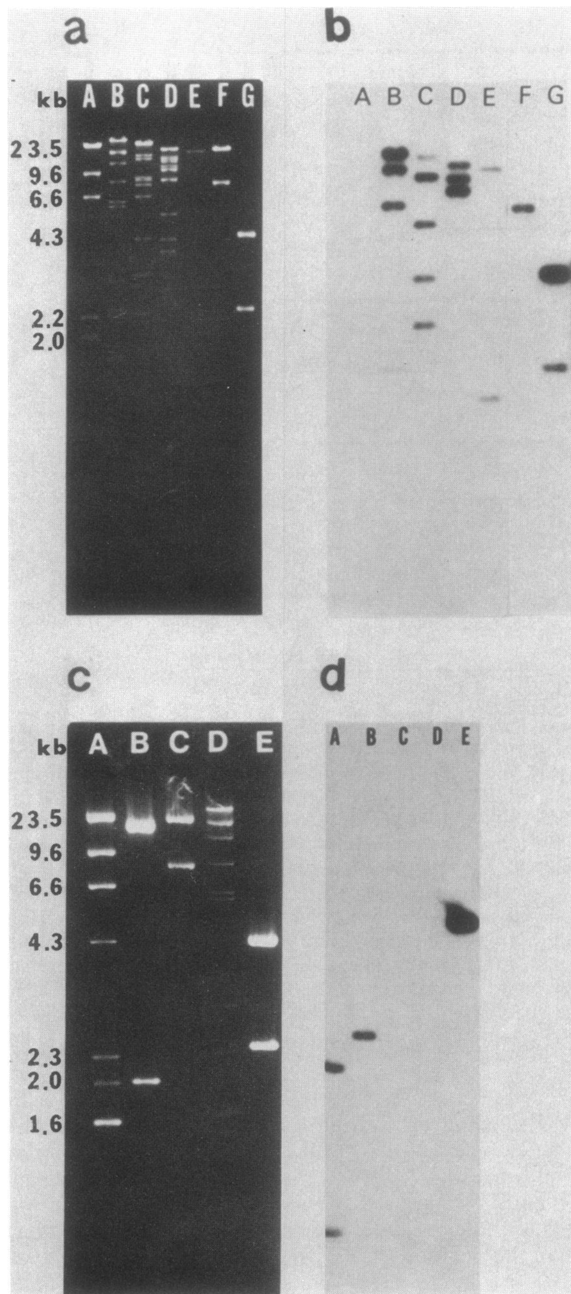

FIG. 2. Southern blot hybridization with pBRG29 (IS*1* probe) and copy number of the repeated sequences present in the pColV-K30 genome. (a) Ethidium bromide-stained 0.8% agarose gel: lanes A, *Hin*dIII-cleaved lambda DNA for size markers; pColV-K30 DNA cleaved with *Hin*dIII (B), *Bam*HI (C), and *Eco*RI (D); *Hin*dIII-cleaved DNA of pABN1 (E), pJHC-V9 (F), and pJHC-P5 (G). (b) Autoradiogragh of a Southern blot hybridization of the gel in (a), using as a probe ^{32}P-labeled pBRG29 DNA (containing IS*1*). To visualize the low-intensity bands in lanes B, C, D, and E, it was necessary to expose the film for a long time, leading to over-exposure of bands in lanes F and G which are homologous to the probe. Thus lanes F and G shown in this photograph were obtained from a shorter exposure of the same Southern blotted gel. (c) Ethidium bromide-stained 0.8% agarose gel: lanes A, *Hin*dIII-cleaved lambda DNA together with *Hin*fI-cleaved pBR322; *Hin*dIII cleaved DNA of pABN1 (B), pJHC-V9 (C), pColV-K30 (D), and pJHC-P5 (E). (d) Autoradiograph of a Southern blot hybridization of the gel in (c), using as a probe ^{32}P-labeled pBR325 DNA.

gy with the 12.5-kb *Hin*dIII fragment, there must be an additional sequence shared by these two restriction fragments but absent from the 62, 16.3, and 2.3-kb fragments. The nature of this sequence, unrelated to the other repeated sequences, is currently under investigation.

Identification, localization, and copy number of the pColV-K30 repeated sequences. Restriction endonuclease-cleaved pColV-K30 DNA was hybridized with ^{32}P-labeled plasmid pBRG29, which contained IS*1* elements. This probe, a pBR322 derivative, carries the transposition sequence Tn*9* which is bordered by IS*1* elements (S. Biel and D. Berg, personal communication). Since the Tn*9* sequence contains the genes for cloramphenicol resistance, we used as a negative control the pBR325 plasmid, in which this portion of the Tn*9* transposon is intact, whereas the IS*1* sequences had been entirely deleted (14). Results (Fig. 2, lanes B, F, G) indicate that the IS*1* probe hybridized specifically with the 62, 16.3, 8.6, and 2.3-kb *Hin*dIII fragments, whereas the control probe, pBR325, only hybridized with the pBR322-type vectors (Fig. 2d, lanes A, B, and E). To determine the number of copies of the IS*1*-like sequence in pColV-K30 DNA, we hybridized the IS*1* probe with pColV-K30 DNA cleaved with *Eco*RI and *Bam*HI, in addition to the *Hin*dIII restriction endonuclease. Since these enzymes do not cleave within the IS*1* element, the number of restriction endonuclease fragments hybridizing with the IS*1* radioactive probe is a measure of the number of copies present. Results (Fig. 2a and b, lanes C and D) suggest that there are at least four copies of IS*1* in pColV-K30. The existence of a fifth copy is suggested by a faint band of homology in the *Bam*HI-cleaved pColV-K30 DNA (Fig. 2a and b, lane C, band at ca. 25 kb), although this band could be the result of an incomplete digestion of the plasmid DNA.

To map the relative positions of the fragments carrying the IS*1*-like elements in plasmid ColV-K30, we cloned *Eco*RI partial digests of pColV-K30 DNA in the cosmid vector pKY2662, which is a ColE1 derivative carrying the lambda *cos* sites and genetic determinants for resistance to ampicillin (12). After in vitro packaging and transduction into *E. coli* LE392, a series of Ap^r clones were obtained. Each of these clones was tested for production of aerobactin and sensitivity to cloacin. Several clones showed cloacin sensitivity; one of them was selected for further examination. This clone contained recombinant plasmid pJHC-P1 which was cleaved with *Eco*RI, *Hin*dIII, and *Bam*HI restriction endonucleases to map the relative positions of the restriction fragments. This plasmid contained the pColV-K30 *Eco*RI fragments of 16 kb, two of 10 kb, and one of 8.6 kb. Bioassays indicated that the strain harboring pJHC-P1 also produced aerobactin. Therefore, plasmid pJHC-P1 must contain the aerobactin iron uptake region. The 16.3-kb *Hin*dIII fragment carrying the aerobactin region and one of the IS*1*-like sequences, is part of the cloned pColV-K30 DNA present in pJHC-P1 (Fig. 3). Other clones containing *Eco*RI partial digests were also generated. Some of these clones did not have the *Eco*RI fragments of 16 kb and the 10-kb fragment located to the right of the aerobactin genes. Strains harboring these clones were, as expected, cloacin resistant, since there is an *Eco*RI site right inside the receptor gene. These strains still produced aerobactin. Further restriction endonuclease analysis of these clones indicated that the *Hin*dIII fragment of 8.6 kb was still present, but only part of the 16.3-kb *Hin*dIII fragment remained intact. Therefore, the 8.6-kb *Hin*dIII fragment which carries one copy of the IS*1*-like sequence is located adjacent and to the left of the 16.3-kb *Hin*dIII fragment (Fig. 3). This location was confirmed by restriction

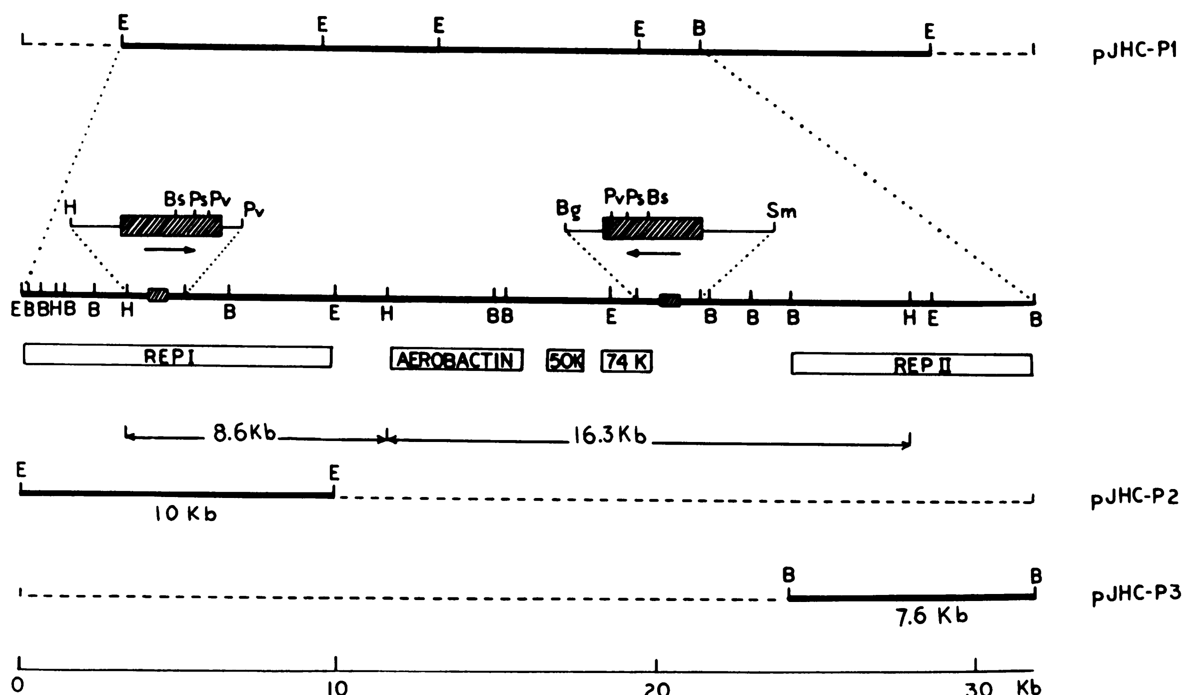

FIG. 3. Genetic and physical map of cloned pColV-K30 DNA. The upper portion of this diagram shows the pColV-K30 *Eco*RI fragments present in pJHC-P1, a recombinant derivative obtained by partial digestion of pColV-K30 with *Eco*RI restriction endonuclease and cloning into the pKY2662 vector. The regions labeled REPI and REPII carry pColV-K30 replication regions, as demonstrated by their ability, upon cloning in ColE1 vectors, to replicate in *E. coli* strains deficient in polymerase I: pJHC-P2, cloned *Eco*RI fragment containing REPI; pJHC-P3, cloned *Bam*HI fragment containing REPII. The regions labeled aerobactin, 50K (50,000 daltons) and 74K (74,000 daltons) are those regions encoding these components of the aerobactin iron uptake system (2, 17). The cross-hatched boxes represent the IS*1*-like sequences flanking the aerobactin region of pColV-K30. A *Hin*dIII-*Pvu*II fragment carrying the left IS*1*-like sequence and a *Bgl*II-*Sma*I fragment containing the right IS*1*-like sequence have been mapped with additional restriction endonucleases. These two fragments have been amplified in the diagram to show the cleavage sites for *Bst*EII (Bs), *Pst*I (Ps), and *Pvu*II (Pv) inside the IS*1*-like elements. The arrows beneath these sequences show that these two sequences are inverted with respect to each other. Thicker lines represent pColV-K30 regions that had been cloned in various cloning vehicles. Dashed lines correspond to regions not included in the clones. The scale at the bottom of the diagram is the coordinate in kb of the amplified region of pJHC-P1 (between *Eco*RI and *Bam*HI sites). The location of the 8.6- and 16.3-kb *Hin*dIII fragments is also shown. The cleavage sites for various restriction endonucleases are shown: H, *Hin*dIII; B, *Bam*HI; Bt, *Bst*EII; Pv, *Pvu*II; E, *Eco*RI; Bg, *Bgl*II; Sm, *Sma*I. The *Pst*I, *Bst*EII, *Bgl*II, and *Sma*I sites in the amplified portion of the pJHC-P1 plasmid are not completely mapped.

endonuclease analysis of several other clones containing restriction fragments in the neighborhood of the boundary between the 8.6 and the 16.3-kb *Hin*dIII fragments.

A restriction endonuclease analysis of the 8.6-kb *Hin*dIII fragment, together with Southern blot hybridizations with the IS*1* probe, plasmid pBRG29, permitted us to map the location and orientation of the IS*1*-like element within this fragment (Fig. 3 and 4). The 8.6-kb *Hin*dIII fragment, cloned in the cosmid vector pVK102 (16), was digested with *Hin*dIII, *Bam*HI, *Pst*I, *Pvu*II, and *Bst*EII in cleavage reactions in which two or three of these enzymes were used successively in double or triple digestions. Analysis of the restriction fragments showing homology with the IS*1* probe allowed us to localize the IS*1*-like sequence on the 8.6-kb *Hin*dIII fragment within a *Hin*dIII-*Pvu*II fragment (Fig. 3). The pVK102 vector used to generate pJHC-V9 has only one site for the restriction endonucleases used to cleave recombinant plasmid pJHC-V9. Furthermore, these enzymes cleave the 8.6-kb *Hin*dIII fragment at various sites; therefore, the fragments hybridizing with the IS*1* probe consist of either sequences within the IS*1* element or those at the ends of this element which are part pColV-K30 DNA and part IS*1* sequences. Therefore, the bands in the autoradiograph in Fig. 4 can be used to map the position of restriction sites on the IS*1* element as well as the relative orientation of this sequence. The results of the analysis of the restriction endonuclease cleavage patterns and Southern blot hybridization strongly suggest that this IS*1*-like sequence is highly related to the IS*1* elements described by Ohtsubo et al. (22). To map the position of the IS*1*-like sequence on the 16.3-kb *Hin*dIII fragment we followed a similar approach, and the order of the restriction sites within this fragment was carried out by analysis of digestions of this fragment with *Eco*RI, *Bam*HI, *Pvu*II, and *Hin*dIII in single and double digestions. The results of this mapping procedure (Fig. 3) indicate that the IS*1*-like sequence is located immediately adjacent and to the right of the genes for the 74,000-dalton outer membrane protein aerobactin receptor, within a *Bgl*II-*Sma*I fragment. Fine restriction endonuclease analysis of this fragment indicates that the orientation of this IS*1*-like element is inverted with respect to that of the IS*1*-like element on the 8.6-kb *Hin*dIII fragment.

Replication regions occur at both ends of the IS*1*-flanked aerobactin region. We began studying the replication properties of plasmid ColV-K30 by cloning essential replication regions of this plasmid. We used as a selection property the ability of this type of plasmid to replicate in *E. coli* 3478 (*polA*), deficient in polymerase I (11). Since ColE1-type

vectors do not replicate in *polA* mutants (25), we used the pBR325 derivative pJHC-P4 as a cloning vector. In transformation experiments, we found that the pJHC-P1 clone carrying the aerobactin regions and the flanking IS*1*-like sequences was capable of replication in strain 3478. To determine the location of the replication regions within the cloned pColV-K30 DNA in pJHC-P1, this plasmid was digested with either *Eco*RI or *Bam*HI restriction endonucleases, ligated into the pJHC-P4 vector, and transformed into the *polA* strain of *E. coli*. Transformant clones were selected as described above.

We obtained clones capable of replicating in the *polA* background. One of these clones, pJHC-P2, carried a pColV-K30 *Eco*RI fragment of 10 kb, whereas the other clone, pJHC-P3, contained a pColV-K30 *Bam*HI fragment of 7.6 kb (Fig. 3). Purified plasmid DNA from these two clones was cleaved with several restriction endonucleases, and their location in the pJHC-P1 map was determined (Fig. 3). These two fragments, containing replication regions, are located adjacent to the ends of the aerobactin region. The replication regions located on the 10-kb *Eco*RI and the 7.6-kb *Bam*HI fragments were designated REPI and REPII, respectively. To confirm these mapping results, we hybridized restriction endonuclease-cleaved DNA of pJHC-P2 and pJHC-P3 with probes containing either the 16.3 or the 8.6-kb *Hin*dIII fragments of pColV-K30. The 8.6-kb *Hin*dIII fragment hybridizes only with pJHC-P2 (Fig. 5a and b, lanes B, C, and D), the clone carrying the 10-kb *Eco*RI fragment, but not with pJHC-P3 (Fig. 5a and b, lanes E and F). These results as well as the hybridization patterns of pJHC-P2 DNA doubly digested with *Bam*HI-*Hin*dIII, *Eco*RI-*Hin*dIII, and *Eco*RI-*Bam*HI, suggest that the 10-kb *Eco*RI fragment containing REPI is actually located to the left of the aerobactin region. This 10-kb *Eco*RI fragment also contains one of the two IS*1*-like elements flanking the aerobactin sequences.

The 16.3-kb *Hin*dIII fragment hybridizes with both the pJHC-P2 (Fig. 5c and d, lanes B, C, and D) and pJHC-P3 (Fig. 5c and d, lanes E and F) clones carrying pColV-K30 replication regions. In the case of pJHC-P2, the homology results are due to the presence of the IS*1*-like sequences in both the 16.3-kb *Hin*dIII fragment and the 10-kb *Eco*RI fragment contained in pJHC-P2 as confirmed by hybridization with the IS*1* probe, plasmid pBRG29 (data not shown). In pJHC-P3, the homology results indicate that the 3.7-kb *Bam*HI-*Hin*dIII fragment located between kb coordinates 24.3 and 28 within the 16.3-kb *Hin*dIII fragment is shared with the 7.6-kb *Bam*HI fragment contained in pJHC-P3 (Fig. 5d, lanes E and G). Thus, these hybridization results confirm that the replication region carried by the 7.6-kb *Bam*HI fragment, designated REPII, is located to the right of the aerobactin region. Since the ColV-K30 plasmid is considered a member of the incompatibility group F1, it was of interest to determine whether one of the two distinct replication regions we found on pJHC-P1 was related to the F replication region contained within the F *Eco*RI fragment f5. Figure 6 shows the results of hybridization experiments in which both pJHC-P2 and pJHC-P3 DNA, cleaved with *Eco*RI and *Bam*HI in single digestions, were hybridized with probes pDF11 and pJHC-P3. Plasmid pDF11 contains the F *Eco*RI fragment f5 cloned in a ColE1 vector harboring Km^r genes (15). pDF11 hybridized only with restriction endonuclease-cleaved pJHC-P3 (Fig. 6b, lane F) and not with pJHC-P2 DNA (Fig. 6b, lane E).

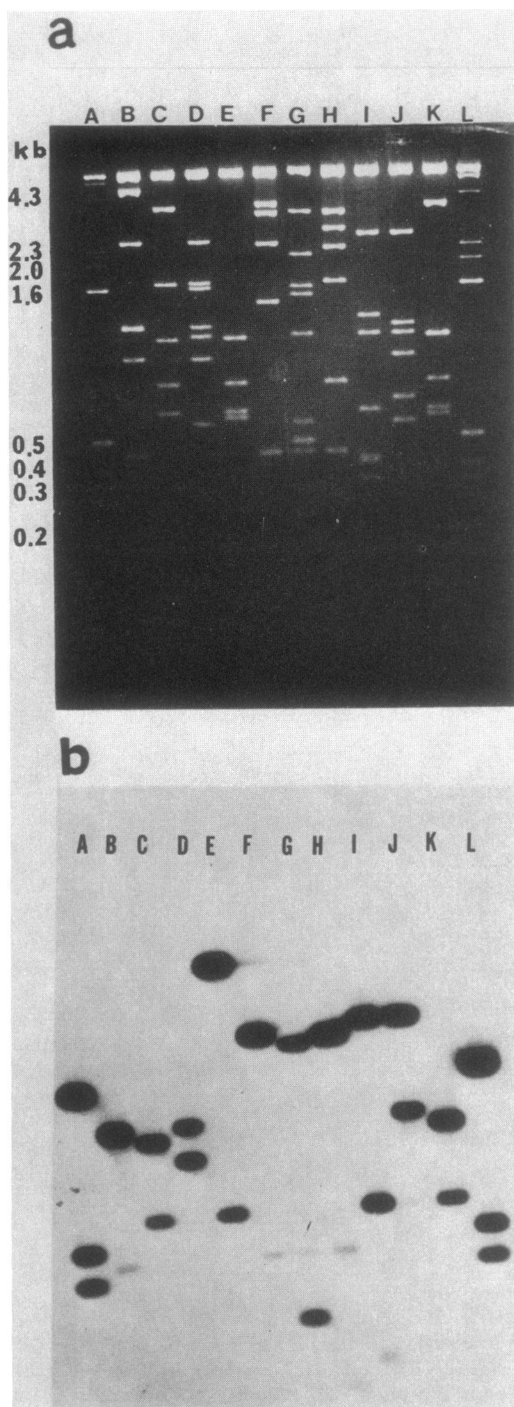

FIG. 4. Hybridization of the IS*1* probe with restriction endonuclease-cleaved pJHC-V9 DNA containing the 8.6-kb *Hin*dIII fragment of ColV-K30. (a) Ethidium bromide-stained 1% agarose gel: lanes A and L, *Hin*dIII-cleaved lambda DNA together with *Hin*fI-cleaved pBR322 DNA as size markers; B to K pJHC-V9 DNA digested successively with *Hin*dIII and *Pvu*II (B), *Hin*dIII and *Pst*I (C), *Hin*dIII and *Bst*EII (D), *Bam*HI and *Pst*I (E), *Bam*HI and *Pvu*II (F), *Pvu*II and *Bst*EII (G), *Pvu*II and *Pst*I (H), *Pst*I and *Bst*EII (I), *Bam*HI and *Bst*EII (J), and *Bam*HI, *Hin*dIII, and *Pst*I (K). (b) Autoradiograph of the Southern blot hybridization of the agarose gel in (a), using as a ^{32}P-labeled probe the pBRG29 plasmid containing the IS*1* sequences.

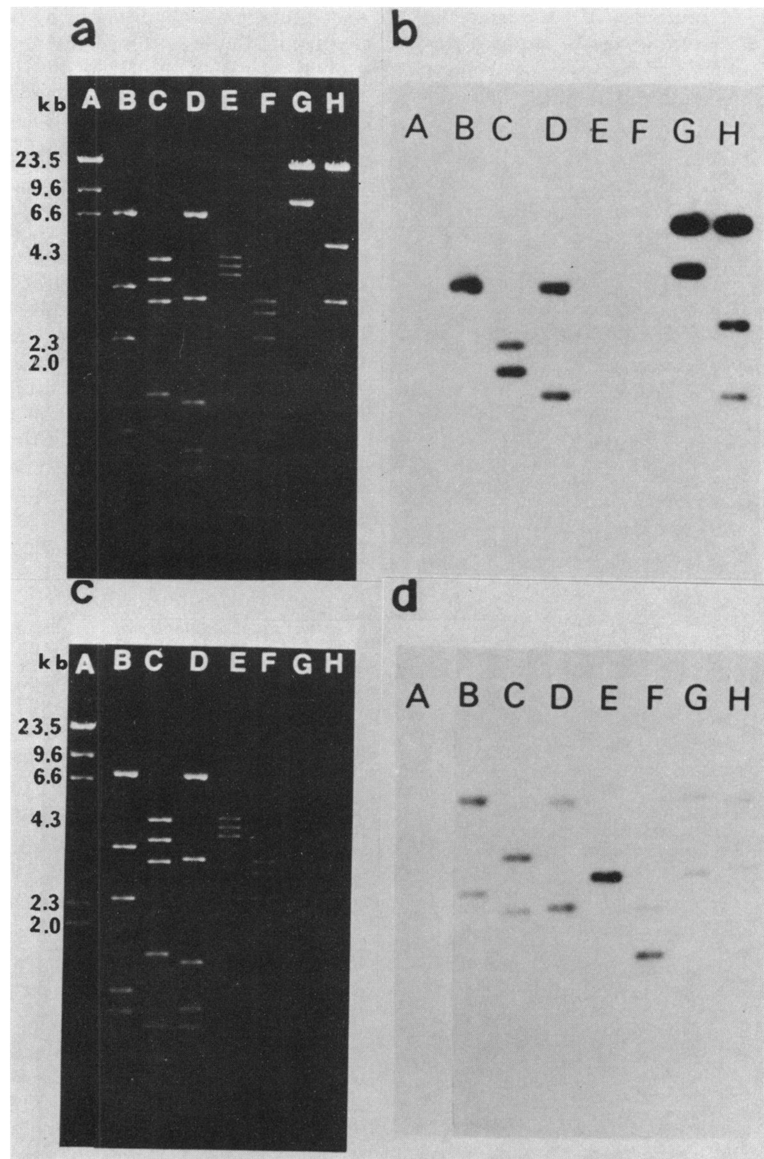

FIG. 5. Localization of the pColV-K30 replication regions. (a) Ethidium bromide-stained 0.8% agarose gel: lanes A, *Hin*dIII-cleaved lambda DNA; pJHC-P2 DNA successively digested with *Eco*RI and *Hin*dIII (B), *Eco*RI and *Bam*HI (C), and *Bam*HI and *Hin*dIII (D); pJHC-P3 DNA successively digested with *Hin*dIII and *Bam*HI (E) and *Pvu*II and *Hin*dIII (F); pJHC-V9 DNA digested with *Hin*dIII (G), and *Bam*HI and *Hin*dIII (H). (b) Autoradiograph of a Southern blot hybridization of the agarose gel in (a), using as a probe ^{32}P-labeled DNA of pJHC-V9. (c) Ethidium bromide-stained 0.8% agarose gel: lanes A, *Hin*dIII-cleaved lambda DNA; B to D, pJHC-P2 DNA successively digested with *Eco*RI and *Hin*dIII (B), *Eco*RI and *Bam*HI (C), and *Bam*HI and *Hin*dIII (D); E and F, pJHC-P3 DNA successively digested with *Hin*dIII and *Bam*HI (E) and *Pvu*II and *Hin*dIII (F); G and H, pABN1 DNA successively digested with *Hin*dIII and *Bam*HI (G) and *Pvu*II and *Hin*dIII (H). (d) Autoradiograph of the agarose gel in (c), using as a ^{32}P-labeled probe DNA from plasmid pABN1; lane A is a result of a longer exposure time of the same Southern blotted gel, to show the lack of homology of the lambda DNA negative control.

The pDF11 probe also hybridized, as did pJHC-P3 (Fig. 6b, lane C), with the same 3.7-kb *Hin*dIII-*Bam*HI fragment (kb coordinates, 24.3 to 28; Fig. 3), within the 16.3-kb *Hin*dIII fragment of pABN1 (Fig. 6c, lane C). Figure 6b shows a hybridization experiment with the pJHC-P3 probe that confirmed the previous findings, since this probe hybridized with the pDF11 DNA carrying the F *Eco*RI fragment f5 (upper band on lane D). Hybridization with the other two *Eco*RI bands of pDF11 is due to sequences present in the cloning vector, a derivative of pBR325 (Fig. 6d). Thus, hybridization of pJHC-P3 with the lower *Eco*RI band of 6.6 kb (Fig. 6c, lane D) is due to the common ColE1 sequences present in pDF11 and pJHC-P4, as assessed by hybridization with pBR322 (Fig. 6d, II, lane B), whereas hybridization with the middle band of 7 kb (Fig. 6c, lane D) is due to the fact that this fragment, which carries the Km^r determinant of pDF11 originally obtained from plasmid R6-5, also contains a segment of the R6-5 Cm^r region (D. Figurski, personal communication) which hybridizes with the Cm^r gene present in the pJHC-P4 vector, as assessed by hybridization with pBR325 (Fig. 6d, III, lane B).

Thus, the results of these experiments indicate that the

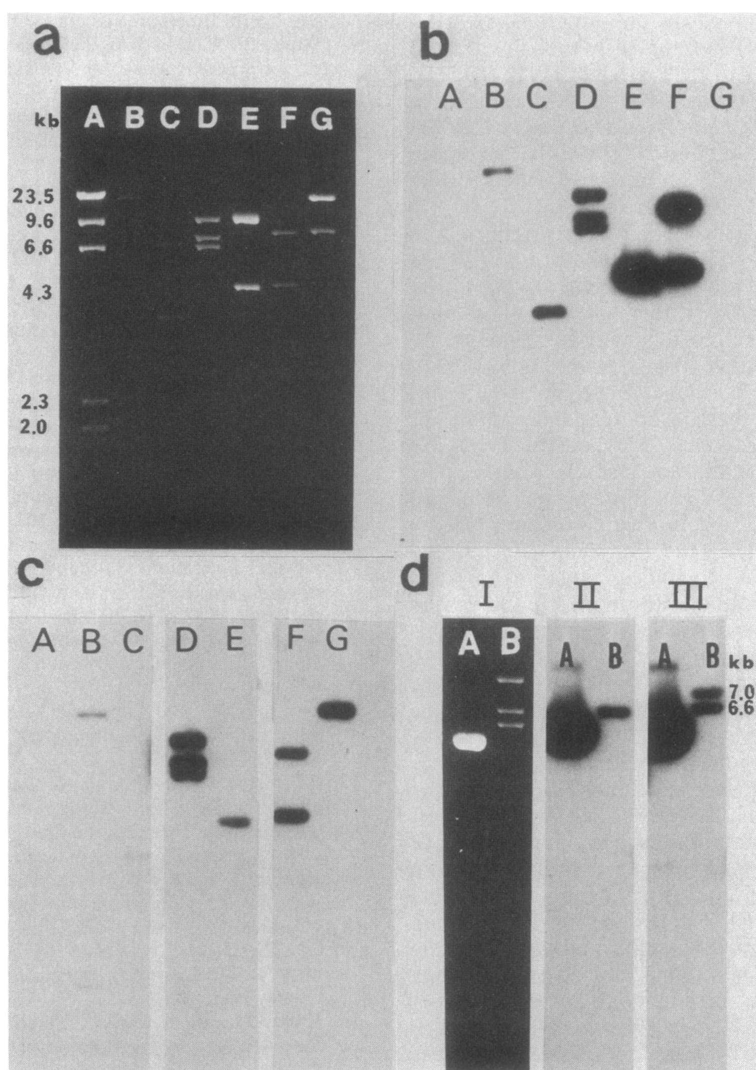

FIG. 6. Relationship between the F and pColV-K30 replication regions. (a) Ethidium bromide-stained 0.8% agarose gel: lanes A, *Hin*dIII-cleaved lambda DNA; B and C, pABN1 DNA digested with *Hin*dIII (B) and *Hin*dIII and *Bam*HI (C); D, *Eco*RI-cleaved pDF11 DNA; E, *Eco*RI-cleaved pJHC-P2 DNA; F, *Bam*HI-cleaved pJHC-P3 DNA; G, *Hin*dIII-cleaved pJHC-V9 DNA. (b) Autoradiograph of a Southern blot hybridization of the agarose gel in (a), using as a ^{32}P-labeled probe pJHC-P3 DNA. (c) Autoradiograph of a Southern blot hybridization of an identical agarose gel to that in (a), using as a ^{32}P-labeled probe pDF11 DNA. To visualize the low-intensity bands in lanes B and C, it was necessary to expose the film for a long time, resulting in overexposure of the DNA in lanes D and E. Consequently, lanes D and E were obtained from a shorter exposure of the same Southern blotted gel. Lane dI, ethidium bromide-stained 0.8% agarose gel: A, *Eco*RI-cleaved pBR325 DNA; B, *Eco*RI-cleaved pDF11 DNA. Lane dII, autoradiograph of a Southern blot hybridization of the agarose gel in lane dI, using as a ^{32}P-labeled probe pBR322 DNA. Lane dIII, autoradiograph of a Southern blot hybridization of an identical agarose gel to that in lane dI, using as a ^{32}P-labeled probe pBR325 DNA.

REPII region of the pColV-K30 plasmid, located to the right of the aerobactin system genes, is related to the replication region carried by the *Eco*RI fragment f5 of plasmid F.

DISCUSSION

Aerobactin sequences can be found on plasmids and chromosomes and have been associated with the ability of invasive strains of *E. coli* to cause disease. The aerobactin regions appeared to be highly conserved in these genomes (19, 21, 30). Thus, the aerobactin genes may be highly mobile as a recombinational unit, and they may have integrated at different sites in various genomes. To determine the extent of conservation of the aerobactin sequences and to assess a possible mechanism by which they become integrated in the different genomes, we initiated an analysis of the sequences flanking the aerobactin genes. A specific sequence present on the 16.3-kb *Hin*dIII fragment (which carries the aerobactin region [3]) was also present on three other *Hin*dIII fragments of 62, 8.6, and 2.3 kb, respectively. These repeated sequences were present in at least four copies on the pColV-K30 molecule. Since the *Hin*dIII fragments of 16.3 and 8.6 kb are contiguous in the pColV-K30 physical map, two of these repeated sequences are located adjacent to both ends of the aerobactin regions (Fig. 3). Restriction endonuclease analysis suggested that these repeated sequences had cleavage sites for certain restriction endonucleases at sites that were reminiscent of those present in the IS*1* element (23). By using a labeled probe consisting of a pBR322

derivative containing the transposon Tn9 which has two IS*1* elements, we demonstrated that the pColV-K30 repeated sequences were homologous to IS*1*, possibly IS*1*R or IS*1*D, a type of IS*1* element that was described in the chromosome of *Shigella dysenteriae* (22). Also, we could assess that the IS*1*-like element flanking the left end of the aerobactin region was in the reverse orientation as compared to the IS*1*-like sequence found on the right end of this region (Fig. 3).

Inverted IS*1* sequences were also recently reported to flank the gene for the heat-stable enterotoxin, another important virulence factor that has spread to various medically important microorganisms (26). In this case the whole unit, heat-stable enterotoxin genes together with the flanking IS*1* elements, was shown to have transposition ability (26). Although transposition of the aerobactin region as such has not been yet demonstrated, there is a potential capability for such an activity, since the genes are bracketed by IS*1* elements, themselves capable of transposition. Another possibility is that the presence of IS*1* elements, per se, could enhance the ability of the aerobactin region to recombine by homologous recombination, via the IS*1* elements, with genomes that also possess IS*1* sequences. Strengthening this hypothesis is our recent finding with an *E. coli* K1 strain isolated from a case of human neonatal meningitis. In this bacterium, the aerobactin regions are located on a 10.5-kb chromosomal *Hin*dIII fragment that also carries IS*1*-like elements (30). Recent related reports also demonstrated the presence of IS*1* elements in pColV-K30 (19, 21) and in the chromosome of *Shigella* strains (19).

Another intriguing result described in this paper is the finding of replication regions, designated REPI and REPII, occurring adjacent to the left and right end, respectively, of the aerobactin region. The REPII region hybridized with a recombinant plasmid, pDF11, carrying the replication region of the F factor, in the F *Eco*RI fragment f5. The REPI region did not hybridize with recombinant plasmids carrying the pColV-K30 REPII region, nor did it show any homology with the F replication probe. It remains to be seen whether the REPI region of pColV-K30 is related to a second F replication region described by Lane (18). Further analysis of the detailed structure and molecular nature of these two pColV-K30 replication regions is currently being done.

The REPI and REPII replication regions that flank the aerobactin system sequences may contribute to their conservation since recombinational events that conserve one of these replication regions may also leave intact the aerobactin system, especially in deletion or insertion events in which new plasmids are generated. Thus, the combination of essential replication regions and insertion sequences adjacent to the aerobactin sequences may have played an important role in their preservation during evolution and may have contributed to their spread.

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