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DNA Environment of the Aerobactin Iron Uptake System Genes in Prototypic ColV Plasmids

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The aerobactin iron uptake system genes in the prototypic plasmid pColV-K30 are flanked by inverted copies of insertion sequence IS*1* and by two distinct replication regions. To address the question of how these flanking regions may facilitate the maintenance and spread of the aerobactin system among the plasmids and chromosomes of enteric species, we investigated the DNA environment of 12 ColV plasmids. We found that the aerobactin system-specific genes are conserved in every plasmid phenotypically positive for the aerobactin system. The upstream IS*1* and its overlapping replication region (REPI) are also conserved. This replication region was cloned from several ColV plasmids and found to be functional by transforming these cloned derivatives into a *polA* bacterial host. In contrast, the downstream flanking region is variable. This includes the downstream copy of IS*1* and the downstream replication region (REPII). We infer from these results that sequences in addition to the two flanking copies of IS*1*, in particular the upstream region including REPI, have been instrumental in the preservation and possible spread of aerobactin genes among ColV plasmids and other members of the FI incompatibility group.

The association of colicin V production and increased pathogenicity of *Escherichia coli* strains was noted in 1949 (14), but not until 1979 did it become clear that factors other than colicin V production caused the increase in virulence when *E. coli* was injected into mice (27). ColV plasmids, encoding colicin V, are actually a heterogeneous group of IncFI plasmids which may also encode virulence determinants such as increased serum survival (4, 23, 28), resistance to phagocytosis (25), adherence to intestinal epithelial cells (10), and the aerobactin-mediated iron uptake system (6, 32, 34). In this work, we address the question of how the aerobactin iron uptake system came to be genetically determined on ColV plasmids. Interest in this question was kindled by the recent demonstration of inverted copies of the insertion sequence IS*1* (20, 22, 26) and of two distinct replication regions, REPI and REPII (26), flanking the aerobactin genes in the prototypic ColV plasmid pColV-K30. This, taken together with the discoveries of the same aerobactin iron uptake genes on the chromosomes of *E. coli* K1 (30) and *Shigella* species (20), on *Salmonella* multiple drug resistance plasmids (11), and on a plasmid of *Enterobacter aerogenes* (22), suggests that the aerobactin operon is genetically mobile. However, despite this ubiquity in nature, the extrachromosomal aerobactin system genes have been found on plasmids, which (when the incompatibility group is known) are always IncFI (11, 12, 33). To gain insight into the reasons for these observations, we surveyed the extent of genetic conservation surrounding the aerobactin operon in 12 ColV plasmids and compared the resulting maps to the corresponding aerobactin regions of IncFI *Salmonella* R plasmids.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Pertinent *E. coli* strains and plasmids and their relevant characteristics are listed in Table 1. *E. coli* 3478 and HB101 were used as the recipients in transformation experiments, and *E. coli* C2110 and LE392 were used as the recipients in the in vitro transductions.

Detection of aerobactin siderophore production and outer membrane receptor. Aerobactin production by bacteria harboring ColV plasmids was determined by a bioassay (32). The presence of the aerobactin outer membrane receptor was assayed by the cloacin sensitivity test (26).

Analysis of plasmid DNA. Plasmid DNA preparation, restriction endonuclease digestion, and Southern blot analysis were performed as previously described (26).

Molecular cloning of ColV plasmid DNA. To obtain clones which included flanking sequences as well as aerobactin-specific sequences, ColV plasmid DNA was partially digested; cosmid vector pHC79 or pVK102 DNA was totally digested and added to the partially digested ColV plasmid DNA at an approximate molar ratio of 1:1. Ligated DNA was packaged in vitro by using commercially available packaging systems (Amersham Corp., Boston, Mass., and Promega Biotech, Madison, Wis.), and transduction was performed in accordance with the protocol recommended by Promega.

RESULTS

Distribution of aerobactin genes among ColV plasmids. With the exception of the plasmid pColV-CA7V, the ColV plasmids studied (Table 1) all engender in their bacterial hosts the capacity to produce the siderophore aerobactin. The molecular sizes of these plasmids, determined by gel electrophoresis (data not shown), ranged from 80 kilobase pairs (kbp) (pColV-B188) to 180 kbp (pColV-K229) (Table 2). However, restriction endonuclease digestion of these various ColV plasmids showed that some fragments appear to be

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

Strain (plasmid)	Genotype	Relevant phenotype conferred by plasmid[a]	Source (reference)
HB101	F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	NA	H. Boyer (5)
LE392	F⁻ *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB2 trpR55*	NA	Bethesda Research Laboratories, Inc., Gaithersburg, Md.
C2110	*polA rha his*	NA	Laboratory stock
3478	F⁻ *polA thy*	NA	Laboratory stock (13)
RW193	*entA proC leu trp tsx thi lacY galK ara mtl xyl azi supE44*	NA	J. B. Neilands (31)
LG1315(pColV-K30)	F⁻ *ara endA lac leu mtl proC rpsL supE fhuA thi trpE xyl*	Iu⁺ Cˢ	P. H. Williams (32)
LG1522(pColV-K30, iuc)	*ara azi fepA lac leu mtl proC rpsL supE fhuA tsx thi*	Iu⁻ Cˢ	P. H. Williams (32)
HB101(pHC79)		Apʳ Tcʳ	Laboratory stock
HB101(pVK102)		Kmʳ Tcʳ	E. Nester (19)
HB101(pJHC-V12)		Iu⁺ Cˢ	Laboratory stock (30)
C600(pBRG29)	F⁻ *thi-1 thr-1 leuB6 lacY1 fhuA21 supE44*	Cmʳ Tcʳ Apʳ	Laboratory stock (1, 30)
3478(pJHC-P1)		Iu⁺ Cˢ Apʳ	Laboratory stock (30)
3478(pJHC-P2)		Iu⁻ Cʳ Apʳ	Laboratory stock (30)
3478(pJHC-P36)[b]		Tcʳ Cʳ	Laboratory stock
3478(pJHC-P3)[c]		Cmʳ Cʳ	Laboratory stock (30)
P72(pColV-P72)		Iu⁺ Cˢ	E. Lederberg (Plasmid Reference Center[d])[e]
B188(pColV-B188)		Iu⁺ Cˢ	D. Savage (23)[e]
292(pColV-292)		Iu⁺ Cˢ	E. Lederberg (Plasmid Reference Center)[e]
H247(pColV-H247)		Iu⁺ Cˢ	D. Savage (23)[e]
CA7V(pColV-CA7V)		Iu⁻ Cʳ	E. Lederberg (Plasmid Reference Center)[f]
F70(pColV-F70)		Iu⁺ Cˢ	E. Lederberg (Plasmid Reference Center)[e]
355(pColV-F70)		Iu⁺ Cˢ	D. Savage (23)
F54(pColV-F54)		Iu⁺ Cˢ	E. Lederberg (Plasmid Reference Center)[e]
291(pColV-F54)		Iu⁺ Cˢ	D. Savage (23)
K229(pColV-K229)		Iu⁺ Cˢ	V. Braun (7)
K311(pColV-K311)		Iu⁺ Cˢ	V. Braun (7)
K328(pColV-K328)		Iu⁺ Cˢ	V. Braun (7)
KB2443(pColV3-K30)		Iu⁺ Cˢ	E. Lederberg (Plasmid Reference Center via P. Bergquist)[g]

[a] NA, Not applicable; Iu, iron uptake status; Cˢ and Cʳ, cloacin sensitivity and resistance, respectively.
[b] Strain containing the cloned deleted version of REPI (see the text).
[c] Strain containing the cloned replication region REPII.
[d] Strain was obtained from the former Plasmid Reference Center.
[e] Originally from the collection of H. W. Smith.
[f] Originally from the collection of A. Gratia.
[g] Although originally (21) the plasmid factor from *E. coli* K30 was called ColV3-K30, the name was apparently shortened to ColV-K30 in many collections.

shared (data not shown). On the basis of restriction enzyme patterns, plasmids pColV-K30 and pColV3-K30 appear to be identical; pColV-292, pColV-F70, and pColV-F54 also appear to be identical. To determine the extent of conservation of aerobactin-related and flanking sequences, we used *Hin*dIII and *Hin*dIII-*Bam*HI digests in Southern blot hybridization experiments. In the hybridization experiments using the aerobactin-specific *Hin*dIII-*Bam*HI probe (probe HB) (Fig. 1), a 3.4-kbp *Hin*dIII-*Bam*HI fragment in all aerobactin-positive plasmids appeared universally conserved and is the equivalent of the 3.4-kbp *Hin*dIII-*Bam*HI cloned sequence of pColV-K30 used here as probe HB (Fig. 2a, lanes K through O and Q through S, and Fig. 3a, lanes F through J). This same probe, which is internal to the aerobactin biosynthetic genes (8, 17), also demonstrated that these 3.4-kbp *Hin*dIII-*Bam*HI fragments are contained within *Hin*dIII fragments with molecular sizes ranging from 14.5 to 45 kbp (Fig. 2a, lanes A through I, and Fig. 3a, lanes A through E). Using the probe HB and other restriction enzymes (data not shown), we confirmed that the regions proximal to the siderophore genes were always conserved in aerobactin-positive ColV plasmids. This region includes genes for the four enzymes needed to construct the hydroxamate siderophore aerobactin and the structural genes for the 74,000-molecular-weight (74K) outer membrane receptor protein (8, 17). The variation in *Hin*dIII fragment size must then reflect changes downstream from the aerobactin genes because the upstream region which encodes the aerobactin system is conserved among these plasmids.

Distribution of IS*1* sequences among ColV plasmids. To assess the degree of conservation of the two flanking IS*1* sequences within the various ColV plasmids, we probed the ColV plasmids with an IS*1*-specific probe, pBRG29. This plasmid is a pBR322 derivative containing the transposon Tn*9*, which is bordered by IS*1* elements. Using as a negative

control probe the pBR325 plasmid which, like Tn*9*, has the genes for chloramphenicol resistance but, unlike Tn*9*, has no IS*1* sequences, we deduced that the ColV plasmid sequences which hybridized to pBRG29 but not to pBR325 were specific for IS*1*. The results of the hybridization with the IS*1* probe are shown in Fig. 2b and 3b. All signals seen in these autoradiographs are IS*1* specific with the exception of a small plasmid, also present in strain B188 (15), which shows vector cross-reaction because it is a ColE1-type plasmid (unpublished results). Since neither *Hin*dIII nor *Bam*HI sites were found within the IS*1* sequence, the minimum number of copies of IS*1* could be counted (Table 2). The IS*1*-containing *Hin*dIII fragment upstream from the aerobactin genes is an 8.6-kbp fragment common to all the aerobactin-positive plasmids (Fig. 2b, lanes A through I, and Fig. 3b, lanes A through E). The downstream IS*1* sequences were within *Hin*dIII fragments varying in size from 14.5 to 45 kbp (Fig. 2b and 3b); these were the same fragments which also hybridized to the aerobactin siderophore probe HB. To more precisely map the location of this IS*1* sequence, presumed to be on the right flank of the aerobactin genes as seen in the pColV-K30 map, we used the restriction enzyme *Bst*EII, which splits the IS*1* sequence approximately in half. Using the probe specific for IS*1* and the *Bam*HI-*Eco*RI probe (Fig. 1), we obtained the following results: the size of the *Bst*EII fragment containing both the 74K aerobactin receptor gene and the proximal half of the flanking IS*1* sequence was 3.40 kbp for pColV-K30, pColV3-K30, pColV-292, pColV-F70, pColV-F54, and pColV-K229 but was 3.50 kbp for pColV-H247 and pColV-P72 and 3.47 kbp for pColV-B188, pColV-K311, and pColV-K328 (data not shown). Therefore, although IS*1* sequences flank the aerobactin operon in all aerobactin-positive plasmids, the distance of the downstream IS*1* sequence from the 74K aerobactin receptor protein gene varies slightly. Using the same strategy to map the upstream IS*1* sequence, we found that its location in all the aerobactin-positive plasmids coincided with the map position of pColV-K30 (Fig. 1), within the upstream 8.6-kbp *Hin*dIII fragment (Table 2).

Distribution of replication regions among ColV plasmids. To test whether one or both of the flanking replication regions of ColV-K30 were conserved in the other ColV plasmids, we probed with REPI and REPII. The REPI probe we used, ΔREPI, had the repeated sequence IS*1* deleted (Fig. 1; J. Perez-Casal and J. H. Crosa, submitted for publication). The REPII probe DNA consists of a region beyond the rightward downstream IS*1* and has no overlap with that IS*1* sequence (Fig. 1). With the ΔREPI probe, the pattern of hybridization was uniform among all the aerobactin-producing plasmids; in every case the homologous *Hin*dIII and *Hin*dIII-*Bam*HI fragments within the ΔREPI region were identical to those of pColV-K30. This conservation of sequences is peculiar to the aerobactin-producing plasmids. In the aerobactin-negative plasmid pColV-CA7V (Fig. 2c, lanes F and P), the conserved fragments are only those which are beyond the upstream IS*1* sequence. The outcome of hybridization experiments with the REPII probe was quite different from that with the ΔREPI probe. REPII sequences are absent in the aerobactin-negative plasmid, but they are also absent in five aerobactin-positive plasmids: pColV-H247, pColV-B188, pColV-K311, pColV-K328, and pColV-P72. (In Fig. 2d, lanes C and M, the hybridization signal is due to vector cross-reaction with a smaller plasmid present in strain B188.) REPII-homologous sequences were only seen after prolonged exposure and within a single 4.0-kbp *Hin*dIII-*Bam*HI fragment of pColV-292, pColV-F70, and pColV-F54, (Fig. 2d, lanes N, R, and S). This weak signal suggests that REPII was not present intact or that considerable divergence had occurred. The plasmid pColV-K229 is the only ColV plasmid other than pColV-K30 which appears to have an intact REPII region. This region is also genetically linked to the REPI-IS*1*-aerobactin system-IS*1* region, but it is more closely linked than is the REPII region of pColV-K30.

Functionality of the REPI region. The functionality of the replication region REPI was assayed by using a cloning vector which cannot replicate in a bacterial host lacking DNA polymerase I and transforming these *polA* bacteria

TABLE 2. Distinguishing characteristics of ColV plasmids

Plasmid	Size (kbp)	No. of copies of IS*1* element	Size (kbp) of *Hin*dIII fragment carrying:	
			IS*1* sequence upstream from aerobactin genes	Downstream IS*1* and aerobactin genes
pColV-P72	137	3	8.6	15.5
pColV-B188	80	2	8.6	45
pColV-292	150	4	8.6	14.5
pColV-H247	137	3	8.6	15.5
pColV-CA7V	98	3	15.8[a]	NA[b]
pColV-K30	144	5	8.6	16.3
pColV-F70	150	4	8.6	14.5
pColV-F54	150	4	8.6	14.5
pColV-K229	180	7	8.6	14.5
pColV-K311	130	2	8.6	45
pColV-K328	140	2	8.6	45
pColV3-K30	144	5	8.6	16.3

[a] In pColV-CA7V all three copies of IS*1* are contained within this 15.8-kbp *Hin*dIII fragment, but the copy which is linked to REPI sequences is partially deleted.

[b] NA, Not applicable since pColV-CA7V is genotypically and phenotypically negative for the aerobactin iron uptake system.

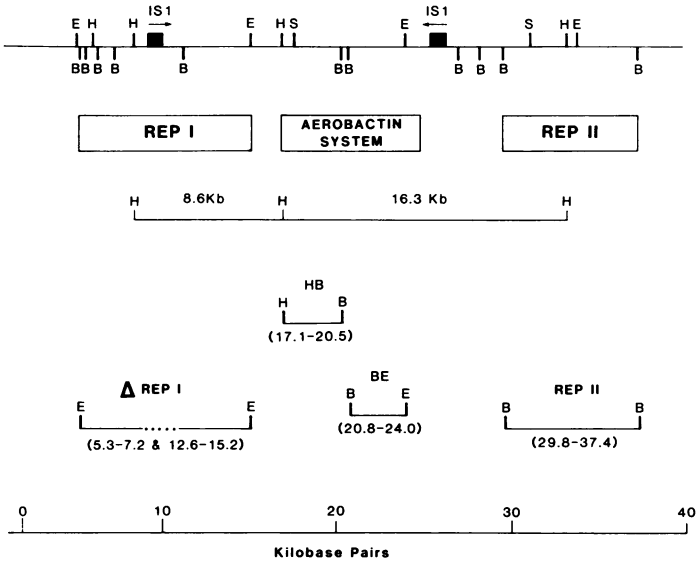

FIG. 1. Partial map of plasmid pColV-K30, indicating fragments used as probes in Southern blot hybridization experiments with ColV plasmids. Map positions of replication regions REPI and REPII, the aerobactin system genes, and the insertion sequence IS*1* are indicated. The cleavage sites for various restriction endonucleases are shown: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; and S, *Sal*I.

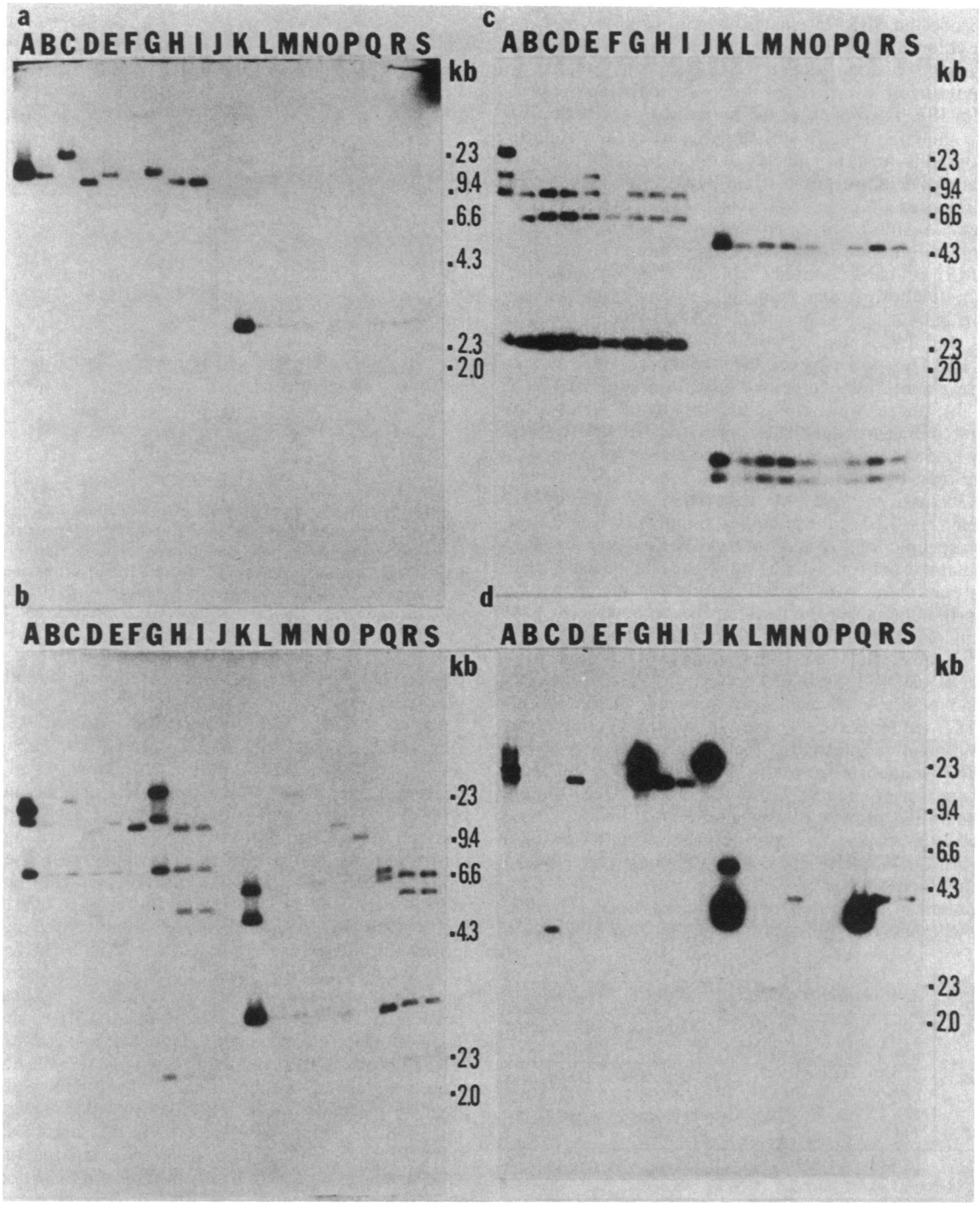

FIG. 2. Hybridization patterns with the following probes: (a) probe HB, which is specific for the aerobactin siderophore genes; (b) pBRG29, which is specific for the insertion sequence IS*1*; (c) pJHC-P36, the ΔREPI probe which is specific for the replication region REPI but with the IS*1* sequence deleted; (d) pJHC-P3, which is specific for replication region REPII. ColV plasmid DNA was digested with *Hin*dIII (lanes A through I) and *Hin*dIII-*Bam*HI (lanes K through S). Lanes: A, pJHC-P1; B, pColV-P72; C, pColV-B188; D, pColV-292; E, pColV-H247; F, pColV-CA7V; G, pColV-K30; H, pColV-F70; I, pColV-F54; J, lambda DNA digested with *Hin*dIII; K, pJHC-P1; L, pColV-P72; M, pColV-B188; N, pColV-292; O, pColV-H247; P, pColV-CA7V; Q, pColV-K30; R, pColV-F70; S, pColV-F54. To demonstrate faintly hybridizing fragments, the exposure in panel d was prolonged such that the two bands (3.4 and 3.6 kbp) in lanes K and Q are indistinguishable. In lanes A and K of panels b, c, and d, the vector-containing fragments of the clone pJHC-P1 (26) show cross-reaction with the cloned probes.

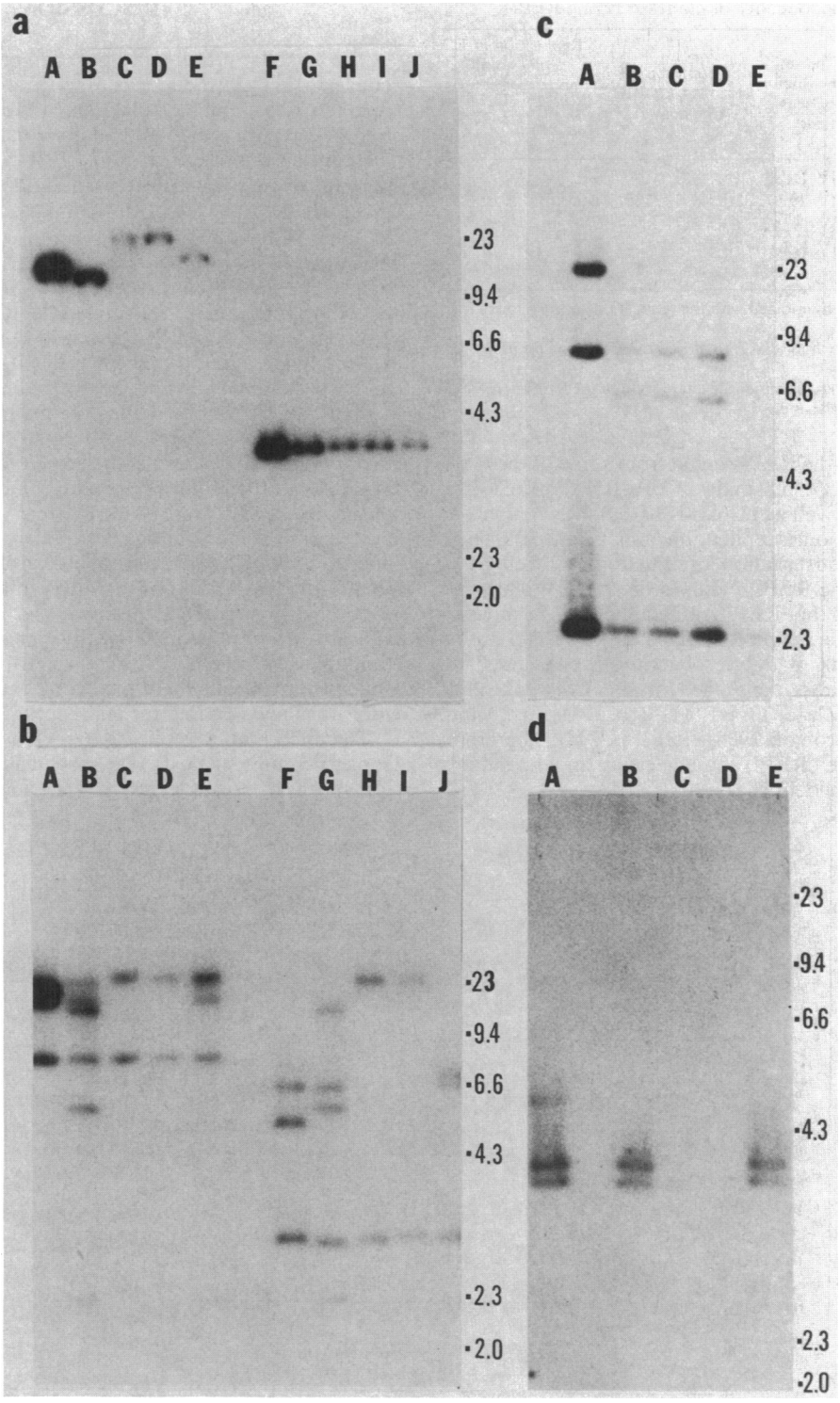

FIG. 3. Hybridization patterns with the following probes (described in the legend to Fig. 2): (a) probe HB; (b) pBRG29; (c) pJHC-P36; (d) pJHC-P3. In panels a and b, ColV plasmid DNA was digested with *Hin*dIII (lanes A through E) and *Hin*dIII-*Bam*HI (lanes F through J). In panel c, ColV plasmid DNA was digested with *Hin*dIII, and in panel d, ColV plasmid DNA was doubly digested with *Hin*dIII and *Bam*HI. Lanes: A, pJHC-P1; B, pColV-K229; C, pColV-K311; D, pColV-K328; E, pColV3-K30; F, pJHC-P1; G, pColV-K229; H, pColV-K311; I, pColV-K328; J, pColV3-K30. In lanes A and F of panels b, c, and d, the vector-containing fragments of the clone pJHC-P1 (26) show cross-reaction with the cloned probes. Fragment sizes in kbp are indicated in the right margins.

TABLE 3. Functionality of clones carrying REPI

Clone	ColV plasmid from which clone was made	Size(s) (kbp) of cloned *Eco*RI fragment(s)	Replication in *E. coli* strain: HB101 (*polA*$^+$)	Replication in *E. coli* strain: C2110 or 3478 (*polA*$^-$)
pJHC-VWB4	pColV-B188	10,[a] 8.6,[b] 10[c]	+	+
pJHC-VW2922	pColV-292	10,[a] 8.6,[b] 13[c]	+	+
pJHC-VWH5	pColV-H247	10,[a] 3.7[c]	+	+
pJHC-P2	pColV-K30	10[a]	+	+
pHC79 (vector)			+	−

[a] *Eco*RI fragment which hybridized with the replication region (cloned from pColV-K30) REPI.

[b] *Eco*RI fragment which hybridized to the aerobactin-specific probe HB (Fig. 3).

[c] *Eco*RI fragments which flank either the left or right of the REPI-aerobactin system unit in the native plasmid.

with clones carrying the REPI region from some of the ColV plasmids. In clones of plasmids pColV-B188, pColV-292, and pColV-H247, as well as pColV-K30 (26), this region was found to be a functional replication unit (Table 3). Thus, there has been a conservation of function, as well as of genetic linkage, of the REPI region in these ColV plasmids. We have determined that REPI is unstable unless accompanied by a maintenance region that maps outside REPI but on the downstream flank of the aerobactin system genes and that this region is also conserved in the ColV plasmids examined (J. Perez-Casal and J. H. Crosa, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, H-103, p. 144). Therefore, those plasmids without REPII sequences are understandably capable of stable replication.

DISCUSSION

The results presented here demonstrate not only the conservation of the genes encoding the aerobactin iron uptake system among ColV plasmids but also the conservation of a region which extends beyond the upstream IS*1* to include the replication region REPI, as originally found on the prototypic pColV-K30 plasmid (26). The functionality of this highly conserved REPI region was established in clones of some of the ColV plasmids and is expected in the other plasmids, based on their identical REPI restriction enzyme maps. The published map of certain *Salmonella* aerobactin-encoding R plasmids (11) similarly shows the restriction site environment of REPI. Note that in the ColV plasmids (Fig. 4) the conservation of sequences begins upstream from REPI and the variability begins downstream from the aerobactin system but before the downstream IS*1* sequence (indicated by dotted lines in Fig. 4 and derived from fine analysis). In the *Salmonella* R plasmids the conservation appears to begin at REPI but then follows the pattern obtained with the ColV plasmids. It therefore appears that the REPI-aerobactin system unit stands out against the two (*Salmonella* plasmid and *E. coli* plasmid) varied backgrounds. We have also found this pattern of conservation in an IncFI, aerobactin-encoding, non-ColV, R plasmid of a clinical *E. coli* strain isolated from a neonate with sepsis and pyelonephritis (unpublished results). The conservation of the upstream sequences appears therefore to be the general rule among IncFI plasmids coding for aerobactin biosynthesis.

The fact that a replication region is part of the large conserved unit suggests that mechanisms other than simple transposition are at work in fostering the ubiquity of the

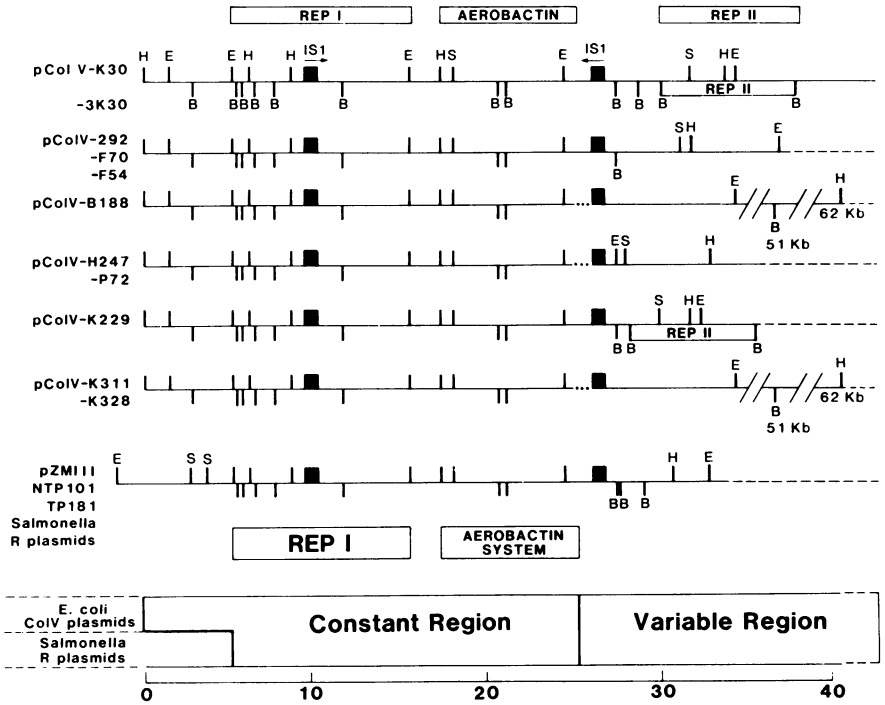

FIG. 4. Mapped regions of ColV plasmids studied and of *Salmonella* drug resistance plasmids pZMIII, NTP101, and TP181 included for comparison (see the text). The genetic regions held in common by all these plasmids and by only the ColV plasmids are delineated in the Constant Region. The genetic regions which vary among the plasmids are delineated in the Variable Region. -----, Unmapped areas; ....., slight variations which have been mapped to show where the Constant Region ends. The cleavage sites for various retriction endonucleases are shown: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; S, *Sal*I. The enzyme site locations which coincide with those of pColV-K30 are indicated without letter designations.

aerobactin genes on the plasmids of enteric bacteria. This proposal is supported by three observations: (i) the size of the IS*1*-bound region is large (16 kbp), giving a low probability of transposition (9); (ii) the orientation of the boundary IS*1* sequences is not the orientation most often associated with IS*1*-mediated transposition, such as the transposition of *E. coli* heat-stable toxin by Tn*1681* (24, 29); and (iii) transposition of the IS*1*-bound aerobactin system genes has not been achieved in the laboratory (unpublished results and S. Payne, personal communication). In support of the transposability of aerobactin genes is the fact that the IS*1*-bound region was found to be in two different orientations among the *Salmonella* R plasmids (11). However, even in these plasmids, flanking sequences beyond the IS*1*-bound region have been conserved, including the restriction enzyme environment of REPI (11; B. Colonna, personal communication). If the IS*1*-bound aerobactin genes were spread by transposition, the flanking genetic environments would be expected to vary. Since such variation is not observed, the inversion event may be less consequential than the preservation of a larger unit containing the aerobactin system genes and REPI sequences. Also, there are other copies of IS*1* in most of the aerobactin-producing ColV plasmids, which may function in the recombination of a larger and possibly more stable aerobactin replication unit (18, 24). This could result in what we call a virulence factor replication unit. A large replicon could undergo deletions, and a deleted segment which includes its own replication region and aerobactin genes could thereby be perpetuated either momentarily or for a longer term. Replicon fusion between independently replicating units could similarly result in the passage of the aerobactin system genes from one replicon to another.

The extrachromosomal aerobactin system genes have thus far only been associated with IncFI plasmids (11, 12, 33), although for plasmid pSMN1 of *E. aerogenes* (22), the incompatibility group is unknown. One important characteristic of most members of the IncFI incompatibility group is the possession of sequences homologous to the secondary replicon of plasmid F present in *Eco*RI fragment f7 (2, 16). These sequences, termed *incE*, are involved in FI-type incompatibility reactions. Detailed mapping and analysis of the REPI region have shown that it carries, in addition to replication-related genes, sequences homologous to *incE* (3; Perez-Casal and Crosa, submitted for publication). The distribution of extrachromosomal aerobactin system genes among IncFI-type plasmids may then simply reflect the fact that these genes are linked to the REPI region.

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