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Plasmid- and Chromosome-Coded Aerobactin Synthesis in Enteric Bacteria: Insertion Sequences Flank Operon in Plasmid-Mediated Systems

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Large plasmids were detected in two aerobactin-producing enteric bacterial species (*Aerobacter aerogenes* 62-I, *Salmonella arizona* SA1, and *S. arizona* SL5301) and designated pSMN1, pSMN2, and pSMN3, respectively. Other *Salmonella* spp., namely, *S. arizona* SL5302, *S. arizona* SLS, *Salmonella austin*, and *Salmonella memphis*, formed aerobactin but contained no detectable large plasmids. *S. arizona* SL5283 made no aerobactin. A probe consisting of the aerobactin biosynthetic genes cloned on plasmid pABN5 hybridized to a *Hin*dIII digest of pSMN1 but not to digests of pSMN2 or pSMN3. A larger probe, the insert of pABN1 containing the complete aerobactin operon, hybridized to four fragments in *Hin*dIII digests of the parent plasmid, pColV-K30. A 2.0-kilobase *Pvu*II fragment responsible for this multiple-hybridization pattern was cloned into vector pUC9 to form pSMN30. The latter was mapped and shown to correspond to either IS*1* or to a closely related insertion sequence.

Iron is essential for the growth of most, if not all, living cells. However, in an aerobic environment at biological pH, iron is quantitatively insoluble or is bound by host iron-binding proteins such as lactoferrin in secretions and transferrin in serum (17). To overcome this iron limitation, bacteria commonly take up iron via high-affinity systems which are comprised of specific low-molecular-weight iron-binding compounds, termed siderophores, and their cognate membrane receptors. The siderophores and receptors, which may be coordinately regulated (12, 16), are induced by iron starvation (17). Many gram-negative bacteria, including *Escherichia coli*, synthesize the catechol type of siderophore, enterobactin (also called enterochelin). Aerobactin, a hydroxymate type of siderophore, was first isolated from *Aerobacter aerogenes* (8). *Shigella flexneri* (22) and *Enterobacter cloacae* (30) also synthesize aerobactin. Aerobactin, the siderophore coded for by the ColV plasmid in *E. coli*, is at least partially responsible for the invasiveness of this strain (29, 33, 34).

We elected to survey a range of aerobactin-forming bacterial species on the premise that at least some of them would program the siderophore from a plasmid. Obviously, a plasmid significantly smaller than the ca.-70-megadalton (Mdal) pColV-K30 would facilitate its genetic analysis. However, among the several species screened in the present work, only the original source of aerobactin, *A. aerogenes* 62-I, was found to encode the system on a plasmid. The latter, designated pSMN1, is a 130-Mdal plasmid.

At this stage of our work, the aerobactin DNA of pColV-K30 became available, as cloned on the small, multicopy plasmids pABN5 and pABN1 (4). These contain, respectively, the biosynthetic and the biosynthetic-plus-transport genes for aerobactin. By means of the differential hybridization patterns of pABN5 and the somewhat larger pABN1 with *Hin*dIII-digested pColV, we obtained evidence for insertional sequences flanking the aerobactin gene complex. A repeated DNA sequence surrounding the aerobactin genes was cloned, mapped, and identified as an IS*1* element.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used in this study (Table 1) were maintained on Luria broth. Iron-limited Tris-buffered medium (26) was supplemented with sodium succinate (10 g/liter), thiamine (2.5 μg/ml), and the appropriate amino acids (20 μg/ml). Vogel-Bonner minimal medium E (32) was used for the enterobactin bioassays (13). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside indicator plates were Luria broth plates supplemented with 40 μg of the substrate per ml, 1 mM isopropyl-β-D-thiogalactopyranoside, and 30 μg of ampicillin per ml.

Bioassays. The aerobactin indicator strain, LG1522, was overlaid in plain top agar at 3×10^7 cells per plate on a Tris-succinate agar base. The strains to be tested were streaked onto the overlayer, and the plates were incubated overnight at 37°C. Growth zones of the indicator strain around the test strain indicated the ability of the test strain to produce aerobactin. The *enb-7* bioassay for enterobactin followed the same procedure as above, except that medium E plates were used.

Chemical tests for siderophore synthesis. The test for catechols by the method of Arnow (1) and the $Fe(ClO_4)_3$ assay for hydroxymates (2) have been previously described.

Plasmid curing. A stationary-phase culture (3×10^7 cells) of *A. aerogenes* 62-I grown in Luria broth was subcultured into 2 ml of Luria broth containing 300 to 500 μg of novobiocin per ml (15). The highest concentration of the drug which still allowed bacterial growth was 300 to 500 μg/ml. The cultures were grown overnight at 37°C in the dark, diluted, plated on Luria broth agar, and incubated overnight at 37°C. The resulting colonies were screened for aerobactin production by both bioassay and ferric perchlorate tests. The loss of plasmid DNA was detected by screening the cultures on agarose gels and by colony hybridization with ^{32}P-labeled pSMN1 as the probe.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or relevant features	Source[a]
E. coli		
AN194	*leu proC trp thi tonA*	I. G. Young
JM105	Δ(*pro-lac*) *thi rpsL hsdR4 endA sbcB* F′ *traD36 proA⁺B⁺ lacI*^{q} Δ*lacZM15*	E. E. Penhoet
LG1315	*ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl* ColV-K30	P. H. Williams
LG1522	*ara azi fepA403 lac leu mtl proC rpsL supE tonA tsx thi* ColV-K30 *iuc*	P. H. Williams
A. aerogenes 62-I	*phe trp tyr*	F. Gibson
S. typhimurium		
TA2443	*enb-7*	Laboratory stock (23)
S. austin	wild type	J. Garibaldi
S. memphis	wild type	J. Garibaldi
S. arizona		
SA1		J. Garibaldi
SLS, SL5301, SL5302, and SL5283		B. A. D. Stocker
Plasmids		
pABN1	Carries the aerobactin biosynthesis and transport genes	(4)
pABN5	Carries the aerobactin biosynthesis genes	(4)
pUC9	Ap^{r} *lacZ* insertion vector[b]	Bethesda Research Laboratories (31)
pSMN30	pUC9 derivative carrying a 2.0-kb *Pvu*II insert from pABN1	This study
pSMN1	From *A. aerogenes* 62-I	This study
pSMN2	From *S. arizona* SA1	This study
pSMN3	From *S. arizona* SL5301	This study

[a] I. G. Young and F. Gibson, Australian National University, Canberra, Australia; E. E. Penhoet and J. A. Garibaldi, this department; P. H. Williams, University of Leicester, Leicester, England; B. A. D. Stocker, Stanford University, Palo Alto, Calif.
[b] Ap, Ampicillin.

Plasmid DNA screens and isolation. The rapid plasmid-screening method of Portnoy et al. (24) was used to screen for high-molecular-weight plasmids. Recombinant DNA plasmids were detected by a minilysate procedure (10). The method of Hansen and Olsen (9) was used for large-scale plasmid DNA isolation. The DNA was further purified by two centrifugations in cesium chloride-ethidium bromide density gradients at 65,000 rpm for 5 h or at 45,000 rpm for 14 h at 15°C in the Sorvall TV865 rotor.

Plasmids and their restriction products were subjected to agarose gel electrophoreses (0.7 and 1%) in a Tris-borate buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA).

Restriction endonuclease digestions. The conditions recommended by the supplier (Bethesda Research Laboratories) were followed for enzyme digestions. For DNA segments smaller than 1 kilobase (kb), the fragment was end labeled with [α-^{32}P]dNTP (ICN Pharmaceuticals, Inc.) by using the T4 DNA polymerase large fragment (New England Biolabs), digested with a restriction endonuclease, and separated by electrophoresis in 4 to 8% polyacrylamide gels (14). Plasmid DNA was mapped by single and double digestions followed by agarose gel (1%) electrophoresis in Tris-borate buffer.

To isolate 2- to 17-kb DNA fragments, the DNA was eluted onto Whatman DE81 DEAE-cellulose paper and recovered by the method of Dretzen et al. (7). DNA fragments isolated in this way were used in ligation or nick-translation reactions.

Transfer of DNA to nitrocellulose and DNA-DNA hybridization conditions. Restriction endonuclease-cleaved plasmid DNA was transferred to nitrocellulose paper by the method of Southern (28). Radioactive DNA probes were obtained by the nick translation of purified DNA restriction fragments with [α-^{32}P]dCTP in the usual way (25). The hybridization was carried out in 50% formamide at 42°C, washed for 2 to 4 h at 65°C in 5× SSC (SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate with three buffer changes, and autoradiographed as previously described (24).

Cloning procedures. The cloning vector used was pUC9 (Bethesda Research Laboratories) which has ampicillin resistance as a selectable marker and *lac* as an insertional detection marker. Restriction endonuclease-digested DNA was mixed and ligated with T4 DNA ligase (Bethesda Research Laboratories). Transformed *E. coli* JM105 were selected by ampicillin resistance on 5-bromo-4-chloro-3-indolyl-β-D-galactoside plates. Plasmid DNA was isolated from white colonies and analyzed for the appropriate insertion.

RESULTS

Siderophore production. The *A. aerogenes* and *Salmonella* spp. strains were screened for their ability to produce

TABLE 2. Siderophore production by enteric bacterial strains

Strain	Production of siderophore[a]	
	Aerobactin	Enterobactin
E. coli LG1315	+	−
A. aerogenes 62-I	+	+
S. arizona spp.[b]	+	+
S. austin	+	−
S. memphis	+	−

[a] Production was measured by bioassay and chemical tests. + Produced; −, not produced.
[b] Strain SL5283 (Table 1) did not produce aerobactin.

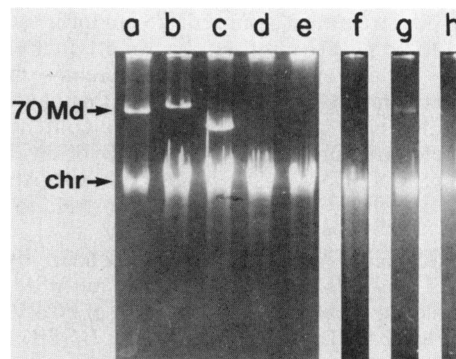

FIG. 1. Agarose gel electrophoretic profiles of plasmid DNA. Lane a, *E. coli* LG1315; lane b, *A. aerogenes* 62-I; lane c, *S. arizona* SA1; lane d, *S. austin*; lane e, *S. memphis*; lane f, *S. arizona* SLS; lane g, *S. arizona* SL5301; lane h, *S. arizona* SL5302. Arrow marked 70 Md indicates pColV-K30; chr indicates chromosomal DNA.

TABLE 3. Effect of pSMN1 curing on aerobactin production[a]

A. aerogenes derivatives	Presence of pSMN1	Aerobactin production
62-I	+	+
AA134	−	−
AA716	−	−
AA736	−	−
AA122	+	+
AA130	+	+
AA142	+	+
AA544	+	+

[a] The derivatives were obtained after treating *A. aerogenes* with novobiocin. Of 528 strains tested, 3 were negative for hydroxymate production. The bottom four derivatives were random hydroxymate-positive clones. +, Presence of pSMN1 or production of aerobactin; −, absence of pSMN1 or no production of aerobactin.

aerobactin and enterobactin (Table 2). One of five strains of *S. arizona* tested did not produce aerobactin. This strain, SL5283, was shown by hybridization studies (S. McDougall, Ph.D. thesis, University of California, Berkeley, 1984) not to carry the aerobactin biosynthesis genes in its genome. The *S. austin* and *S. memphis* strains do not synthesize or utilize enterobactin, which is thought to be unusual for a *Salmonella* species. However, a probing experiment with a plasmid carrying three enterobactin genes indicates that at least part of the enterobactin gene complex is present in these bacteria (unpublished data).

Plasmid content. *A. aerogenes* 62-I, *S. austin*, *S. memphis*, and the four *S. arizona* spp. which were positive for aerobactin were analyzed for their plasmid content by the rapid alkaline lysis procedure. Agarose gel electrophoresis profiles of the resulting plasmid preparations are shown in Fig. 1. The diffuse bands seen in all lanes are chromosomal DNA which was not completely removed in the miniscreen. A large plasmid of about 130 Mdal, pSMN1, was observed in *A. aerogenes* 62-I. The plasmid content of the *S. arizona* strains varied with the strain. SA1 has a plasmid of about 45 Mdal (pSMN2), and strain SL5301 has a plasmid of about 70 Mdal (pSMN3). Strains SL5302 and SLS did not contain visible plasmid DNA bands. Plasmid DNA was not observed in *S. austin* or *S. memphis*.

Correlation between the aerobactin genes and high-molecular-weight plasmids. Curing of the *A. aerogenes* plasmid, pSMN1, was carried out by growing the culture in novobiocin. Novobiocin treatment afforded 528 colonies, of which 3 were found to be negative for aerobactin production. These three strains along with four control colonies that were subjected to the novobiocin treatment but that gave positive bioassay results were analyzed for plasmid content both by agarose gel electrophoresis and by colony hybridization with ^{32}P-labeled pSMN1 DNA as the probe (Table 3). Absence of the plasmid correlated with inability of the strain to produce aerobactin.

These results led us to isolate and characterize by restriction analysis plasmids pSMN1, pSMN2, and pSMN3. The *Hin*dIII profiles of these three plasmids and of pColV-K30 are shown in Fig. 2A. To determine which *Hin*dIII band of pSMN1 carried the aerobactin genes and to determine whether pSMN2 and pSMN3 carried the aerobactin genes, we probed the plasmids with the cloned *E. coli* pColV-K30 aerobactin genes. The probe, the 6.8-kb *Hin*dIII-*Eco*RI insert of pABN5, codes for the aerobactin biosynthesis genes and has been previously described (4). The results are in Fig. 2B. The *S. arizona* plasmids pSMN2 and pSMN3 did not show any hybridization with the aerobactin gene probe. The same result was obtained when the hybridization was carried out under low-stringency conditions (unpublished data). The probe did hybridize to a 16-kb *Hin*dIII band of the *A. aerogenes* plasmid, pSMN1. These results agree with the curing experiments for this strain.

Analysis of the aerobactin gene cluster flanking DNA in pColV. During the course of Southern blot hybridization studies with pABN1, which carries a 16.3-kb *Hin*dIII insert coding for both the aerobactin biosynthesis and transport genes (Fig. 3) (4), it was observed that the insert hybridized to not only the 16.3-kb *Hin*dIII fragment of pColV, from which it originated, but also to three additional *Hin*dIII fragments of that plasmid. This pattern was not observed with the smaller clone in pABN5 when used as a probe. Since pABN1 contains 7.9 kb of DNA previously not thought to be associated with the aerobactin genes, this segment on the right side of the operon was studied further. Figure 3 shows the four fragments which were isolated from pABN1, one of which carries the 74-kilodalton (K) aerobactin receptor gene, and which were used to probe *Hin*dIII-digested pColV. The autoradiogram in Fig. 4 showed that the 2.0-kb *Pvu*II fragment was solely responsible for the additional hybridization pattern. The faint signal seen for the 2.5-kb band is probably due to less DNA in that band, relative to the higher-molecular-weight bands, and little homology with the probe. In addition, this 2.0-kb *Pvu*II fragment hybridized to five bands in *Hin*dIII-digested total DNA isolated from *E. coli* AN194 (unpublished data). This afforded the first experimental evidence for an insertion sequence associated with the aerobactin operon.

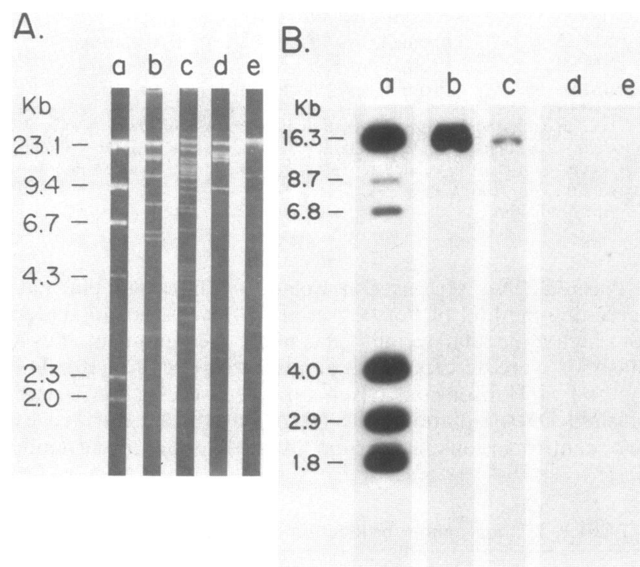

FIG. 2. (A) *Hin*dIII restriction endonuclease profiles of plasmid DNA. Lane a, *Hin*dIII-digested lambda DNA which are molecular weight markers; lane b, pColV; lane c, pSMN1; lane d, pSMN2; lane e, pSMN3. (B) Autoradiograph of *Hin*dIII-digested plasmid DNA hybridized with the ^{32}P-labeled, 6.8-kb *Hin*dIII-*Eco*RI insert of pABN5. Lane a, molecular weight markers consisting of a mixture of restriction endonuclease fragments of pABN1 which hybridized with the probe; lane b, pColV; lane c, pSMN1; lane d, pSMN2; lane e, pSMN3.

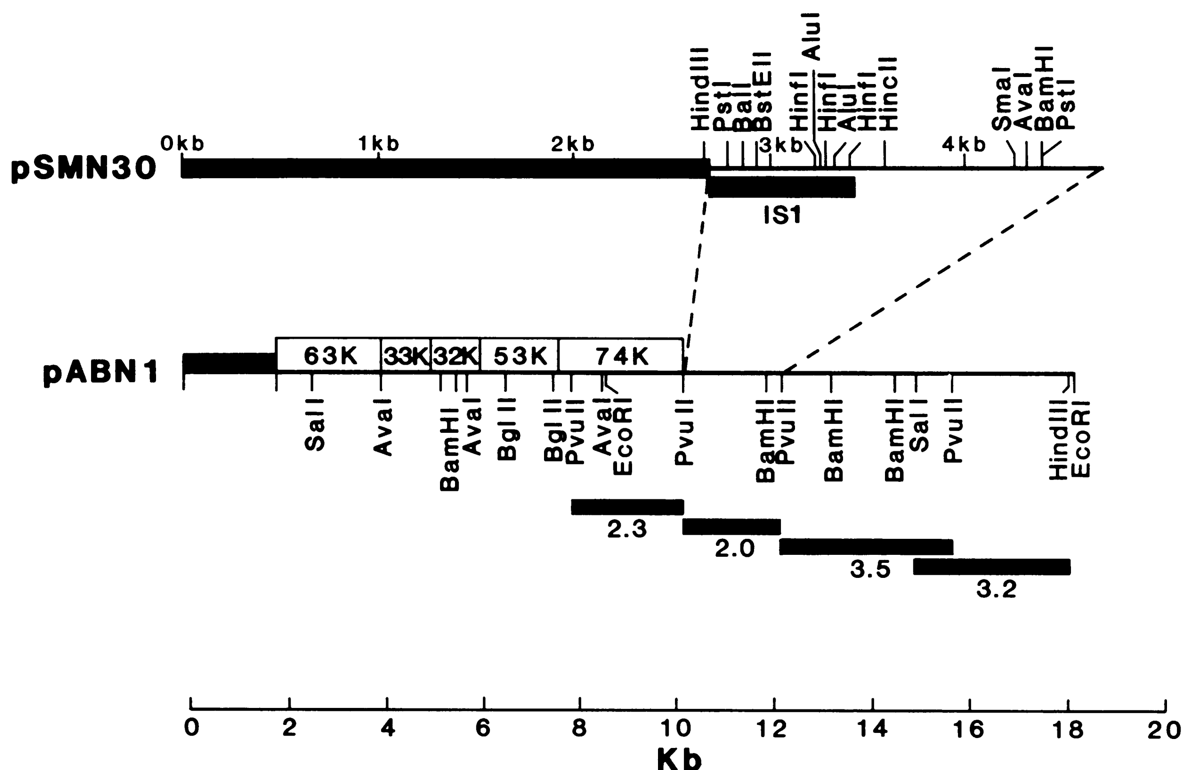

FIG. 3. Restriction maps of pABN1 and pSMN30. The solid lines indicate the vector sequences, pPlac for pABN1 and pUC9 for pSMN30. The location of the insertion sequence IS*1* (21) in pSMN30 is indicated by the heavy line below pSMN30. The approximate locations of the proteins encoded by pABN1 are shown. The 2.3-kb *Pvu*II-*Pvu*II, 2.0-kb *Pvu*II-*Pvu*II, 3.5-kb *Pvu*II-*Pvu*II, and 3.2-kb *Sal*I-*Hin*dIII fragments derived from pABN1 are indicated by the heavy lines below pABN1.

If the aerobactin genes were flanked by insertion sequences, we would expect to find a second similar sequence in close proximity to the left side of the aserobactin gene cluster. A map of the aerobactin genes and the surrounding DNA is given in Fig. 5. Through a series of Southern blot hybridizations, with *Ava*I-, *Bam*HI-, *Eco*RI-, *Hin*dIII- and *Sal*I-digested pColV-K30 DNA and with the 2.0-kb fragment used as the probe, the latter was found to hybridize to the 3.4-kb *Hin*dIII-*Bam*HI fragment located 5.3 kb to the left of the transcriptional start site of the aerobactin genes (A. Bindereif and J. B. Neilands, unpublished data). The total DNA between the left *Hin*dIII-*Bam*HI fragment and the right *Pvu*II fragment is 13.3 kb.

Cloning and characterization of the 2.0-kb *Pvu*II fragment. For further analysis of the 2.0-kb *Pvu*II fragment, it was cloned into the insertion vector pUC9. A restriction map of the resulting plasmid, pSMN30, is given in Fig. 3. Comparison of this map to the map of IS*1* (21) allowed the preliminary identification of this sequence of DNA as IS*1* or a closely related derivative. The majority of the 768-base-pair sequence is contained on the *Hin*dIII-*Hin*cII fragment of pSMN30 (Fig. 3). Approximately 80 base pairs map to the left of the *Pvu*II site which is next to the 74K receptor gene.

Presence of the flanking sequences of pColV in other aerobactin-producing bacteria. To determine whether the pColV aerobactin-gene-flanking sequence was present in other aerobactin-producing bacteria, the pSMN30 insert was used as a probe, along with the 2.3-kb receptor fragment as a control. Figure 6 demonstrates that the 2.0-kb flanking fragment of pColVK30 is present on the 16-kb *Hin*dIII fragment of pSMN1 which also carries the aerobactin genes. The probe did not hybridize to total DNA isolated from *S. austin* and *S. memphis*, whereas the control probe (the 2.3-kb fragment coding for part of the 74K receptor) did hybridize (data not shown). These results agree with the general distribution pattern of IS*1* in enteric bacteria (19).

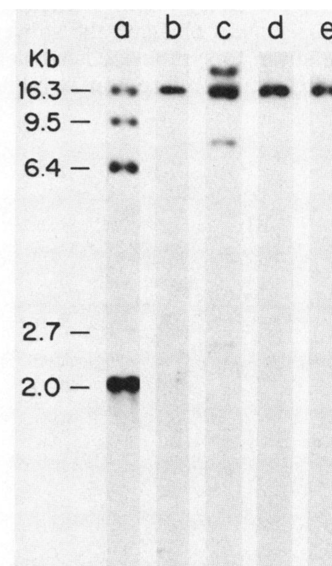

FIG. 4. Autoradiograph of *Hin*dIII-digested pColV hybridized with the ^{32}P-labeled probes diagrammed in Fig. 3. Lane a, molecular weight markers consisting of a mixture of restriction endonuclease fragments of pABN1 which hybridized with the probe; lane b, 2.3-kb *Pvu*II-*Pvu*II probe; lane c, 2.0-kb *Pvu*II-*Pvu*II probe; lane d, 3.5-kb *Pvu*II-*Pvu*II probe; lane e, 3.2-kb *Sal*I-*Hin*dIII probe.

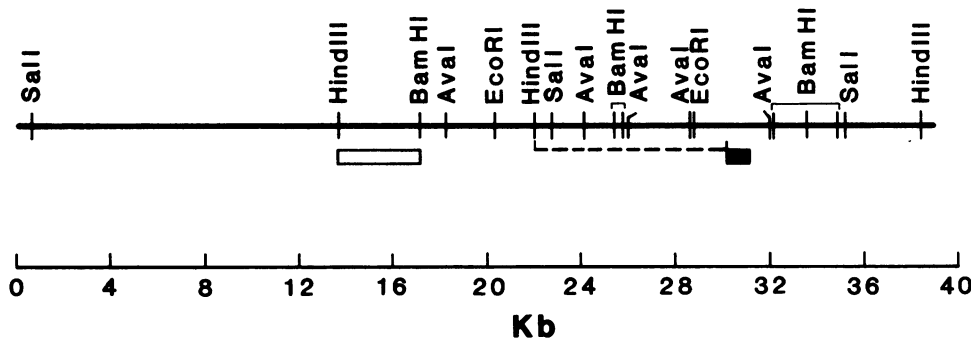

FIG. 5. Restriction map of the aerobactin genes of ColV-K30 and flanking region. The dashed line indicates the aerobactin genes. The right two *Hin*dIII sites correspond to the insert of pABN1. The closed box represents the right insertion sequence, and the open box indicates the left sequence. The *Ava*I sites are not completely mapped. The left *Hin*dIII site is from reference 5.

DISCUSSION

When this study was initiated, *E. coli* pColV-K30 represented the sole example of an aerobactin-mediated iron assimilation system confirmed to be plasmid encoded. Although other aerobactin-producing bacteria often harbor high-molecular-weight plasmids, the results of the present survey indicate that the aerobactin genes may be both chromosome coded and plasmid coded.

Large plasmids were found in two of the four aerobactin-producing strains of *S. arizona*. Hybridization studies show that the genes are not located on these plasmids. No plasmids were detected in the *S. austin* and *S. memphis* strains. In all six *Salmonella* species examined by us, the aerobactin genes were found to be associated with the chromosome. This result differs from an earlier hypothesis by our laboratory that aerobactin synthesis might be plasmid mediated in all cases (3).

However, for *A. aerogenes* 62-I a large plasmid, pSMN1, was isolated and shown by hybridization studies to carry the aerobactin genes on a 16-kb *Hin*dIII fragment. Curing of pSMN1 from *A. aerogenes* 62-I and the subsequent loss of the ability of the strain to synthesize aerobactin confirmed these results. Therefore, there are now two known cases of plasmid-mediated aerobactin production, with pColV and pSMN1.

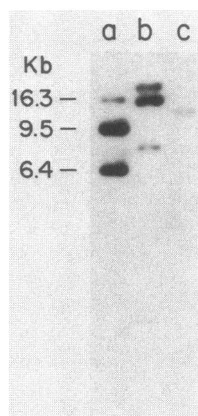

FIG. 6. Autoradiograph of *Hin*dIII-digested plasmid DNA hybridized with the ^{32}P-labeled, 2.0-kb *Pvu*II-*Pvu*II fragment of pABN1. Lane a, molecular weight markers consisting of a mixture of restriction endonuclease fragments of pABN1 which hybridized with the probe; lane b, pColV; lane c, pSMN1.

In recent years, plasmid-mediated determinants, aside from antibiotic resistance, have been found to be on transposons. These include heat-stable enterotoxin (27) and utilization of lactose (6) and citrate (11). In support of an earlier speculation (18), we now report similar results for the plasmid-mediated aerobactin gene complex.

An insertion sequence has been cloned, mapped, and identified from the ColV-K30 plasmid. This sequence is located directly adjacent to the 74K receptor DNA, which is the last known gene on the right side of the aerobactin operon. This sequence has also been located on a 3.9-kb *Hin*dIII-*Bam*HI fragment which is 5.3 kb to the left of the transcriptional start site. The total amount of DNA carried between these two sequences is 13.3 kb. There are probably two more copies of the insertion sequence or closely related sequences on the ColV plasmid. Comparison of the restriction map of the sequences with those of known insertion sequences has identified the right sequence as IS*1* or a closely related sequence, IS*1D*, which is usually found in *Shigella* spp. (20). Further characterization of the left repeated sequence will be done to determine its exact location and orientation relative to the right IS*1*. The latter insertion sequence has also been found on the 16-kb *Hin*dIII fragment of pSMN1 and will be mapped relative to the aerobactin genes in this plasmid. The sequence is not found in *S. austin* or *S. memphis*. Shelley M. Payne (personal communication) has found the same sequence associated with the aerobactin genes in *Shigella flexneri*. Whether the IS*1* actually has a role in the mobility of the aerobactin gene cluster has not yet been demonstrated, but it seems reasonable to postulate that IS*1* may have such a function.

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