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A Cluster of Five Genes Specifying the Aerobactin Iron Uptake System of Plasmid ColV-K30

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The genetic determinants for the aerobactin iron uptake system of plasmid ColV-K30, cloned as recombinant plasmid pABN1, were mapped by insertional inactivation using Tn*1000* (γδ). Sites of insertion resulting in loss of aerobactin biosynthesis spanned ca. 5.5 kilobase pairs of cloned ColV-K30 DNA contiguous with a 2-kilobase-pair region in which transposon insertion resulted in loss of the outer membrane ferric-aerobactin receptor protein. Translation products of plasmid pABN1, and of subclones specifying siderophore biosynthesis alone or receptor activity alone, were analyzed by using the maxicell and minicell expression system. Four polypeptides (M_r = 62,000, 35,000, 45,000, and 50,000) are required for biosynthesis of aerobactin. A fifth product (M_r = 74,000) of plasmid pABN1 represents the outer membrane receptor protein. The linear order of genes for these polypeptides was determined by comparing translation products of a series of smaller derivative plasmids and of a number of mutant plasmids carrying Tn*1000* at known locations.

Plasmids specifying the antibacterial protein colicin V (ColV plasmids) were reported several years ago to be associated with isolates of *Escherichia coli* from bacteremia in humans and domestic animals (20, 21). These plasmids significantly enhanced the virulence of host bacterial strains in experimental infections of several laboratory and domestic animals (21). Several phenotypic properties of ColV plasmids, including epithelial adherence (7) and serum resistance (3), as well as direct effects of colicin V itself (14, 15), have been proposed to contribute to pathogenesis of plasmid-bearing strains. Of major importance, however, are the determinants of a specific high-affinity iron uptake mechanism which confers selective advantage on host bacteria in conditions of iron limitation (22, 26, 27), such as are encountered in the tissues and fluids of infected animals (25). The system has two components, the hydroxamate siderophore aerobactin (4, 24) and an inducible outer membrane protein which is the receptor for ferric-aerobactin (1, 10). A *Hin*dIII restriction fragment which encodes the entire aerobactin system has been cloned from the prototype ColV plasmid ColV-K30 into a high-copy-number vector plasmid, and a preliminary restriction map of the resultant recombinant plasmid, pABN1, was published previously (2). In this paper we report the localization of the region of plasmid pABN1 encoding the aerobactin system by insertional inactivation using Tn*1000* (γδ, see reference 11), and we present a polypeptide map based on the use of maxicell and minicell expression systems to determine translation products.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are shown in Table 1. Plasmid pABN1 (2) was kindly provided by J. B. Neilands and A. Bindereif. The following were derived from pABN1 (Fig. 1): a plasmid comprising the 8.6-kilobase pair (kb) *Eco*RI fragment of pABN1 is identical to the previously described subclone pABN5 (2); plasmids pLG141 and pLG142 consist of the 6.5-kb *Bam*HI fragment of pABN1 cloned in vector pACYC184 (6) in opposite orientations; plasmid pLG152 is the 9.7-kb *Eco*RI fragment of pABN1 in pACYC184; plasmids pLG153, pLG154, pLG155, pLG156, pLG157, and pLG158 are subclones derived from Tn*1000* insertion mutants of pABN1 as described in Fig. 1 and below.

Culture media. Strains were routinely cultured in nutrient broth or M9 minimal salts medium (18) supplemented with glucose (0.4% [wt/vol]), thiamine (50 μg/ml), and required amino acids, usually as Casamino Acids (0.5% [wt/vol]). The iron chelating agent α,α'-dipyridyl (200 μM) was added to M9 medium to induce siderophore and receptor synthesis. Antibiotics (either ampicillin [25 μg/ml] or chloramphenicol [20 μg/ml]) were added as appropriate to maintain the plasmids.

Biological assay for siderophore production. Lawns of the bacterial strain LG1522 (ca. 10^7 bacteria per plate) were made on appropriately supplemented M9 minimal agar containing α,α'-dipyridyl. Strains to be tested were inoculated onto the lawns (15 to 20 per plate) and incubated at 37°C overnight. Aerobactin production was detected as a halo of growth of the indicator strain around an inoculum.

Biological assay for receptor activity. The ferric-aerobactin receptor is also the receptor for the *Enterobacter cloacae* bacteriocin, cloacin DF13 (1, 23). Therefore, strains of *E. coli*, which is naturally insensitive to the cloacin, are rendered sensitive by the presence of ColV plasmids that specify the aerobactin system. Strains to be tested were grown in M9 minimal salts medium containing α,α'-dipyridyl and spread as lawns onto nutrient agar. A crude preparation of cloacin DF13 (10 to 20 μl) was spotted onto each lawn and incubated at 37°C overnight. Strains expressing the outer membrane receptor protein were indicated by a clear killing zone in the lawn. Cloacin was prepared by adding mitomycin C (0.2 μg/ml) to a growing nutrient broth culture (cell density, 10^9 cells per ml) of *Enterobacter cloacae* N4049 and incubating with aeration at 37°C overnight. Cells were pelleted by centrifugation, and the supernatant fluid, containing cloacin DF13 activity, was sterilized by membrane filtration.

Plasmid preparation. Plasmid DNA for restriction enyzme analysis was prepared by the procedure of Klein et al. (12). Cells were harvested from overnight cultures (10 ml) and lysed with lysozyme and phenol-chloroform in a final vol-

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TABLE 1. Bacterial strains

Microorganism and strain	Characteristics	Source (reference)
Escherichia coli K-12		
AN263	*ara fepA lac leu mtl proC rpsL supE thi tonA trpE xyl*	I. G. Young
LG1522	As AN263, carrying plasmid ColV-K30 *iuc*	This paper; (27)
RB308	F^+ *deoC lacY thyA*	R. Buxton
GB1	*cir his lys rpsL trp*	B. Wilkins
CSH26 ΔF6	*ara* Δ(*recA srl*)F6 Δ(*lac pro*) *rpsL thi*	P. Oliver
DS410	*ara azi lacY* λ^- *malA minA minB rpsL* sup^0 *tonA*	(17)
Enterobacter cloacae N4049	Carries plasmid CloDF13	E. Veltkamp

ume of 0.5 ml. Phenol was extracted from the aqueous phase with chloroform-isoamyl alcohol (24:1), and plasmid DNA was recovered by precipitation from ethanol.

Restriction enzyme analysis and cloning procedures. Plasmid DNA was restricted by using the enzymes *Eco*RI, *Hin*dIII, *Bam*HI, and *Sal*I (Bethesda Research Laboratories, Inc.), and restriction fragments were analyzed by electrophoresis through agarose gels (0.7 to 1.0%) with *Hin*dIII-generated fragments of phage λ DNA as size markers. Ligation with T4 DNA ligase (New England Biolabs) was carried out at 4°C for 16 h in the presence of 1 mM ATP. Transformation was performed according to the method of Cohen et al. (8); transformants were selected on nutrient agar supplemented with appropriate antibiotics.

Preparation and labeling of maxicells and minicells and analysis of plasmid-specified polypeptides. Maxicells were prepared from plasmid-containing derivatives of the strain CSH26 ΔF6 according to the procedure of Sancar et al. (19). Logarithmically growing cells (2 ml) were UV-irradiated and incubated overnight at 37°C in the dark with cycloserine (100 μg/ml) to kill survivors. Cells were washed, suspended in M9 medium containing α,α′-dipyridyl, and labeled with [^{35}S]methionine for 1 h at 37°C, followed by 10 min of incubation with excess unlabeled methionine. Minicells were prepared from strain DS410 and labeled according to the method of Reeve (17). Cells from a stationary culture (400 ml) were pelleted and then fractionated on a 10 to 30% sucrose density gradient; the minicell band was repurified on a second gradient, washed, suspended in minimal medium containing α,α′-dipyridyl, and labeled with [^{35}S]methionine for 1 h at 37°C, followed by 10 min of incubation with excess unlabeled methionine. Labeled polypeptides from maxicell and minicell expression systems were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 11 or 13% gels and by autoradiography or fluorography. Labeled molecular size marker proteins were myosin (200,000 daltons), phosphorylase *A* (100,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

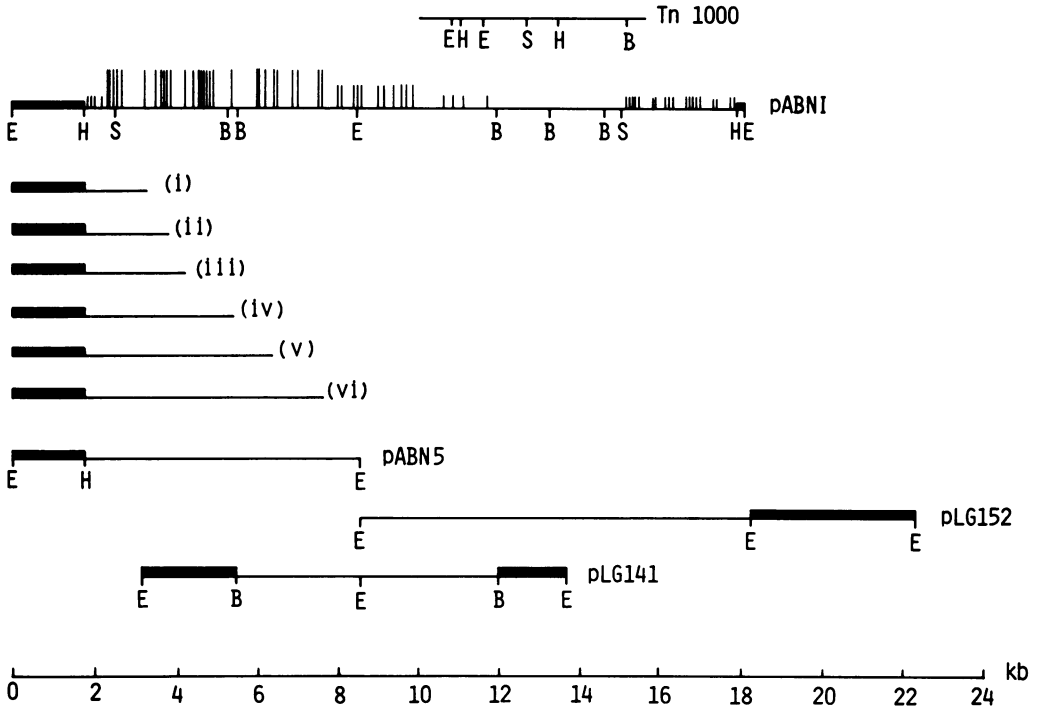

FIG. 1. Physical and genetic map of pABN1 and of derived subclones. Restriction sites for the enzymes *Eco*RI (E), *Hin*dIII (H), *Bam*HI (B), and *Sal*I (S) are indicated for pABN1 and Tn*1000*. Long stalks on pABN1 indicate insertions of Tn*1000* that inactivated aerobactin production, whereas the intermediate-length stalks represent insertions which result in cloacin resistance (i.e., inactivation of receptor activity). Short stalks indicate insertions which leave the whole iron uptake system intact. Maps i through vi describe plasmids pLG153 through pLG158, respectively, which were derived (as described in the text) from pABN1::Tn*1000* mutants defective in aerobactin production; the section of transposon included in these plasmids has been omitted from the diagrams. Plasmids pABN5, pLG152, and pLG141 are subclones of pABN1. The vector (represented by thick lines) in plasmids pABN1 and pABN5 and derivatives pLG153 through pLG158 is pPlac (2); that in plasmids pLG152 and pLG141 is pACYC184 (6).

RESULTS

Generation of Tn*1000* insertion mutants of plasmid pABN1. Plasmid pABN1 is nonmobilizable because the vector, like many vector plasmids, lacks sites or functions essential for single-strand transfer from a donor strain also harboring a conjugative plasmid. However, sex factor F, which carries Tn*1000* ($\gamma\delta$), mediates low-frequency transfer of recombinant plasmids by a process that involves plasmid cointegrate formation promoted by Tn*1000* (11). Subsequent resolution of cointegrates after conjugal transfer to a recipient cell results in duplication of Tn*1000* so that each regenerated component plasmid carries a copy of the transposon sequence. Tn*1000* insertion mutants of plasmid pABN1 were isolated as follows. Strain RB308 (F^+) was transformed with pABN1 DNA and mated with recipient strain GB1 for 2 h at 37°C in nutrient broth with gentle shaking, followed by selection for ampicillin- and streptomycin-resistant transconjugants.

Characterization of aerobactin-deficient Tn*1000* mutants of pABN1. Of 450 such strains assayed for aerobactin production with indicator strain LG1522, 220 (49%) were found to be defective in siderophore production. The transposon insertion site in 30 of these was determined by restriction analysis of plasmid DNA. Digestion with *Eco*RI localized all insertions to the smaller (8.6-kb) *Eco*RI fragment of pABN1, whereas *Bam*HI digestion assigned them to either the 8.9-kb or the 6.5-kb *Bam*HI fragments. *Sal*I, an enzyme with a unique central site in Tn*1000*, was used finally to locate the precise position of the insertion, whereas analysis with *Eco*RI and *Bam*HI (which cleave Tn*1000* assymetrically) allowed us to determine the orientation of the transposon. The sites of insertion in aerobactin-deficient mutant plasmids (indicated by the long stalks in Fig. 1) define a region of ca. 5.5 kb, representing 34% of the ColV-K30-derived *Hin*dIII fragment of pABN1. This localization based on transposon mutagenesis is consistent with the observation that strains carrying plasmid pABN5, a derivative comprising only the 8.6-kb *Eco*RI fragment of pABN1 (Fig. 1), produce aerobactin (as determined in the biological assay with strain LG1522), and so clearly contain all genes required for its biosynthesis.

Ten mutants defective in aerobactin biosynthesis, with transposon insertions at various positions, were assayed for the presence of the aerobactin receptor protein as described above. All were sensitive to cloacin DF13, indicating that insertion of Tn*1000* into the biosynthesis genes does not completely abolish expression of the receptor gene.

Characterization of receptor-deficient Tn*1000* mutants of pABN1. Of 70 aerobactin-producing strains similarly assayed for the presence of the receptor protein, 11 (16%) were cloacin DF13 resistant. Sites of Tn*1000* insertion in the plasmids of these and of 35 cloacin-sensitive strains were determined as described above, by using restriction enzymes *Eco*RI and *Sal*I. In cloacin-resistant strains, insertion sites (indicated by the intermediate-length stalks in Fig. 1) spanned a region of ca. 2 kb which includes the central *Eco*RI site of pABN1. This is consistent with the observation that neither *Eco*RI fragment of pABN1, when cloned alone, confers cloacin DF13 sensitivity on the host cell (2). Furthermore, the defined region is within the 6.5-kb *Bam*HI fragment of pABN1, which, when cloned into a vector plasmid, was shown to specify receptor activity as indicated by sensitivity to cloacin DF13 (13; this paper).

Expression of pABN1 in maxicells and minicells. The cloned fragment in pABN1 specified the synthesis of five polypeptides, with apparent molecular weights of 74,000, 62,000, 50,000, 45,000, and 35,000, in both the minicell (Fig. 2) and maxicell (data not shown) expression systems. All except the 74,000-dalton polypeptide were also produced by pABN5 DNA; however, pABN5 encodes an additional polypeptide of 34,000 daltons which may be a truncated form of the 74,000-dalton product. Note that the 45,000-dalton polypeptide appears with varying intensity, although always relatively weakly. A time course of expression of this polypeptide in minicells showed no evidence of significant instability, and the omission of sample boiling before SDS-PAGE also had no obvious effect on the intensity of the polypeptide band (data not shown). Plasmid pLG152, comprising the large *Eco*RI fragment of pABN1 (Fig. l), apparently encodes no major polypeptide species. The 6.5-kb *Bam*HI fragment of pABN1, which specifies receptor activity when cloned in either orientation in vector plasmid pACYC184 (as in pLG141 and pLG142), encodes two polypeptides (M_r = 74,000 and 50,000).

Expression of pABN1::Tn*1000* plasmids and their derivatives. Two approaches were used to determine the linear order of genes specifying the polypeptides required for aerobactin biosynthesis. The first involved construction of derivatives of selected pABN1::Tn*1000* plasmid mutants defective in aerobactin production. Plasmid DNA was restricted with *Eco*RI and then ligated to generate subclones extending from the *Eco*RI site in the vector moiety to the nearer *Eco*RI site within the transposon. Thus, a series of plasmids, designated pLG153 through pLG158, was obtained which contained varying lengths of the insert of pABN1 and a portion of Tn*1000* 0.9 or 4.0 kb in size, depending on the orientation of the transposon in the parent plasmid. Figure 1 shows these plasmids (maps i through vi) with the Tn*1000* portion omitted for clarity. Polypeptides expressed in minicells harboring these plasmids were analyzed by SDS-PAGE (Fig. 3).

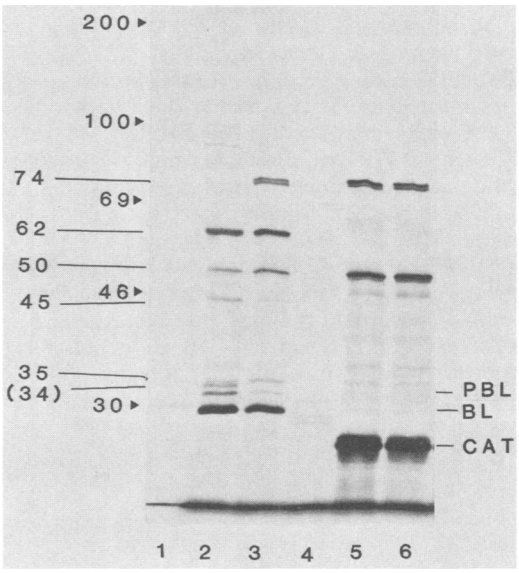

FIG. 2. SDS-PAGE of polypeptides expressed in minicells harboring plasmid pABN1 and derivatives. Lane 1 contains standard molecular size markers; size are indicated in kilodaltons. Translation products of pABN5 (lane 2), pABN1, (lane 3), pLG152 (lane 4), pLG141 (lane 5), and pLG142 (lane 6) are shown with an indication of their molecular size. The 74,000-dalton polypeptide is visible in its mature and precursor forms. BL indicates pPlac-encoded β-lactamase; PBL indicates pre-β-lactamase, the precursor form; and CAT is pACYC184-encoded chloramphenicol acetyltransferase.

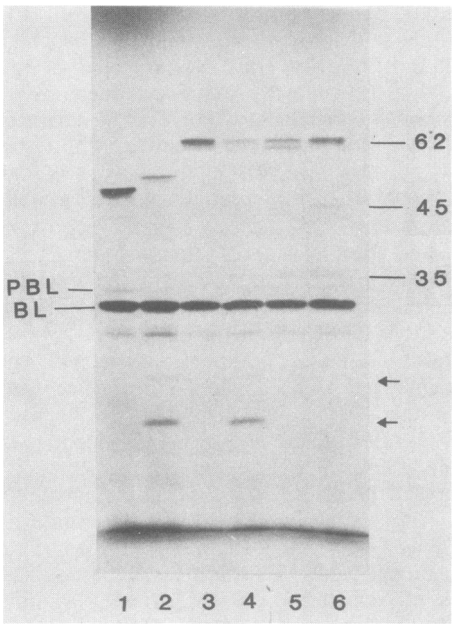

FIG. 3. SDS-PAGE of polypeptides expressed in minicells harboring subclone derivatives of pABN1::Tn*1000* mutant plasmids. Translation products of plasmids pLG153 (lane 1), pLG154 (lane 2), pLG155 (lane 3), pLG156 (lane 4), pLG157 (lane 5), and pLG158 (lane 6) were analyzed; molecular sizes are indicated in kilodaltons. BL and PBL represent β-lactamase and pre-β-lactamase, respectively. Polypeptides marked by arrows are probably Tn*1000* specified; plasmids pLG154 and pLG156, which express the polypeptides, contain a 4.0-kb portion of the transposon, whereas the others contain only 0.9 kb of Tn*1000* (see the text).

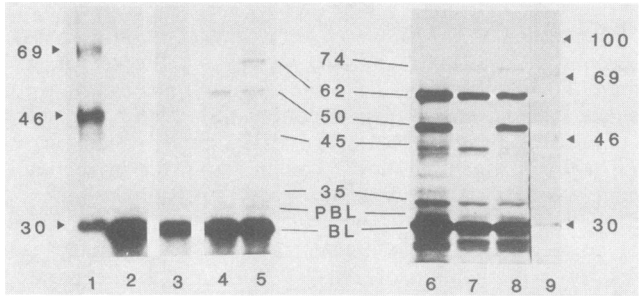

FIG. 4. SDS-PAGE of polypeptides expressed in maxicells carrying pABN1::Tn*1000* mutant plasmids. Lanes 1 and 9 contain molecular size markers; sizes are given in kilodaltons. Lanes 5 and 6 show polypeptides specified by plasmid pABN5. Also shown are translation products of pABN1 derivatives with Tn*1000* inserted at sites a (lane 2), b (lane 3), c (lane 4), e (lane 7), and d (lane 8) indicated in Fig. 5.

Plasmid pLG158 (map vi) encodes three major products with apparent molecular weights of 62,000, 45,000, and 35,000. No polypeptide band of 50,000 daltons was observed, confirming evidence from plasmids pLG141 and pLG142 that the coding sequence for this product is adjacent to the receptor gene. It is possible that the 45,000-dalton band in this track contains a truncated form of the 50,000-dalton product. Plasmid pLG156 (map iv) encodes two polypeptides with apparent molecular weights of 62,000 and 35,000; plasmid pLG155 (map iii) encodes only the former. Plasmids pLG153 and pLG154 (maps i and ii) specify products of ca. 47,000 and 52,000 daltons, respectively, which presumably represent truncated forms of the 62,000-dalton polypeptide. These data indicate that the coding sequence for this protein lies at the extreme left of the region required for aerobactin synthesis, with that for the 35,000-dalton polypeptide adjacent to it. Definitive evidence for the location of the gene for the 45,000-dalton protein was not obtained in this experiment. We assume that it lies between those of the 35,000- and 50,000-dalton products; the observation that plasmid pLG157 (map v) apparently specified no more polypeptides than pLG156 did (map iv) may be due to weak and variable expression of the 45,000-dalton polypeptide in minicells.

A second means of determining a polypeptide map of pABN1 was to express selected aerobactin-deficient pABN1::Tn*1000* mutant plasmids in maxicells and analyze the products by SDS-PAGE (Fig. 4). The sites of transposon insertion in the mutant plasmids tested are indicated in Fig. 5 (positions a through e). Of the five polypeptides specified by pABN1, Tn*1000* insertion at positions e and d abolished expression of the 50,000- and 45,000-dalton polypeptides, respectively, thus confirming our assumption about the relative positions of the coding sequences for these polypeptides. However, these insertions also reduced the level of expression of the 74,000-dalton polypeptide relative to the other products of the system. Insertion at position c abolished expression of the 62,000-dalton polypeptide only, confirming the location of its gene to the left of the required region. Furthermore, expression of all the other polypeptides was reduced relative to that of the β-lactamase protein of the vector (so that the 74,000-dalton band is not visible in this particular track). Insertions at positions a and b, on the other hand, completely prevented expression of all five polypeptides of pABN1; it is possible that these insertions identify an essential control region of the aerobactin system.

Taken together, the two approaches indicate a linear map of the translation products of plasmid pABN1 (Fig. 5). The presence in some gels of putative truncated forms of the

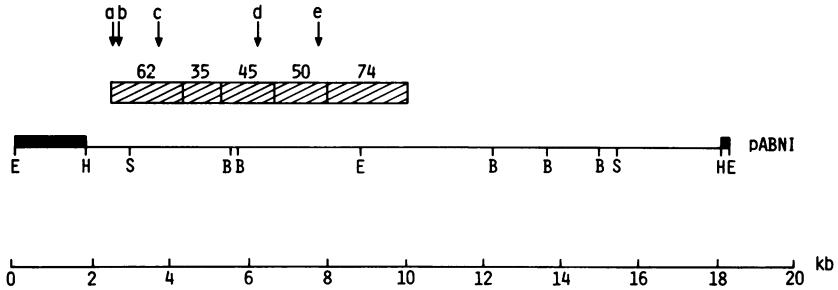

FIG. 5. Translational map of the iron uptake region of plasmid pABN1. Numbers above the shaded boxes denote polypeptide sizes in kilodaltons. Sites of Tn*1000* insertion in mutant plasmids expressed in maxicells (Fig. 4) are designated a through e.

74,000-, 62,000-, and 50,000-dalton polypeptides suggests that the direction of transcription, at least for three of the genes, is from left to right of the map as drawn (Fig. 5).

DISCUSSION

We have used transposon insertion mutagenesis to map a region of plasmid ColV-K30, cloned as recombinant plasmid pABN1, which specifies the high-affinity aerobactin iron uptake system. Derivatives of pABN1 were obtained by F-mediated "illegitimate" conjugal transfer, a process which depends on cointegrate formation promoted by Tn*1000*. Resolution of transferred cointegrates involves duplication of the Tn*1000* sequence, so that, in theory, each selected transconjugant will carry pABN1 with the transposon inserted into it. Tn*1000* is thought to insert randomly into DNA sequences (16); in this study, however, 49% of Tn*1000* insertions were mapped to a region comprising only 34% of the cloned fragment of pABN1, suggesting that insertion may not in fact be totally random.

By mapping sites of transposon insertion in characterized pABN1::Tn*1000* plasmids, we have localized the aerobactin system of ColV-K30 to a segment of pABN1 DNA of ca. 7.5 kb. This region specifies five polypeptides, four of which are involved in aerobactin biosynthesis and the fifth of which is the 74,000-dalton outer membrane protein that serves as the receptor for ferric-aerobactin. The order of genes encoding these polypeptides, which probably represent the total coding capacity of the region, has been determined by comparing translation products of defined pABN1::Tn*1000* mutants and other derivative plasmids (Fig. 5). The aerobactin molecule comprises two *N*-acetylated, *N*-hydroxylated lysine derivatives linked to the α and γ carboxyl groups of a single residue of citric acid (9). Nothing is known about the specific pathway of synthesis from existing lysine and citric acid, but in the light of our evidence that four genes are required, it is not unreasonable to speculate that two specific enzymes modify lysine, and two more effect condensation of these derivatives to the citric acid moiety. The precise biochemical roles of the four polypeptides we have identified is not yet known, but it is hoped that a greater understanding of their functions will allow the development of inhibitors or analogs which may be of therapeutic value in the control of invasive bacterial infections.

A striking feature of the data presented here is that insertions of 5.7-kb Tn*1000* showed variable polar effects on the expression of other genes. For example, insertions into a small region apparently at the proximal end of the gene encoding the 62,000-dalton polypeptide have a strong polar effect, abolishing synthesis of all five polypeptides of the system. However, insertion within the gene itself appears only to reduce, rather than abolish, expression of all the other genes, and insertions in the genes encoding the 45,000- and 50,000-dalton polypeptides reduce synthesis of the 74,000-dalton product. Unless Tn*1000* carries promoter sequences which initiate transcription beyond the site of insertion (and it is known that no such sequences lie within the terminal 50 nucleotides; see reference 16), these data may suggest the presence of weak secondary promoters within the system which allow limited expression of downstream genes. Braun et al. (5) reported that insertion of Tn*5* or of the transposon-like bacteriophage Mu dl (Ap *lac*) into the equivalent iron uptake system of plasmid ColV-K311 exerted a strong polar effect on the expression of downstream genes, particularly of the 74,000-dalton receptor protein. Based on the apparent equivalence of restriction sites, it is probable that the aerobactin genes are identical in plasmids ColV-K311 and ColV-K30. However, the evidence from ColV-K311 is purely of a biological nature, whereas the data from plasmid ColV-K30 presented in this study include observations at a molecular level also. The nature of the regions involved in the expression of the aerobactin system, and their possible roles in regulation, are currently under investigation.

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