RNA-DNA Hybridization Analysis of Transcription of the Plasmid ColV-K30 Aerobactin Gene Cluster

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Plasmid pABN1 contains the genetic determinants for the aerobactin iron uptake system of plasmid Colv-K30. Transposon Tn1000 mutants of pABN1 defective in synthesis of a 50,000-dalton polypeptide were found neither to secrete nor to accumulate aerobactin, but were not impaired in iron transport functions, clearly indicating a role for this polypeptide in aerobactin biosynthesis. RNA-DNA hybridization studies with probes spanning the entire aerobactin gene cluster showed that the system is regulated at the transcriptional level by the availability of iron in the external medium. When induced by low-iron stress, all five genes of the cluster were transcribed at a uniformly high level. When repressed by excess iron, transcripts of the four biosynthesis genes were some 30-fold less abundant in the case of the parental Colv-K30 plasmid and 10-fold less for the recombinant plasmid pABN1, whereas the receptor gene in either plasmid was transcribed at only about a third of the induced level.

Aerobactin is a hydroxamate siderophore that is synthesized by strains of Aerobacter (now Enterobacter) aerogenes (for which it was named [11]), Enterobacter cloacae (28), Escherichia coli (3, 13, 30), and species of Klebsiella (16), Salmonella (8, 21), and Shigella (20, 23). In the case of E. coli, it is most frequently found among isolates from human urinary tract infections (5) and from septicemia of humans and domestic animals (5, 22, 27), and it has been proposed that the aerobactin iron uptake system can be considered to be a virulence determinant in such strains. Presumably efficient iron sequestration by aerobactin is essential for bacterial proliferation at extraintestinal sites of infection where high levels of the iron-binding glycoproteins transferrin and lactoferrin are present in unsaturated form (31).

The first reports of the aerobactin system in E. coli indicated its association with ColV plasmids in invasive isolates (30, 32). The genetic determinants of one such plasmid, Colv-K30, were subsequently cloned as a 16-kilobase (kb) HindIII restriction fragment in a small multicopy vector plasmid (1), and transposon mutagenesis and deletion analysis of the recombinant plasmid, designated pABN1, have been used to locate a cluster of five genes and to identify their products (6). The system is probably organized as a single operon (2, 14) encoding, in order of transcription, polypeptides of apparent molecular weights 62,000, 35,000, 45,000, 50,000, and 74,000 (6). Other workers (9, 15, 21) have reported slightly different sizes for some of the five gene products. The 74,000-dalton protein is located in the outer membrane and acts as the receptor for ferric aerobactin (12, 29). The remaining four genes encode enzymes necessary for biosynthesis of the siderophore (6, 9, 14, 15). In this paper we present further evidence that the 50,000-dalton polypeptide has a role in aerobactin biosynthesis, and we also demonstrate significant constitutive expression of the receptor gene, the most distal of the aerobactin gene cluster, even in iron-rich conditions that more completely repress expression of the four biosynthesis genes of the operon.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are described in Table 1, and the structures of some of the plasmids relevant to this work are illustrated in Fig. 1. Plasmids pABN1 and pABN5 (1) were generously provided by Albrecht Bindereif and Joe Neillands. Plasmid pLG141, comprising a 6.5-kb BamHI fragment of pABN1 cloned in vector pACYC184, has been described previously (6). In addition to these, we used plasmid pMC1871 (7) as a source of lacZ DNA probe to standardize RNA samples in RNA-DNA hybridization experiments. Biological assays for aerobactin (with strain LG1522 as an indicator) and for ferric aerobactin receptor activity (with cloacin DF13) have been described previously (6).

Analysis of plasmid-specific polypeptides expressed in minicells. Minicells prepared by the method of Reeve (24) were incubated with [35S]methionine as described previously (6). Labeled polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by autoradiography (6).

Aerobactin labeling by the addition of labeled precursor. Uniformly 14C-labeled l-lysine (Amersham International Plc.; >300 mCi/mmol, 0.1 μCi/ml) was added to early exponential M9 minimal medium (26) cultures of bacterial strains and incubated overnight with aeration at 37°C. The cells were harvested, washed twice with 5 mM sodium phosphate buffer (pH 7.0) suspended in 50% ethanol, and disrupted by ultrasonication. Cell debris was pelleted by centrifugation, and supernatant material was analyzed by chromatography on Whatman 3MM paper after the addition of 50 μg of unlabeled aerobactin (purified as previously described [11]) as an internal standard. Chromatography was

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for 16 h with an ascending solvent system of n-butanol–acetic acid–water (60:15:25), after which the paper was dried and sprayed with acidic FeCl₃ (1.25%, wt/vol, in 1 M HCl) to locate the internal standard as two pink spots with Rf of 0.56 (feric aerobactin) and 0.71 (iron-free aerobactin). The chromatogram was again thoroughly dried, and radiolabeled spots were located by autoradiography. Individual spots were subsequently excised for quantitation in a Packard scintillation counter with Optiscint T scintillation fluid.

**Isolation of total cellular RNA.** M9 minimal medium (24) containing 2 g of glucose per liter, 1 mM isopropyl-β-D-thiogalactopyranoside, and either 10 μM FeCl₃ or 200 μM α,ω-dipryridyl was used to grow cultures for RNA preparation. Since β-galactosidase activity was found to be identical for all strains used in both conditions of iron availability, isopropyl-β-D-thiogalactopyranoside-induced transcription of the lacZ gene could be used as an internal standard for all other measurements of specific mRNAs. Overnight cultures were diluted into prewarmed fresh medium (1 liter) to an optical density of 0.1 (at 450 nm) and grown to an optical density of 0.5 to 0.6. NaN₃ (10 mM) was added, and the cultures were diluted into an equal volume of cold medium to bring the aerobic bacteria to 4°C. Cells were harvested by centrifugation at 0°C, suspended in 4 ml of ice-cold 20 mM sodium acetate–1 mM EDTA, and added to 8 ml of boiling 20 mM sodium acetate–1 mM EDTA–3% sodium dodecyl sulfate in large siliconized glass tubes. Nonvicious lysates were brought to 0.5 M NaCl and extracted twice with phenol (once at 65°C and again at room temperature) and twice with chloroform–isoamyl alcohol (24:1). Nucleic acids were precipitated with an equal volume of isopropanol, and contaminating DNA was removed from redissolved samples by ultracentrifugation through CsCl.

**Preparation of DNA probes.** Restriction fragments of plasmid DNA required as probes were purified by electrophoresis into Whatman DE81 paper inserted into the gel (10), eluted, and labeled by nick translation with [α-32P]dCTP (Amersham; 3,000 Ci/mmol) and the Klenow fragment of *E. coli* DNA polymerase I (25).

**RNA-DNA hybridization.** Samples of RNA (50 to 150 μg) were spotted onto 25-mm-diameter nitrocellulose disks, dried, and baked at 80°C for 2 h. Filters were incubated at 42°C overnight in prehybridization buffer (50% formamide, 50 mM sodium phosphate, 5 × SSC [1 × SSC is 0.15 M NaCl–0.015 M sodium citrate], 250 μg of denatured salmon sperm DNA per ml, 0.2 mg of bovine serum albumin per ml, 0.2 mg of Ficoll–400 per ml, 0.2 mg of polyvinylpyrrolidone per ml, 25 μg of polyadenylic acid [pH 6.5] per ml) and then transferred to hybridization buffer (4 parts of the prehybridization buffer, with polyadenylic acid omitted, mixed with 1 part 50% dextran sulfate) to which the heat-denatured labeled DNA probes were added. Incubation was continued at 42°C overnight, and then the filters were washed extensively with 0.1 × SSC at 50°C. The filters were blotted dry, and the level of hybridization was measured by counting bound radioactivity in a Packard liquid scintillation spectrometer.

### RESULTS

**Mutants of pABN1 defective in synthesis of the 50,000-dalton protein.** Four independent derivatives of pABN1 with the transposon Tn1000 inserted within a 1.4-kb region adjacent to the receptor gene (Fig. 1) were unable to secrete sufficient aerobactin to support the growth by cross-feeding of tester strain LG1522, which cannot synthesize aerobactin but can utilize it if it is provided exogenously (6). All four mutants, however, were complemented for this function by the presence of the same recombination-deficient strain of plasmid pLG141, which carries the complete coding sequence of the 50,000-dalton protein as well as that for the receptor (6). The amount of aerobactin specified by the complementing plasmids was somewhat less than with pABN1 itself, as judged by the size of the zone of growth of LG1522 around the inoculum. This may be due to significant loss of one or the other plasmid during growth on the bioassay plates, since it was not feasible to add selective antibiotics.

Strains harboring the four pABN1::Tn1000 mutants were sensitive to killing by cloacin DF13, although the test was highly variable for any particular strain, in some cases giving a turbid killing zone rather than the clear zones always observed with pABN1 or CoV-K30. This indicates that the receptor gene was being expressed by these plasmids, albeit sometimes at reduced levels, even though transposons were present in the adjacent gene. Consistent with this is our
The previous observation (6) that maxicells carrying pABN1::Tn1000-7 synthesized no 50,000-dalton product but significant, though somewhat reduced, levels of 74,000-dalton receptor protein. Mutant pABN1::Tn1000-11 (in which the transposon is inserted in the opposite orientation from mutant 7) also lacked the 50,000-dalton protein (Fig. 2) when the plasmid was expressed in minicells, although in this case the receptor protein, seen here in both precursor and processed forms, did not appear to be significantly reduced by comparison with pABN1.

Effect on aerobactin biosynthesis of transposon insertion in the 50,000-dalton gene. To determine whether these mutants were in fact able to synthesize aerobactin, but not able to secrete it into the medium, we attempted to detect intracellular accumulation of the siderophore. Strains were grown in appropriately supplemented minimal medium; cells were harvested by centrifugation and, after extensive washing to remove any cell-associated aerobactin, disrupted by ultrasonication. Cell debris was pelleted by centrifugation, and supernatant fractions were tested for their ability to support growth of indicator strain LG1522 (Table 2), an assay that will detect as little as 0.2 μM aerobactin. Strains harboring any of the pABN1::Tn1000 mutants were negative in this test, whereas a similar preparation of a strain carrying pABN1 supported strong growth of LG1522, indicating the presence of aerobactin even in the soluble fraction of cells that are actively secreting the siderophore.

In addition, strain GB1 harboring pABN1::Tn1000-7 was grown in the presence of [14C]lysine, sonicated, and analyzed by paper chromatography and autoradiography as described in Materials and Methods. Of the total radioactivity applied at the origin (56,000 cpm), most was seen as unreacted lysine (Rf 0.18), whereas only background levels of activity (<400 cpm per spot) cochromatographed with purified aerobactin (Rf 0.56 and 0.71) added as an internal standard. Plasmid mutant pABN1::Tn1000-11 gave a similar result.

Effect on iron transport of transposon insertion in the 50,000-dalton protein gene. If the 50,000-dalton polypeptide were involved in transport of iron from ferric aerobactin into the cell, mutants lacking this gene product should not grow in low iron conditions when supplied with the siderophore. However, strain AN1937 (entA) carrying any one of the four mutant plasmids defective in synthesis of the 50,000-dalton protein grew strongly in the presence of purified aerobactin or by cross-feeding around the point of inoculum with a strain carrying pABN1 (Table 2). It is interesting to note that plasmid pLG141 also permitted aerobactin-promoted growth of AN1937; this result contrasts with an observation of Krone et al. (18) on plasmid pFS8, which comprises the same BamHI restriction fragment of ColV-K30 inserted in the vector pBR322.

Expression of the aerobactin receptor gene in pABN1 and ColV-K30. It has been suggested that the five genes of the aerobactin gene cluster of ColV plasmids are organized as a single operon (2, 14). The results shown in Table 2, however, as well as the results for maxicell and minicell expression systems (6) imply that transposon Tn1000 inserted into the coding sequence of the 50,000-dalton protein does not totally abolish the expression of the promoter-distal receptor gene. Thus there are still sufficient receptor molecules present in the outer membrane to allow aerobactin-promoted growth of the strain harboring the mutant plasmids. To investigate this, we isolated total RNA from strains containing ColV-K30 or pABN1 and analyzed samples for the presence of specific transcripts of the aerobactin gene cluster by RNA-DNA dot-blot hybridization with the probes indicated in Fig. 1. It should be noted that RNA was isolated from bacterial cultures in which transcription of the lacZ gene was induced by the presence of isopropyl-β-D-thiogalactopyranoside, and which all synthesized the same high level of β-galactosidase activity. Thus, by also probing RNA samples with lacZ DNA prepared from plasmid pMC1871 (7) we were able to detect and allow for variations in recovery of total RNA from the various strains and growth conditions. The relative numbers of counts bound to purified plasmid pABN1 DNA and to RNA isolated from strain GB1(pABN1) grown in the presence of 200 μM α,α'-dipyridyl were very similar (Table 3). In the experiment shown, there was a slight deficit of

![FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of polypeptide products of the aerobactin gene cluster expressed in minicells. Labeled molecular size marker proteins (track b) were phosphorylase (100,000 and 92,500), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000). Polypeptides encoded by pABN1 are shown in tracks c and d, and the positions of the five bands of the aerobactin system are indicated; numbers denote molecular masses in kilodaltons. Polypeptides expressed by plasmids pABN1::Tn1000-11 and pLG141 are shown in tracks a and e, respectively. Vector proteins β-lactamase (BL), pre-β-lactamase (PBL), and chloramphenicol acetyltransferase (CAT) are indicated.]

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**TABLE 2.** Aerobactin biosynthesis and uptake by strains carrying derivatives of plasmid pABN1

<table>
<thead>
<tr>
<th>Plasmid present</th>
<th>Ability to promote growth of LG1522a</th>
<th>Growth in the presence of aerobactinb</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pABN1</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>pLG141</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>pABN1::Tn1000-16</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pABN1::Tn1000-7</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>pABN1::Tn1000-11</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>pABN1::Tn1000-15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pABN1::Tn1000-23</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Samples (100 μl) of sonicates of strain GB1 harboring the plasmids indicated were placed in 5-mm-diameter wells cut in bioassay plates seeded with strain LG1522. +, Growth; -, no growth.

b Bioassay plates seeded with strain AN1937 harboring the plasmids indicated were point inoculated with the aerobactin-producing strain GB1(pABN1). Also, purified aerobactin (1 mg) was added to 5-mm-diameter wells cut in the agar. +, Growth; -, no growth.

NA, Not applicable, since AN1937 carrying pABN1 is able to grow on bioassay plates.
TABLE 3. Hybridization to DNA probes of the aerobactin gene cluster

<table>
<thead>
<tr>
<th>Probe</th>
<th>pABN1 DNA</th>
<th>GB1(pABN1) RNA</th>
<th>GB1 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>Ratio</td>
<td>cpm</td>
</tr>
<tr>
<td>1</td>
<td>24,358</td>
<td>1.0</td>
<td>5,767</td>
</tr>
<tr>
<td>2</td>
<td>8,893</td>
<td>0.36</td>
<td>2,134</td>
</tr>
<tr>
<td>3</td>
<td>20,590</td>
<td>0.85</td>
<td>4,231</td>
</tr>
<tr>
<td>4</td>
<td>60,540</td>
<td>2.49</td>
<td>12,770</td>
</tr>
</tbody>
</table>

a 32P-labeled restriction-nuclease fragments spanning the aerobactin gene cluster (Fig. 1) were used as DNA probes.

b Purified plasmid pABN1 DNA (1 μg per filter) was used to determine the relative efficiency of labeling of the four DNA probes.

cRNA was isolated from strains grown in the presence of α, α'-dipyridyl.
d Ratios indicate hybridization relative to that of probe 1.

Transcripts corresponding to probes 3 and 4, but this was not consistently observed with all batches of RNA, and the data indicate that transcription of the various regions of the aerobactin gene cluster defined by the four probes was essentially uniform. Preparations of RNA from a strain lacking a plasmid showed virtually no hybridization to any of the aerobactin gene probes.

Transcription of the aerobactin system in pABN1 is regulated by external iron levels; transcripts of the aerobactin biosynthesis region (probes 1 through 3) were approximately 10-fold less abundant in RNA prepared from GB1(pABN1) grown in the presence of 10 mM FeCl3 (Table 4). Interestingly, however, transcripts homologous with the receptor gene (probe 4) were only threefold less abundant than in RNA samples from iron stress conditions. In a strain harboring ColV-K30, transcription of the aerobactin biosynthesis genes was similarly induced by conditions of iron stress (Table 4), in this case some 25- to 30-fold, although the overall induced level was about one-sixth that observed with pABN1, presumably because of the different plasmid copy numbers. Again, however, receptor gene activity was induced about threefold by iron stress from a high constitutive basal level in iron-rich conditions.

Expression of the receptor gene in pABN1::Tn1000 plasmids mutant in biosynthesis genes. Further evidence for constitutive expression of the receptor gene comes from the analysis of RNA samples from strains carrying pABN1::Tn1000 mutants. In mutant 16 the site of transposon insertion lies slightly to the right of the AvaI site that defines the left-hand end of probe 3 (Fig. 1), within the coding sequence of the 45,000-dalton protein (6). Note that transcription upstream of the insert (corresponding to probes 1 and 2) was regulated normally, showing an approximately 10-fold enhancement in conditions of iron stress compared with when iron was readily available (Table 4). On the other hand, transcription corresponding to probe 3 was drastically reduced, indicating the strong polar effect of Tn1000 insertion on downstream transcription. However, transcription of the receptor gene (probe 4) occurred at the same basal level as observed for plasmid pABN1 and was virtually unaffected by imposition of iron stress conditions. Similar unregulated constitutive expression of the receptor gene was seen with pABN1::Tn1000-7, in which the transposon is again inserted in the region spanned by probe 3, but in this case toward the promoter-distal end. All three biosynthesis gene probes detected 10-fold inducibility of transcription upstream of the site of insertion.

DISCUSSION

There has been particular interest recently in the hydroxamate siderophore aerobactin, not least because it has been found to be widespread among clinical isolates of E. coli and may be crucial in the pathogenesis of these strains in extraintestinal infections of humans and domestic animals (5, 22, 27). In this paper, we present observations that bear on an understanding of the structure and regulation of the genetic determinants for the biosynthesis of aerobactin and for the uptake of iron complexed to it. We have confirmed that a polypeptide with an apparent molecular weight of 50,000 is required for aerobactin biosynthesis and apparently not for transport of iron from ferric aerobactin bound to outer membrane receptors to the intracellular compartment. Mutants of plasmid pABN1 having transposon Tn1000 inserted within a 1.4-kb stretch of DNA immediately adjacent to the gene for the 74,000-dalton receptor protein were unable to synthesize only the 50,000-dalton polypeptide of the five gene products in the aerobactin system. None of these mutants was able either to secrete or accumulate aerobactin. Krone et al. (17) had initially reported that the 50,000-dalton protein was necessary for iron transport. They showed that plasmid pSF8 (which carries the 6.5-kb BamHI fragment of ColV-K30 DNA, encoding the 50,000- and 74,000-dalton polypeptides) promoted radioactive iron uptake by an enterohaemolysin-deficient mutant, whereas a deletion derivative that was unable to synthesize the 50,000-dalton protein did not allow internalization of 55Fe bound to surface receptors in complex with aerobactin. It is, of course, possible that the 50,000-dalton protein is bifunctional and is required for both biosynthesis and transport. However, we show here that an entA strain harboring pABN1 mutants defective in synthesis of this protein grows well in iron-restricted medium when provided with aerobactin, implying normal uptake of iron. Moreover, Gross et al. (15) have recently repeated the uptake experiments of Krone et al. (17) with identically derived plasmids, but using growing rather than iron-starved cells, and obtained results consistent with ours. We also found that AN1937 harboring plasmid pLG141 grew readily

TABLE 4. RNA-DNA hybridization analysis of transcription of the aerobactin systema

<table>
<thead>
<tr>
<th>Probe</th>
<th>GB1(pABN1)</th>
<th>GB1(pABN1::Tn1000-16)</th>
<th>GB1(pABN1::Tn1000-7)</th>
<th>GB1(ColV-K30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe3+ +Dipyridyl</td>
<td>Fe3+ +Dipyridyl</td>
<td>Fe3+ +Dipyridyl</td>
<td>Fe3+ +Dipyridyl</td>
</tr>
<tr>
<td>1</td>
<td>9.4</td>
<td>100</td>
<td>7.3</td>
<td>8.9</td>
</tr>
<tr>
<td>2</td>
<td>9.3</td>
<td>100</td>
<td>6.1</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>10.8</td>
<td>100</td>
<td>&lt;0.5</td>
<td>6.6</td>
</tr>
<tr>
<td>4</td>
<td>34.4</td>
<td>100</td>
<td>98.7</td>
<td>75.5</td>
</tr>
</tbody>
</table>

a Strains were grown in the presence of 10 μM iron (Fe3+) or 200 μM α, α'-dipyridyl for preparation of total RNA.

b Results for each probe are given as percentages of hybridization to RNA from the pABN1-containing strain grown in the presence of α, α'-dipyridyl. All data are normalized with respect to lacZ expression.
on bioassay plates when supplied with aerobactin, a result which, in combination with data from the pABN1::Tn1000 mutants, indicates that the 74,000-dalton outer membrane receptor protein is the sole ColV-K30 plasmid-specified function necessary for assimilation of iron from ferric aerobactin. Thus, the data presented here, together with the work of others (9, 15), confirm our original proposal that the 50,000-dalton protein is involved in biosynthesis of aerobactin (6).

There is good evidence that the five genes of the aerobactin gene cluster form a single operon. Polar effects of transposon insertion on expression of promoter-distal genes have been reported by several workers (2, 4, 6, 14). In this paper we confirm that the expression of all of the genes is regulated by the availability of iron in the external medium and show that control is exerted at the level of transcription. In ColV-K30, transcription of the biosynthesis genes is induced about 30-fold from a low basal level by the imposition of iron stress. With plasmid pABN1, however, the basal level when iron was freely available was about 20 times higher than with the parental plasmid, probably because of titration of a chromosomally determined repressor by multiple copies of regulatory regions in the recombinant plasmid. Even so, transcription was enhanced a further 10-fold by iron stress to give an overall induced level about six times higher than that of the biosynthesis genes of plasmid ColV-K30. Transcription of the receptor gene of ColV-K30 and pABN1, on the other hand, was induced only threefold from a comparatively high basal level, the actual levels reflecting different plasmid copy numbers. Thus, in iron-replete conditions, only the receptor gene was transcribed to any measurable extent. The possibility that there is a minor constitutive promoter for this gene, as suggested by our data, awaits further analysis, although it should be noted that no promoterlike sequences have yet been detected immediately upstream of the coding region (19). On the other hand, in conditions of iron stress all five genes of the cluster were transcribed at a high level from the major promoter (2) adjacent to the gene encoding the 62,000-dalton polypeptide, probably as a single polygenic message and possibly inhibiting initiation from the putative minor unregulated promoter of the receptor gene.

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LITERATURE CITED


