INFECTION AND IMMUNITY, Aug. 1981, p. 540-545
0019-9567/81/080540-06$02.00/0

Vol. 33, No. 2

ColV Plasmid-Specified Aerobactin Synthesis by Invasive Strains of *Escherichia coli*

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Received 11 March 1981/Accepted 10 May 1981

Certain strains of *Escherichia coli* associated with bacteremia of humans and domestic animals harbor plasmids that promote efficient iron uptake. The mechanism, which is an important component of the virulence of invasive strains, is independent of the enterobactin system for iron uptake. Plasmid-specified siderophore was assayed by its ability to support the growth of a chelator-deficient mutant in conditions of iron deprivation. The chelator, which was chemically determined to be a hydroxamate compound, was identical on the basis of field desorption mass spectrometry with aerobactin, a siderophore synthesized by *Aerobacter aerogenes*. In conditions of iron stress, aerobactin is secreted into the culture medium of plasmid-bearing *E. coli* strains. Reconstruction experiments involving a chelator-deficient mutant growing with exogenous chelator suggested that association of a small fraction of the total siderophore synthesized with cellular material is due to transient binding of aerobactin to membrane receptors during active bacterial growth.

Iron is an essential trace element for microbial growth. In nature, however, it exists either as insoluble high-molecular-weight polynuclear aggregates (12) or complexed with iron-binding proteins such as transferrin in body fluids and tissues (14). Bacterial growth, therefore, depends upon successful sequestering of iron from these sources and subsequent efficient transport into bacterial cells. Enteric bacteria secrete the catechol siderophore enterobactin (enterochelin), and ferric enterobactin complex formed in the external medium is actively transported across cell membranes (8). Certain invasive strains of *Escherichia coli* synthesize, in addition, a noncatechol iron-chelating compound (15), the genetic determinants for which reside on plasmids identified in the laboratory by their ability to promote the production of the specific antibacterial protein colicin V. Such ColV plasmids are common in strains of *E. coli* isolated from cases of bacteremia and meningitis of humans and domestic animals (1, 10), and the presence of a plasmid is crucial to the survival and proliferation of invasive bacteria within an infected body (10, 11), and therefore to the development of disease symptoms. The selective advantage conferred by ColV plasmids is abolished when iron is freely available for bacterial growth (15).

Strains of *E. coli* harboring ColV plasmids synthesize hydroxamate material in response to iron deprivation (13). Since hydroxylamine nitrogen was chemically detectable in pellets of colicinogenic bacteria, it has been suggested that plasmid-specified hydroxamate siderophore molecules are components of the bacterial cell membrane (13). Genetic data, on the other hand, indicate that the ColV plasmid-specified chelator is a secreted product which requires both plasmid- and chromosome-encoded functions for its transport into the cell (16). This paper reports the identification of the hydroxamate siderophore synthesized by invasive strains of *E. coli* as aerobactin (Fig. 1), and provides further evidence for its cell-free mode of action.

A preliminary report of this work was presented at the International Conference on Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids, Santo Domingo, Dominican Republic, January 1981.

MATERIALS AND METHODS

Bacterial and culture media. Bacterial strains used in this study are listed in Table 1. Defined bacterial culture media were phosphate-buffered M9 salts medium (7) or tris(hydroxymethyl)aminomethane-buffered minimal medium (9) supplemented with glucose (0.2 to 0.4%, wt/vol), thiamine (50 μg/ml), and required amino acids either individually (20 μg/ml) or as Casamino Acids (0.5%, wt/vol). Siderophore synthesis and secretion were enhanced by addition of either α,α'-dipyridyl (160 μM), to chelate available iron, or sodium succinate (1%, wt/vol) as sole carbon source.

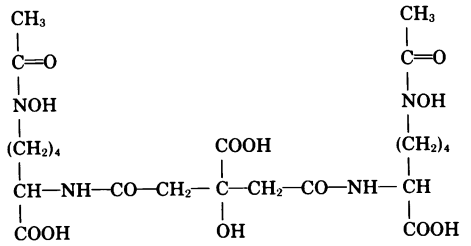

FIG. 1. *Structural formula of E. coli aerobactin.*

TABLE 1. *Bacterial strains*

Strain designation	Characteristics	Reference
E. coli		
AN1937[a]	*ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl*	15, 16
LG1315	Derivative of AN1937, carrying plasmid ColV-K30 Iu^{+} [b]	15, 16
LG1418	Derivative of AN1937, *nalA*, carrying mutant plasmid ColV-K30 *iuc*[b]	16
LG1419	Derivative of AN1937, *nalA*, carrying mutant plasmid ColV-K30 *iut*[b]	16
A. aerogenes 62-1	Requires phenylalanine, tryptophan, tyrosine	5

[a] AN1937 is the same as AN193 isolated by I. G. Young.

[b] Iu^{+} indicates plasmid-determined iron uptake; *iuc* and *iut* designate plasmid mutations leading to defects in chelator synthesis and transport function, respectively.

Ion-exchange chromatography. Columns of Dowex-1 (approximately 4-ml bed volume) were washed extensively with 2 N acetic acid and then with distilled water. Samples of bacterial culture supernatant fluid (pH 7) were applied to the resin at a flow rate of 15 ml/h. Elution of adsorbed material was effected by increasing concentrations of ammonium chloride solution, either stepwise or as a gradient at the same constant flow rate.

Gel filtration. Samples (1 ml) were applied to Sephadex G-50 columns (25 by 1 cm) and eluted with 100 mM phosphate buffer (pH 7.0) at a flow rate of 15 ml/h.

Biological detection of plasmid-specified siderophore. Lawns of bacterial strain LG1418 (approximately 10^7 bacteria per plate) were made on appropriately supplemented M9 minimal agar containing α,α'-dipyridyl (160 μM). Samples (0.1 ml) of serial dilutions of preparations containing siderophore activity were placed in wells (5-mm diameter) cut in the agar. Zones of growth of the indicator strain around a well showed the presence of iron-chelating activity in the sample. Activity was arbitrarily defined as the reciprocal of the highest dilution of a sample at which growth of the indicator strain was detectable.

Chemical assay for hydroxamate. Hydroxylamine nitrogen was assayed by the method of Csáky (3). Samples in 3 M sulfuric acid were autoclaved (121°C for 30 min) and then neutralized with sodium acetate (35%, wt/vol). Sulfanilic acid and iodine (both in 30% [vol/vol] acetic acid) were added to final concentrations of 0.15 and 0.2%, respectively, and mixtures were allowed to stand for 5 min to allow oxidation to nitrite of hydroxylamino groups released by acid hydrolysis. Sodium arsenite was added (0.25% [wt/vol] final concentration) to destroy excess iodine, and nitrite was estimated colorimetrically (absorbance at 526 nm) after addition of α-naphthylamine (0.03% [wt/vol], final concentration) and allowing 30 min for the development of coloration. The test is not strictly quantitative, but is adequate for comparison of consecutive column fractions (4).

Determination of secreted iron-binding capacity. Carrier-free $^{55}FeCl_3$ (1 μCi) was added to 1-ml samples of supernatant fluid of bacterial cultures. The presence of low-molecular-weight compounds with the capacity to chelate iron was determined by gel filtration through Sephadex G-50. Unbound iron was solubilized by the addition of excess human transferrin to samples before gel filtration. Samples (5 μl) of column fractions were spotted onto Whatman no. 1 filter paper disks, air dried, and counted in a Packard model 3255 liquid scintillation counter. A toluene-based scintillation fluid containing 2,5-diphenyloxazole (5 g/liter) and *p*-bis-2-(5-phenyloxazolyl)benzene (33 mg/liter) was used.

Thin-layer chromatography. Samples were analyzed on Macherey-Nagel Polygram silica gel precoated plastic (Sil-G) with the solvent system *n*-butanol-acetic acid-water (4:1:1). A 1% (wt/vol) solution of $FeCl_3$ in methanol was used as a detection aid.

Mass spectrometry. A Kratos/AEI M59 instrument equipped with a field desorption ion source was used.

RESULTS

Identification of the plasmid-specified siderophore as hydroxamate. The supernatant fraction of a culture of strain LG1315 (*entA*, Iu^{+}) grown in appropriately supplemented M9 medium containing 160 μM α,α'-dipyridyl promotes the growth of mutant strain LG1418 (deficient in ColV plasmid-mediated siderophore synthesis, *iuc*) when added to similar medium. This biological activity, which is dependent on iron limitation during bacterial growth, adsorbed to the anion-exchange resin Dowex-1 and was desorbed as a single peak during ammonium chloride concentration gradient elution (Fig. 2). Hydroxylamine nitrogen was not detectable by the method described by Csáky (3) in biologically active culture supernatant fractions of strain LG1315. However, because of the inherent concentration effect of loading large volumes of culture supernatant onto Dowex-1 it could be shown that chemically detectable hydroxamate coeluted precisely with biologically determined activity from the ion-exchange resin (Fig. 2). There was also precise coelution of biologically and chemically detectable material as a single peak from a Sephadex G-50 column (Fig. 3a);

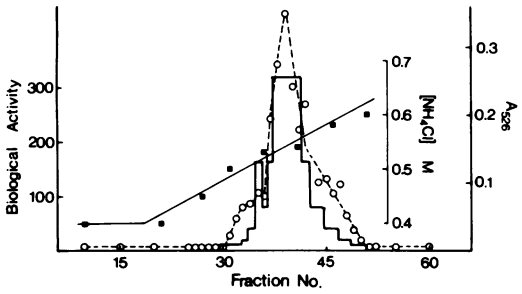

FIG. 2. *Ion-exchange chromatography of ColV plasmid-specified siderophore. Culture supernatant fluid (50 ml, pH 7) of strain LG1315 was applied to a Dowex-1 column (3.5 by 1 cm) and eluted with a 100-ml 0.4 to 1.0 M ammonium chloride gradient (■) at a flow rate of 15 ml/h. Fractions (1 ml) were collected and tested for biological activity against strain LG1418 (histograms) and hydroxylamine nitrogen (absorbance at 526 nm [A_{526}]; ○).*

this material represents the sole ^{55}Fe-binding capacity secreted by strain LG1315 (Fig. 3b). These data, which indicate that the activities measured in biological and chemical assays are identical on the basis of both charge and size, strongly suggest that biological activity secreted by the ColV plasmid-carrying strain LG1315 is due to a hydroxamate siderophore.

Support for this suggestion comes from analysis of mutants of strain LG1315 defective in plasmid-specified iron uptake (16). The mutant strain LG1418 was proposed on the basis of cross-feeding tests to be deficient in chelator synthesis (16); this strain did not secrete hydroxamate compounds at levels that were detectable even after ion-exchange chromatography with Dowex-1 that would have effected 50-fold concentration of material in the culture medium. On the other hand, strain LG1419, postulated to be defective in the transport of iron (*iut*) into the cell (16), produced 10 to 100 times more chelator (depending on phase of growth) than Iu^+ strain LG1315, on the basis of both biological and chemical assays.

Identification of the hydroxamate siderophore as aerobactin. Strain LG1315 was cultured for 3 to 4 days at 37°C with vigorous aeration in 1-liter volumes of tris(hydroxymethyl)aminomethane minimal medium supplemented with sodium succinate (1%, wt/vol). Cells were removed by centrifugation, and siderophore was isolated from the supernatant fluid by a modification of the method described for the isolation of aerobactin (4) from *Aerobacter aerogenes* 62-1 (5). The modified method was developed for isolating hydroxamate siderophore from *Salmonella* spp. and will be reported separately (A. Bindereif, J. A. Garibaldi, M. A. McIntosh, and J. B. Neilands, manuscript in preparation). Thin-layer chromatographic analysis of purified siderophore from *E. coli* LG1315 showed it to have the same R_f (0.54) as authentic aerobactin derived from *A. aerogenes* 62-1. Positive identification of the product from strain LG1315 as aerobactin was achieved by field desorption mass spectrometry. At lower emitter temperatures the most prominent feature of the spectrum was an intense line at mass number 565. A similar natural-abundance ^{13}C isotope peak was present at mass 566, and a sodium-cationized satellite line was evident at 587. At higher emitter temperatures the elimination of labile oxygen as an element of water was observed. These findings are compatible with the anticipated behavior of aerobactin (mass 564.54) in the field desorption mass spectrometer. In addition, we have observed that authentic aerobactin from *A. aerogenes* 62-1, like the ColV plasmid-specified siderophore, permits growth in conditions of iron stress of *E. coli* LG1418, but not of the plasmid-free strain AN1937.

Cell association of aerobactin. Biological assays using strain LG1418 as indicator have confirmed the previous observation (13), based on chemical analysis of hydroxylamine nitrogen,

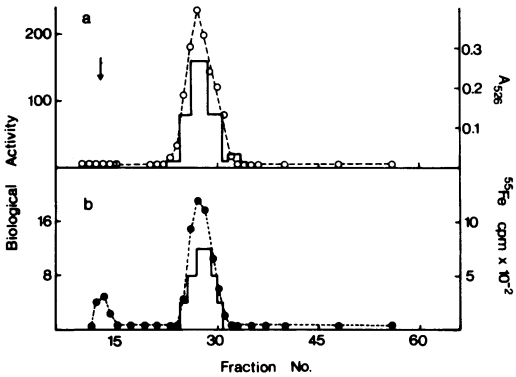

FIG. 3. *Gel filtration of ColV plasmid-specified siderophore. (a) A sample (1 ml) of biologically active eluant (0.6 M NH_4Cl) from Dowex-1 chromatography of strain LG1315 culture supernatant fluid (approximately 20-fold concentration) was applied to a Sephadex G-50 column (25 by 1 cm) and eluted with 100 mM phosphate buffer (pH 7.0) at a flow rate of 15 ml/h. Fractions (1 ml) were collected and tested for biological activity against strain LG1418 (histograms) and hydroxylamine nitrogen (absorbance at 526 nm [A_{526}]; ○). The void volume is marked by the arrow. (b) To a sample (1 ml) of strain LG1315 culture supernatant fluid were added 1 μCi of carrier-free $^{55}FeCl_3$ and excess human transferrin. The mixture was applied to Sephadex G-50 as described above. Fractions were tested for biological activity (histograms) and ^{55}Fe radioactivity (●). Ferric transferrin eluted in the void volume.*

that hydroxamate material exists in the cellular fraction of growing colicinogenic cultures (Fig. 4). Bacteria were harvested from exponential cultures of strain LG1315 in M9 medium containing α,α'-dipyridyl and washed several times with 0.1 M phosphate buffer (pH 7.0) until no further biologically active material could be eluted from the cells. Suspensions of washed cells were sonicated (four periods of 30 s, 6-μm amplitude) and assayed for biological activity. Approximately 10% of the total detectable siderophore synthesized by a culture of strain LG1315 was present in the sonicated cell pellet (Fig. 4). Cell-associated activity in sonicated pellets and secreted activity in the supernatant fraction of cultures of strain LG1315 eluted identically from Sephadex G-50 columns.

The nature of the association of siderophore with cellular material is suggested by reconstruction experiments involving *iuc* mutant LG1418 growing in conditions of iron deprivation because of the addition of aerobactin to the growth medium (Fig. 4a). The source of aerobactin was the supernatant fluid of a stationary culture of strain LG1315. Supplementation with 10, 1, or 0.1% (vol/vol) of this material allowed growth of strain LG1418 in M9 medium containing α,α'-dipyridyl at the same rate as the Iu^+ strain LG1315. Supplementation with 0.01% (vol/vol) also allowed growth, but at a much reduced rate. Biological activity was observed in well-washed sonicated cell pellets of strain LG1418 growing under these conditions (Fig. 4b); at concentrations of exogenous siderophore that were not growth rate limiting, the absolute level of cell-associated activity was approximately the same as that seen in the aerobactin-producing strain LG1315. Furthermore, when well-washed cells that had been growing with 10% aerobactin supplementation were suspended in minimal medium without aerobactin, the cell-associated material allowed continued, but limited, bacterial growth during which a significant amount of the siderophore was released from the cellular fraction (Fig. 4). These data strongly suggest that plasmid-specific hydroxamate material associated with cell pellets was actively involved in iron transport into growing cells at the time of sampling.

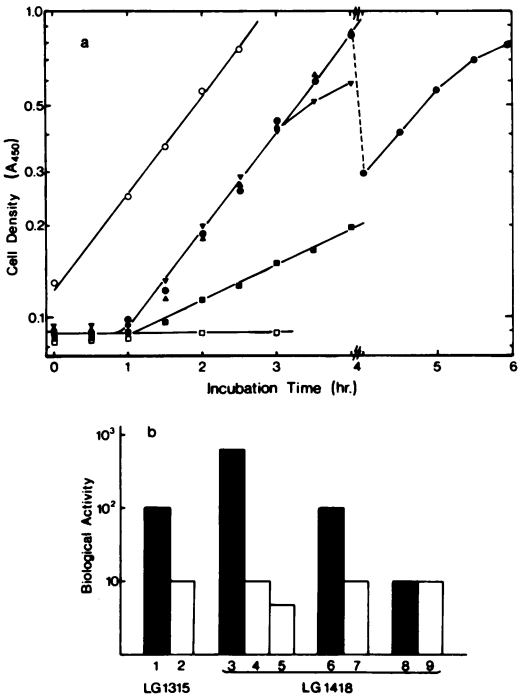

FIG. 4. *Association of aerobactin with growing bacteria. (a) Growth kinetics (absorbance at 450 nm [A_{450}]) were determined in appropriately supplemented M9 minimal medium containing 160 μM α,α'-dipyridyl (37°C): strain LG1315 (○); strain LG1418 in the presence of 10% (●), 1% (▲), 0.1% (▼), or 0.01% (■) supplementation (vol/vol) with a stationary-phase culture supernatant fluid of strain LG1315, or in the absence of supplementation (□). Growing bacteria were harvested from one of the cultures (10% supplementation, ●), washed extensively, and incubated in the absence of added supernatant fluid. (b) Biological activity in both supernatant (black histograms) and cellular fractions (white histograms) was determined for strain LG1315 (columns 1 and 2) and for strain LG1418 growing with 10% (columns 3, 4, and 5), 1% (columns 6 and 7), or 0.1% (columns 8 and 9) supplementation (vol/vol) with LG1315 culture supernatant fluid. Histogram 5 shows the level of cell-associated activity after subsequent incubation in the absence of aerobactin. All cultures were harvested in midlogarithmic growth phase (absorbance at 450 nm, 0.6 to 0.7). Supernatant fractions were sterilized by membrane filtration, and biological activity was determined using strain LG1418 as indicator. Cell pellets were washed extensively with 0.1 M phosphate buffer (pH 7), sonicated, and assayed for biological activity.*

DISCUSSION

The characterization of mutants deficient in ColV plasmid-specified iron uptake led to the suggestion that colicinogenic cells secrete a siderophore which requires plasmid-encoded functions for uptake (16). One class of mutant, designated *iuc* and exemplified by strain LG1418, is defective in siderophore synthesis, but can grow in conditions of iron deprivation by taking up exogenously supplied siderophore. Thus these strains can be used as indicators in a sensitive biological assay for the presence of plasmid-specified iron chelator. The data presented here indicate that this chelating agent is a hydroxamate

compound, and so confirm the previous observation of Stuart et al. (13). In terms of binding to and elution from anion-exchange resin, and on the basis of molecular size as suggested by gel filtration, there was complete coincidence of activity in biological assays and chemical analysis of hydroxylamine nitrogen. Furthermore, there was a good correlation between biological and chemical tests of the behavior of plasmid mutants; strain LG1418 gave negative results in both assays, whereas a mutant defective in siderophore transport (LG1419 *iut*; 16) was seen in both tests to overproduce chelator. Overproduction of siderophore by mutants defective in transport or assimilation of iron has also been reported for the enterobactin system of iron uptake (2). The ColV plasmid-specified hydroxamate siderophore has been identified by field desorption mass spectrometry as aerobactin, a compound originally shown to be secreted by *A. aerogenes* 62-1 (4). Authentic aerobactin was capable of supporting the growth of *E. coli* mutant strain LG1418 in conditions of iron deficiency.

It has been proposed that ColV plasmid-specified hydroxamate iron-chelating compounds are cell bound (13) and thus functionally different from the enterobactin system for iron uptake. This conclusion, which contrasts with the cell-free mode of action suggested by genetic data (16), is based on the observation that hydroxamates were chemically detectable in cell pellets of colicinogenic bacteria (13). We have also detected biological activity in cell pellets; active material released by sonication was identical in size (as indicated by gel filtration) to secreted siderophore. Moreover, biological activity was also detected in sonicated cell pellets of chelator-deficient mutant strain LG1418 which had been growing in the presence of exogenous chelator; cell-associated siderophore was released into the medium during subsequent growth in the absence of supplementation. These data suggest that there is transient association of aerobactin with bacterial membrane receptors rather than, as suggested by Stuart et al. (13), more permanent involvement of siderophore molecules as components of the bacterial membrane.

Aerobactin is synthesized by a number of enteric bacterial species. As well as strain 62-1 of *A. aerogenes* (for which the siderophore was named) and ColV plasmid-bearing invasive strains of *E. coli*, as reported here, clinical isolates of *Salmonella austin* and *Salmonella memphis*, a strain of *Arizona hinshawii* (Bindereif et al., manuscript in preparation), and strains of *Shigella flexneri* (6) have all been shown to secrete aerobactin. It is not known whether siderophore synthesis is plasmid encoded in all cases, but clearly there may be evolutionary or epidemiological relationships between the genetic determinants for aerobactin biosynthesis in these organisms. Furthermore, it is likely that mechanisms of iron transport across cell membranes may be similar among bacteria which utilize aerobactin as siderophore. These aspects are currently under investigation in our laboratories. Although aerobactin is the sole siderophore synthesized by some *Salmonella* and *S. flexneri* strains, other species are able to secrete both hydroxamate and catechol compounds. In the case of invasive strains of *E. coli* it is interesting to consider how aerobactin can confer marked selective advantage on a bacterial cell that also synthesizes the relatively high-affinity siderophore enterobactin. Catechol siderophores are, in general, chemically and enzymically less stable than hydroxamates; in addition, they are less soluble, and their iron complexes are very pH dependent. Furthermore, catechols are antigenic. It should also be noted that the biosynthesis of enterobactin and its hydrolytic cleavage to release iron within the cell require much metabolic energy. We may speculate, therefore, that in the conditions of extreme iron stress existing in the body fluids of a healthy individual the operation of the aerobactin iron uptake system, which requires little energy, may be crucial to the survival and proliferation of an invading bacterial cell.

ACKNOWLEDGMENTS

We thank A. Burlingame, Division of Research Resources, University of California, Berkeley, for mass spectrometry analysis.

This work was supported by Public Health Service grant RR00719 from the National Institutes of Health. Additional support was provided by Medical Research Council grant G979/461/C, Public Health Service grants AU04156 and AM17146 from the National Institutes of Health, and National Science Foundation grant PMC 78-12198.

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