Transport of ferric-aerobactin into the periplasm and cytoplasm of *Escherichia coli* K12: role of envelope-associated proteins and effect of endogenous siderophores

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Purified [^{14}C]aerobactin, supplied exogenously to non-growing bacteria, was translocated via the periplasm into the cytoplasm of *Escherichia coli* K12 strains expressing the aerobactin receptor protein IutA. No significant uptake was observed into either compartment of strains lacking the *iutA* gene or specifically defective in *tonB*. Uptake into both compartments was markedly reduced, but not abolished, in an *exb* mutant. Accumulation of [^{14}C]aerobactin in the periplasm of *fhuD*, *fhuB* or *fhuC* mutant strains was not significantly lower than in the wild-type strain, but entry into the cytoplasm was greatly reduced in all cases. Uptake of aerobactin by strains wild-type for all transport functions occurred most efficiently in strains either lacking or specifically defective in the genetic determinants for aerobactin biosynthesis; significantly lower levels of exogenous ^{14}C -labelled siderophore were observed in both compartments of strains producing aerobactin. Aerobactin-mediated ^{59}Fe uptake, however, was not inhibited by the presence of endogenous aerobactin. Endogenous enterochelin did not affect aerobactin uptake.

Introduction

Siderophore-mediated uptake of iron by *Escherichia coli* is initiated by binding of ferric-siderophore complexes to cognate outer-membrane receptor proteins (Braun & Hantke, 1981; Neilands, 1982). Transport of iron into the periplasmic space then requires the activity of the cytoplasmic membrane protein TonB, which is itself stabilized by ExbB, one of the products of the *exb* locus (Fischer *et al.*, 1989; Neilands, 1982). Subsequent passage of iron through the periplasm and across the cytoplasmic membrane requires the participation of several gene products which resemble periplasmic transport mechanisms described for some nutrients (Ames, 1986). These systems comprise a hydrophilic protein located in the periplasm, one or two very hydrophobic proteins in the cytoplasmic membrane, and a relatively hydrophilic protein, which is nonetheless also associated with the cytoplasmic membrane, containing regions of extensive homology with ATP-binding proteins. In *E. coli*, specific systems of this general type are involved in the transport of iron by enterochelin (Ozenberger *et al.*, 1987), citrate (Staudenmaier *et al.*, 1989), and hydroxamate siderophores (Burkhardt & Braun, 1987; Köster & Braun, 1989).

Aerobactin-mediated uptake of iron into *E. coli* cells requires the specific outer-membrane receptor protein IutA (Bindereif *et al.*, 1982; Grewal *et al.*, 1982) for initial binding of the ferric siderophore complex, and the common inner membrane protein TonB (and therefore presumably its functionally associated protein ExbB) for release of iron into the periplasm (Williams, 1979). Analysis of mutants (Braun *et al.*, 1982, 1983; Hantke, 1983), and characterization and localization of proteins (Fecker & Braun, 1983; Köster & Braun, 1989), suggest that the products of the *fhuB*, *fhuC* and *fhuD* genes form a common periplasmic binding system for uptake of iron via various hydroxamate siderophores, including aerobactin. FhuD is located in the periplasm, while the hydrophobic protein FhuB and the hydrophilic putative ATP-binding protein FhuC are located in the cytoplasmic membrane and are thought to comprise a cytoplasmic membrane permease complex. However, there are no reports of direct measurements of the uptake of any siderophore into well-defined cellular compartments of *E. coli*, nor physical confirmation of the assumed role of periplasmic binding protein systems in the translocation of siderophores to the cytoplasm. Indeed similar genetic evidence exists for the citrate-mediated iron-uptake system of *E. coli* (Frost & Rosenberg, 1973; Staudenmaier *et al.*, 1989; Wagegg & Braun, 1981), and

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yet citrate appears not to be transported across the cytoplasmic membrane (Hussein *et al.*, 1981).

Previously a sensitive binding assay for IutA activity involving radiolabelled aerobactin has been described (Roberts *et al.*, 1989). In this paper, the subsequent subcellular localization of receptor-bound aerobactin in wild-type *E. coli* and in mutants lacking each of the necessary envelope-associated functions is reported. Furthermore, the rate of uptake of exogenous aerobactin and of exogenous ferric iron was studied in the presence and absence of endogenous aerobactin.

Methods

Bacterial strains and plasmids. Characteristics of all strains and plasmids used in this study are described in Table 1. Recombinant plasmid pABN1 contains the entire iron-regulated aerobactin operon of the prototype ColV plasmid ColV-K30, cloned in the vector pPlac (Bindereif & Neilands, 1983). Plasmid pLG141 is a subclone of pABN1 carrying the receptor gene *iutA*, but lacking most of the siderophore biosynthesis (*iuc*) genes, cloned in the vector pACYC184 (Carbonetti & Williams, 1984); only polypeptides corresponding to the *iutA*, *iucD* and *cat* genes are expressed in minicells harbouring this plasmid. Plasmid ColV-K30*iuc* was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of a strain harbouring plasmid ColV-K30 (Williams & Warner, 1980). Plasmid pFB102 contains a *Pst*I-*Eco*RI fragment of *E. coli* chromosomal DNA that includes the entire *fhu* region containing a Tn5 insertion in *fhuD*; the fragment is cloned into the ampicillin-resistance gene of pBR322 (Fecker & Braun, 1983). Plasmid pBR322 was introduced into plasmid-bearing strains as a source of periplasmic β -lactamase (Sutcliffe, 1979).

Media and growth conditions. Bacteria were grown either in nutrient broth (Oxoid no. 2) or in M9 minimal salts medium (Roberts *et al.*, 1963) supplemented with 0.2% glucose, 0.5% Casamino acids and 20 μ g L-tryptophan ml⁻¹, and containing 200 μ M-2,2'-dipyridyl to induce

iron-regulated genes. Ampicillin (100 μ g ml⁻¹) was included in media for the growth of strains carrying plasmids pABN1 and/or pBR322; tetracycline (15 μ g ml⁻¹) was added to maintain plasmid pFB102 in strain LF940. Chloramphenicol (20 μ g ml⁻¹) was added to media for growth of strains harbouring pLG141. Cultures were grown aerobically with vigorous agitation at 37 °C.

Preparation of [¹⁴C]aerobactin. [¹⁴C]-labelled aerobactin was purified from culture supernatants of *Aerobacter aerogenes* (*Klebsiella pneumoniae*) strain 62-I incubated at 37 °C for 4 h in 10 ml M9 salts medium containing 185 kBq [¹⁴C]lysine (specific activity 11.99 GBq mmol⁻¹, Amersham). After removal of cells by centrifugation, culture fluid was lyophilized, redissolved in 0.5 ml distilled water and subjected to ascending chromatography on Whatman 3MM paper with a butanol/acetic acid/water (12:3:5, by vol.) solvent system. Labelled products were located by autoradiography, and material of *R_F* = 0.53 (Gibson & Magrath, 1969) was eluted in 1 ml distilled water. Aerobactin concentrations were determined from the molar absorption coefficient for ferric aerobactin as reported by Harris *et al.* (1979).

Preparation of [⁵⁹Fe/¹⁴C]aerobactin. [¹⁴C]Aerobactin prepared as above was dissolved in 1 mM-EDTA before chromatography, and labelled material migrating with *R_F* = 0.3 (apo-aerobactin) was eluted in 400 μ l distilled water. Siderophore concentrations were determined as described above (Harris *et al.*, 1979). To 400 μ l (313 nmol at 147 MBq mmol⁻¹) of this were added 40 μ l 1 M-Tris/HCl (pH 7.6), 4 μ l 20 mM-2,2'-dipyridyl, 3.7 μ l ⁵⁹Fe (3.45 nmol; 46 kBq, as Fe(II)SO₄ in 0.05 M-H₂SO₄, Dupont de Nemours, and 31 μ l 10 mM-FeCl₃. The mixture was incubated at room temperature for 30 min before use to allow the formation of [⁵⁹Fe/¹⁴C]aerobactin complex.

Cell fractionation. After incubation of bacterial suspensions with [¹⁴C]aerobactin or [⁵⁹Fe/¹⁴C]aerobactin, cells were separated into periplasmic and cytoplasmic fractions by a modification of the cold osmotic shock method of Nossal & Heppel (1966). Inner and outer membranes were separated by differential solubility in Triton X-100 (Schnaitman, 1971). Briefly, bacteria from 1 ml reaction mixtures were washed thoroughly in 10 mM-Tris/HCl (pH 7.5), 10 mM-EDTA containing 25% (w/v) sucrose, resuspended in 0.4 ml ice-cold water, and incubated on ice for 10 min. Cells were recovered by centrifugation.

Table 1. *Bacterial strains and plasmids*

Strain/plasmid	Relevant properties	Reference
Bacteria		
<i>E. coli</i> K12		
W3110	Wild-type	Bachmann (1972)
W3110-6	As W3110 but <i>exb</i>	Fischer <i>et al.</i> (1989)
AN1937	<i>ara entA lac leu mtl proC rpsL supE thi fhuA xyl</i>	Williams (1979)
LG1316	As AN1937 but <i>tonB</i>	Williams (1979)
LF940	<i>recA hsdM hsdR supE lacY leu thi pro aro fhuB fhuD</i>	Fecker & Braun (1983)
LF947	<i>recA minA minB lacY xyl thi mtl hsdM hsdR fepA fhuC12</i>	Fecker & Braun (1983)
BU736	<i>aroB cir tsx malT thi fhuB</i> ColV-K229	Braun <i>et al.</i> (1982)
LG1706	As BU736 but cured of plasmid ColV-K229	This study
<i>Aerobacter aerogenes</i> (<i>Klebsiella pneumoniae</i>)		
62-I	Aerobactin producer	Gibson & Magrath (1969)
Plasmids		
ColV-K30	IutA ⁺ Iuc ⁺ Cva ⁺	Williams (1979)
ColV-K30 <i>iuc</i>	IutA ⁺ Cva ⁺ <i>iuc</i>	Williams & Warner (1980)
pABN1	IutA ⁺ Iuc ⁺ Bla ⁺ (Ap ^R)	Bindereif & Neilands (1983)
pLG141	IutA ⁺ Cat ⁺ (Cm ^R)	Carbonetti & Williams (1984)
pBR322	Bla ⁺	Sutcliffe (1979)
pFB102	<i>fhuB fhuC fhuD::Tn5</i>	Fecker & Braun (1983)

Table 2. Uptake of [^{14}C]aerobactin into *E. coli* K12 strains lacking specific gene products involved in aerobactin uptake

Strain	Relevant characteristics	[^{14}C]Aerobactin uptake [pmol (mg protein) $^{-1}$]*			
		Periplasm	Cytoplasm	Inner membrane	Outer membrane
W3110(pLG141)	Wild-type	25.4 (60.7)	15.3 (36.6)	0.5 (1.2)	0.6 (1.4)
W3110	<i>IutA</i> $^{-}$	2.33 (37.4)	1.3 (20.9)	1.7 (27.3)	0.9 (14.4)
LG1316(pLG141)	<i>tonB</i>	3.22 (46.5)	1.1 (15.9)	1.7 (24.6)	0.9 (13.0)
W3110-6(pLG141)	<i>exb</i>	6.76 (45.5)	5.7 (38.4)	1.6 (10.8)	0.8 (5.4)
LF940(pFB102, pLG141)	<i>fhuD</i>	25.1 (93.3)	0.9 (3.3)	0.3 (1.1)	0.6 (2.2)
LG1706(pLG141)	<i>fhuB</i>	17.8 (75.7)	4.0 (17.0)	0.4 (1.7)	1.3 (5.5)
LF947(pLG141)	<i>fhuC</i>	21.8 (75.2)	3.7 (12.8)	1.6 (5.5)	1.9 (6.6)
AN1937(pLG141)	<i>entA</i>	33.3 (61.2)	20.0 (36.8)	0.5 (0.9)	0.6 (1.1)
W3110(pABN1)	<i>Iuc</i> $^{+}$	11.6 (77.9)	3.3 (22.1)	—	—

—, Not determined.

* Values in parentheses are percentages of total cell-associated label in each compartment.

Supernatant (periplasmic) fractions were removed and spheroplast pellets were resuspended in 0.4 ml 10 mM-Tris/HCl (pH 7.5). Spheroplasts were disrupted by sonication and subjected to ultracentrifugation (100000 *g* for 10 min). Supernatant (cytoplasmic) fractions were removed and pellets (membranes) were resuspended in 0.4 ml 10 mM-Tris/HCl (pH 7.5), containing 2% (v/v) Triton X-100 and incubated at room temperature for 30 min. After ultracentrifugation, supernatant fractions (cytoplasmic membranes) were removed and pellets (Triton-insoluble outer membranes) were resuspended in 0.4 ml of the same buffer. Radioactivity associated with each fraction was measured after addition of 4 ml Optiphase X scintillation cocktail (Pharmacia) in a Packard Tri-Carb 2000A liquid scintillation analyser. In single-label experiments ^{14}C emissions were measured in a window of 0–156 keV. For measurement of double-labelled cell fractions, ^{59}Fe activity was calculated from emissions measured in a window of 80–2000 keV (accounting for 25% of total ^{59}Fe emissions), and ^{14}C activity was calculated from the emissions measured in a window of 0–10 keV, after subtraction of the ^{59}Fe contribution (12% of total ^{59}Fe emissions); this accounts for 25% of ^{14}C emissions.

Enzyme assays of subcellular fractions. To determine the purity of periplasmic and cytoplasmic fractions, enzyme activities characteristic of the two cellular compartments were determined. The periplasmic enzyme β -lactamase, encoded by the *bla* gene of pBR322, was assayed by conversion of the chromogenic substrate nitrocefing (Glaxo) to a product quantifiable by absorbance measurements at 482 nm. The cytoplasmic marker was the chromosomally encoded enzyme malate dehydrogenase, assayed by conversion of oxaloacetic acid to a product quantifiable by absorbance measurements at 340 nm. Cytoplasmic fractions routinely contained less than 8% of total cellular β -lactamase activity, and periplasmic fractions consistently contained only 3% of total cellular malate dehydrogenase activity.

Results

Cellular localization of aerobactin

Various types of cells carrying plasmid pLG141 and expressing *IutA* protein constitutively (Carbonetti & Williams, 1984), were grown overnight in nutrient broth containing 200 μM -dipyridyl, washed and resuspended in

phosphate-buffered saline containing 200 μM -dipyridyl to an OD₆₀₀ of 1. ^{14}C -labelled aerobactin (approximately 4.5 nmol, specific activity 414 MBq mmol $^{-1}$) was added to 1 ml samples of cell suspension and incubated at 37 °C for 90 min. Uptake of radiolabelled aerobactin into the four cellular fractions of strains with various defined mutations affecting utilization of aerobactin as an iron source was determined as described in Methods (Table 2). Radioactivity associated with membrane fractions of all strains tested was negligible, suggesting only transient association of aerobactin. However, significant levels of radioactivity were observed in both the periplasmic and cytoplasmic fractions of the wild-type strain W3110(pLG141), but not of strain W3110, which lacks the plasmid-encoded *IutA* gene, nor of the *tonB* strain LG1316(pLG141). These data confirm the requirement of aerobactin iron transport for *IutA* and *TonB* functions (Bindereif *et al.*, 1982; Braun *et al.*, 1982; Williams, 1979). Uptake into both cellular compartments of the *exbB* strain W3110-6(pLG141) was also markedly reduced compared with W3110(pLG141), but was nonetheless significantly higher than into *tonB* cells. This is consistent with earlier findings that requirement for *exb* gene products is less stringent than for *TonB* in *TonB*-dependent uptake mechanisms (Fischer *et al.*, 1989).

Accumulation of [^{14}C]aerobactin in the periplasmic compartments of the three *fhu* mutant strains LF940(pFB102, pLG141), LG1706(pLG141) and LF947(pLG141) was similar to that in the periplasm of W3110(pLG141). However, uptake into the cytoplasm of the *fhuD* mutant LF940(pFB102, pLG141) was negligible, confirming that the periplasmic binding protein *FhuD* is absolutely required for translocation of aerobactin across the cytoplasmic membrane. By contrast, the *fhuB* and *fhuC* strains LG1706 and LF947 harbour-

ing pLG141 consistently showed low (compared with the wild-type) but significant levels of uptake of aerobactin into the cytoplasmic fraction. It is possible that defects in either of the cytoplasmic membrane functions involved in periplasmic transport of ferric-hydroxamate compounds are partially complemented by equivalent components of other systems. Alternatively, if the mutations do not result in complete absence of gene products, mutant proteins may have residual activity which could account for the observed leakiness. The precise nature of the genetic lesions in these mutants has not been determined.

Effect of endogenous siderophores on aerobactin uptake

Strain AN1937 is an *entA* mutant strain that is unable to synthesize enterochelin. A derivative of AN1937 harbouring plasmid pLG141 accumulated levels of radioactive aerobactin in the periplasmic and cytoplasmic fractions similar to those observed for the *Ent*⁺ strain W3110(pLG141) (Table 2). Thus, the ability to make enterochelin has no effect on the rate or extent of uptake of aerobactin from the medium. On the other hand, the presence of endogenous aerobactin appears to inhibit markedly the uptake of exogenously supplied aerobactin; strain W3110 carrying plasmid pABN1, which specifies aerobactin biosynthesis as well as receptor activity (*Iuc*⁺ *IutA*⁺), showed significantly lower levels of [¹⁴C]aerobactin in both cellular compartments compared with W3110(pLG141) (Table 2). Similar effects were observed with a strain containing the entire aerobactin plasmid ColV-K30 (data not shown); ¹⁴C levels associated with the periplasm and cytoplasm of strain W3110(ColV-K30) were two-thirds and half, respectively, of those observed for the corresponding compartments of strain W3110(pLG141). It should be stressed, however, that these plasmids are not directly comparable. First, the aerobactin operon in plasmids pABN1 and ColV-K30 is iron regulated (Roberts *et al.* 1986). Second, ColV-K30 has a copy number of one or two per cell, while pLG141 and pABN1 are multicopy plasmids.

To determine whether variations in levels of *iutA* transcription or gene dosage could account for these observations, the uptake of radiolabelled siderophore into isogenic W3110 strains containing either the prototype aerobactin plasmid ColV-K30, or a mutant derivative ColV-K30*iuc* (Williams & Warner, 1980) was compared. Bacteria were grown overnight in minimal medium containing dipyriddy to induce maximal transcription of the aerobactin operon and full expression of the *iutA* gene in both plasmids. Cells were incubated with [¹⁴C]aerobactin for 90 min as described above. The *iuc* mutant strain consistently accumulated significantly

more exogenously supplied aerobactin in both cellular compartments than did the wild-type *Iuc*⁺ strain ($P < 0.025$): 15.2 pmol (mg protein)⁻¹ compared with 5.4 pmol (mg protein)⁻¹ into the periplasm, and 12.6 pmol (mg protein)⁻¹ compared with 5.6 pmol (mg protein)⁻¹ into the cytoplasm. Values obtained for uptake into each compartment are means of readings from three independent experiments and were compared for significance using a test based on the Student-*t* distribution (Walpole, 1982).

A possible explanation for this difference is that the specific activity of [¹⁴C]aerobactin was reduced by unlabelled siderophore secreted by producing strains. Since less than 2.5% of the total radioactivity in a reaction mixture was actually taken up by receptive bacteria, the level of residual radioactivity in the reaction mixture was not measurably altered and buffers recovered after uptake assays could therefore be reused. Incubation buffers used to determine [¹⁴C]aerobactin uptake by strains W3110(ColV-K30) and W3110(ColV-K30*iuc*) were therefore subsequently used to label nutrient-broth-cultured W3110(pLG141) cells over a further 90 min incubation period. Virtually identical levels of uptake were observed [740 c.p.m. in cells incubated in buffer pre-incubated with W3110(ColV-K30) compared with 654 c.p.m. in cells incubated in buffer pre-incubated with W3110(ColV-K30*iuc*)], indicating that reduced accumulation of radioactivity by the strain carrying ColV-K30 was not due to significant changes in specific activity of external [¹⁴C]aerobactin. Values for uptake into W3110(pLG141) cells are means of two independent experiments.

The fate of iron taken up as ferric-aerobactin

To determine whether uptake of iron complexed with aerobactin, like uptake of aerobactin itself, was inhibited by the presence of endogenous siderophore, double label experiments using [⁵⁹Fe/¹⁴C]aerobactin were carried out. Bacteria were grown overnight in nutrient broth containing dipyriddy, washed and resuspended in phosphate-buffered saline containing dipyriddy and [⁵⁹Fe/¹⁴C]aerobactin (8 mmol, specific activity 14 MBq mmol⁻¹) to an OD₆₀₀ of 0.1. At intervals during incubation at 37 °C, duplicate 1 ml aliquots were removed for cell fractionation and determination of ⁵⁹Fe and [¹⁴C]aerobactin associated with subcellular compartments (Fig. 1). Accumulation of ⁵⁹Fe into the cytoplasmic fraction of W3110(pABN1) cells exceeded that of [¹⁴C]aerobactin by more than twofold over a 2 h period, while in the periplasm the reverse was the case (Fig. 1*a*). This is consistent with a model in which iron is removed, possibly at the cytoplasmic membrane, and aerobactin is rapidly recycled in aerobactin-producing

Fig. 1. Uptake of [⁵⁹Fe/¹⁴C]aerobactin by bacteria expressing the aerobactin receptor IutA. (a) The aerobactin-producing strain W3110(pABN1); (b) the aerobactin-deficient strain W3110(pLG141). Accumulation of ¹⁴C (open symbols) and ⁵⁹Fe (filled symbols) activity in the periplasmic (O, ●) and cytoplasmic (Δ, ▲) fractions was determined at intervals during incubation at 37 °C as described in Methods.

cells. The siderophore returns via the periplasm to the external medium. By contrast, uptake of aerobactin and iron into the non-aerobactin-producing cells of strain W3110(pLG141) occurred at similar levels into both compartments (Fig. 1b). These data strongly suggest that ferric-aerobactin enters the cytoplasm as an intact complex. Interestingly, at later time points, when significant levels of exogenously supplied iron may have accumulated, similar patterns of differential uptake of the two labels were observed in these cells also, albeit at a much lower magnitude than with the aerobactin-producing strain. No significant uptake into either compartment of cells of strain W3110 was observed (data not shown), ruling out the possibility that enterochelin (which all these strains are able to make) may be contributing to the observed assimilation of aerobactin-bound iron.

Discussion

The genes whose products make up periplasmic iron transport systems have been identified on the basis of the inability of mutant strains to use a particular siderophore as a sole iron source (Braun *et al.*, 1982, 1983; Hantke, 1983; Ozenberger *et al.*, 1987; Pierce *et al.*, 1983; Pressler *et al.*, 1988; Staudenmaier *et al.*, 1989). In general, constituent proteins were localized by analysis of particular subcellular compartments (Fecker & Braun, 1983; Köster & Braun, 1989; Ozenberger *et al.*, 1987; Pressler *et al.*, 1988; Staudenmaier *et al.*, 1989). Despite apparent similarities in genetic and physical organiz-

ation, however, the fate of the siderophore-iron complex as it traverses the cell envelope is not necessarily the same in each case. On the one hand, with ferric-dicitrate, iron accumulates in *E. coli* cells but citrate does not, suggesting that the complex is not transported intact into the cytoplasm, but rather that iron is removed within the cell envelope (Hussein *et al.*, 1981). On the other hand, the observation that some siderophores are chemically modified and then excreted after delivering their iron to *E. coli* cells provides indirect evidence for uptake into the cytoplasm. The hydroxamate siderophore ferrichrome, for example, is acetylated (Hartmann & Braun, 1980). Despite previous observations that acetylase activity could be demonstrated in cell-free membrane preparations (Schneider *et al.*, 1981), it is generally assumed that ferrichrome is modified after transport across the cytoplasmic membrane (Fischer *et al.*, 1989; Köster & Braun, 1989).

The catechol siderophore enterochelin is hydrolysed by a cytoplasmic esterase, possibly as a pre-requisite for release of iron, the reduction potential of ferric-enterochelin at neutral pH being outside the range of biological reductants (O'Brien *et al.*, 1971). It has also been suggested, however, that the relatively low pH of the periplasmic compartment may allow reductive removal of iron from enterochelin before passage into the cytoplasm (Ecker *et al.*, 1986). Indeed, in faintly acidic solutions, the siderophore itself tends to reduce ferric iron (Hider, 1984). Further support for the suggestion that cytoplasmic ferric-enterochelin esterase may not in fact be required for removal of iron *in vitro* comes from the observation that synthetic analogues of enterochelin

that are not susceptible to hydrolysis are nonetheless biologically active in growth promotion assays (Heidinger *et al.*, 1983). Thus, it is still unclear whether ferric-enterochelin is delivered as an intact complex to the cytoplasm, or if iron is removed by reduction in the periplasm.

Unlike ferrichrome and enterochelin, aerobactin seems not to be modified after assimilation, and may be recycled for subsequent rounds of iron uptake (Braun *et al.*, 1984). In this paper direct evidence for accumulation of aerobactin in the periplasm and cytoplasm of non-growing bacteria that do not themselves make aerobactin is provided. In general, higher absolute levels were observed in the periplasm, despite the fact that the volume of this compartment under normal conditions is significantly less than that of the cytoplasm (Stock *et al.*, 1977). This is not surprising, however, since it has been known for some time that siderophore uptake across the outer membrane may be more rapid than across the cytoplasmic membrane (Fecker & Braun, 1983). The physiological relevance of this observation is confirmed by the behaviour of various mutants unable to grow with aerobactin as an iron source. Thus, *iutA*, *tonB* and *exb* mutant strains exhibited significantly reduced levels of uptake of radiolabelled aerobactin into the periplasm. On the other hand, *fhuB*, *fhuC* and *fhuD* mutant cells showed normal accumulation of aerobactin in the periplasm, but were defective in transport of the siderophore into the cytoplasm. Presumably, aerobactin bound to IutA is released into the periplasm by a TonB(Exb)-dependent mechanism that does not require the periplasmic FhuD protein. FhuD subsequently binds aerobactin in the periplasm and delivers it to the cytoplasmic membrane permease complex FhuBC, which in turn catalyses transport into the cell. This is in contrast to the uptake of maltodextrins, which are taken up by a process of facilitated diffusion via the outer-membrane protein LamB (Wandersman *et al.*, 1979). In this case the periplasmic binding protein MalE seems to be required for transport across both outer and cytoplasmic membranes.

Uptake of aerobactin by aerobactin-producing strains was significantly lower than by strains that expressed only the aerobactin receptor gene *iutA*. A similar observation was previously reported for schizokinin-mediated iron uptake into the Gram-positive organism *Bacillus megaterium* (Arceneaux *et al.*, 1973); exogenously supplied ferric-schizokinin remained associated with bacteria in which intracellular siderophore concentrations were low, but was rapidly lost from schizokinin-producing cells. By contrast, however, uptake into the cytoplasm of the iron component of ferric-aerobactin was unaffected by the presence of endogenous siderophore. Preferential accumulation of

iron over aerobactin in the cytoplasm might result from rapid re-excretion of siderophore after removal of iron within the cytoplasm. If this were the case a similar effect would be expected in cells that do not produce aerobactin; however, iron and aerobactin accumulate at comparable levels in the cytoplasm of strain W3110(pLG141). Another possibility is that iron is removed from the labelled aerobactin in the periplasm by endogenous aerobactin and delivered to the cytoplasm. Levels of endogenous aerobactin found in the periplasm are not significantly greater, however, than the levels of aerobactin taken up, and unless one proposes a very rapid exchange of iron between aerobactin molecules this could not account for the observed reduction of uptake of labelled aerobactin across the cytoplasmic membrane. Kinetic exchange between siderophore molecules has in fact been reported to be extremely slow (Tufano & Raymond, 1981).

It is therefore proposed that when endogenous aerobactin levels are high some of the receptor-bound exogenous aerobactin is released from the cytoplasmic membrane, perhaps following reduction of ferric iron, or exchange of ferric ions with internal siderophore molecules. The mechanism by which such exchange could occur, however, is not known. Previously it has been proposed that iron supplied to iron-stressed bacteria by aerobactin, unlike that delivered by enterochelin, exists in an intracellular complexed form rather than being released into a cytoplasmic pool, since cells using aerobactin for growth were insensitive to the iron-dependent antibiotic streptonigrin (Williams & Carbonetti, 1986). One possibility suggested by the data presented here is that aerobactin may have a role in intracellular storage and mobilization of ferric iron within the cytoplasm.

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