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Iron, Siderophores, and the Pursuit of Virulence: Independence of the Aerobactin and Enterochelin Iron Uptake Systems in *Escherichia coli*

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Many strains of *Escherichia coli* isolated from extraintestinal infections of humans and domestic animals are able to synthesize two siderophores, aerobactin and enterochelin. Although aerobactin has a dramatically lower affinity for iron than enterochelin, it has been shown to provide a significant selective advantage for bacterial growth in conditions of iron limitation, such as in the body fluids and tissues of an infected animal. We have used streptonigrin, which is bactericidal in the presence of iron, as a probe to determine levels of free intracellular iron during bacterial growth promoted by the two siderophores. A strain with only enterochelin remained sensitive to the bactericidal action of streptonigrin, suggesting that assimilated iron was contributed to an intracellular pool from which the rate of its withdrawal for growth is probably concentration dependent. On the other hand, a strain that synthesized aerobactin alone became resistant to streptonigrin, indicating that iron complexed with aerobactin was not made accessible to streptonigrin and suggesting that it may be channeled directly to where it is required for growth. Aerobactin, probably because it is repeatedly reusable, efficiently stimulated bacterial growth at external concentrations some 500-fold lower than those of enterochelin. Moreover, the effective concentration, and thus the siderophore activity, of enterochelin but not of aerobactin was significantly reduced by the presence of human serum in the medium. Differential regulation of the genetic determinants of the two siderophores resulted in preferential induction of the aerobactin system in the presence of unsaturated levels of transferrin and lactoferrin.

Iron is essential for bacterial growth. However, although it is a very abundant element in nature, the very low solubility of the ferric form renders it virtually unavailable without the activity of specific high-affinity mechanisms for its transport across bacterial membranes (19). Enteric bacteria synthesize the catechol iron-binding compound (siderophore) enterochelin (also called enterobactin), which is secreted into the surrounding medium and then actively taken up, complexed with ferric ions, via specific inner and outer membrane components (24). Some strains of *Escherichia coli*, in particular those isolated from extraintestinal infections of humans and domestic animals, also secrete the hydroxamate siderophore aerobactin (25). This property confers on them a significant selective advantage for growth in conditions of iron limitation (28, 29) such as exist in the body of an infected animal (26) because of the presence at only partial iron saturation of the iron-binding glycoproteins transferrin (in serum) and lactoferrin (in secretions). We demonstrate in this paper that, despite a markedly lower affinity for iron than enterochelin and indeed than transferrin (19), aerobactin possesses physiological and regulatory features which result in more effective provision of ferric ions for bacterial growth.

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MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains used in this study are listed in Table 1. Cells were cultured at 37°C with aeration by shaking in Oxoid nutrient broth or in M9 minimal salts medium (23) as indicated in the text. Iron-chelating agents were added to the media as required to induce the expression of iron transport systems. Human serum transferrin, purified by passage through a column of specific antiserum linked to Sepharose, was generously provided by Robert Evans, Division of Biochemistry, Guy's Hospital, London, England; human lactoferrin, purified by the method of Johansson (14) and shown to be pure by immunoelectrophoresis with antiserum raised against whole human milk, was kindly donated by Jeremy Brock, Department of Bacteriology and Immunology, University of Glasgow, Scotland. α,α′-Dipyridyl was purchased from Sigma Chemical Co.

Streptonigrin sensitivity. Samples of bacterial cultures were harvested by centrifugation, washed, and suspended to an optical density at 450 nm of 0.5 in prewarmed (37°C) phosphate-buffered saline (pH 7). Streptonigrin (Rhône-Poulenc Centre de Recherches de Vitry; batch VIL 571A) was added to a final concentration of 40 μM, and samples were taken at intervals during incubation at 37°C for estimation of viable cell counts on nutrient agar.

Siderophore preparation and estimation. Enterochelin was prepared from culture supernatants of strain AN263 by anion exchange chromatography on DEAE-cellulose (22) and quantified with the nitrite-molybdate reagent of Arnow (1), with dihydroxybenzoic acid as a standard. Aerobactin was prepared from culture supernatants of strain LG1419 by anion-exchange chromatography on Dowex-1 (25) and estimated by the method of Csáky (8) slightly modified as previously described (25), with hydroxylamine hydrochloride as a standard. The fact that parallel preparations from mutant strains defective in siderophore biosynthesis yielded no detectable positively reacting material in either test

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TABLE 1. Bacterial strains

Strain	Characteristics[a]	Reference or source
W3110	*rpsL* (Ent$^+$)	28
KH576	W3110, *nalA* carrying plasmid ColV-K30 (Iu$^+$)	28
AN1937	*ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl*	28, 29
LG1315	AN1937, carrying plasmid ColV-K30 (Iu$^+$)	28, 29
LG1418	AN1937, *nalA* carrying plasmid ColV-K30 *iuc*	29
LG1419	AN1937, *nalA* carrying plasmid ColV-K30 *iut*	29
AN263	*ara fepA lac leu mtl proC rpsL supE thi tonA trpE xyl*	I. G. Young

[a] Ent$^+$ represents the phenotype enterochelin biosynthesis and utilization; *entA* and *fepA* are chromosomal mutations causing defective enterochelin biosynthesis and ferric-enterochelin receptor activity, respectively. Iu$^+$ represents the phenotype aerobactin biosynthesis and utilization; *iuc* and *iut* are ColV-K30 plasmid mutations resulting in defective aerobactin biosynthesis and ferric-aerobactin receptor activity, respectively.

indicates that the two chemical assays could be used for these estimations, despite their obvious lack of specificity. Note that both of the producing strains synthesize a siderophore that they cannot take up; this is particularly important in the case of enterochelin, whose utilization in iron metabolism results in excretion of degradation products which would be detected in the Arnow reaction (17).

^{55}Fe uptake. Uptake of radioactive iron into nongrowing bacterial cells was determined as described previously (28). Medium containing approximately 0.4 μM iron was made with double glass-distilled deionized water and ultrapure chemicals, and all glassware was washed with 0.5% (wt/vol) EDTA and rinsed with double-distilled water before use (17). Carrier-free $^{55}FeCl_3$ (specific activity, >50 mCi/mg of Fe) was supplied by Amersham International UK.

RESULTS

Streptonigrin as a probe for free intracellular iron. Streptonigrin is an aminoquinone antitumor agent. It can kill bacterial cells by degrading the DNA, a process which depends upon the presence of iron (27, 30), and we have exploited this bactericidal effect to attempt to monitor levels of free intracellular iron. Strain AN1937 is defective in the biosynthesis of enterochelin (*entA*). Therefore, unless it is cultured in an iron-rich medium, in which nonspecific or passive uptake of ferric ions can occur (18), AN1937 can acquire iron only if provided exogenously with citrate (9) or siderophores (including, of course, enterochelin) which it is able to utilize. Figure 1a shows the growth curve of strain AN1937 in appropriately supplemented M9 minimal medium; the culture was inoculated with nutrient broth-grown cells and subsequently subcultured into fresh minimal medium to maintain logarithmic growth for as long as possible. Samples of the culture were removed at the times indicated, washed, suspended in phosphate buffer, and tested for sensitivity to the bactericidal activity of streptonigrin (Fig. 1b).

The culture grew with a mass doubling time of approximately 40 min for about 4 h. Prior growth in an iron-rich medium, such as nutrient broth, undoubtedly allows accumulation of intracellular pools of iron that permit several generations of growth upon subculture into iron-limited minimal medium (approximately 2 μM). Eventually, growth of the culture ceased, presumably when the intracellular iron supply was depleted, although addition of sodium citrate to the static culture promoted rapid restoration of active cell division. Consistent with these proposed physiological changes is the observation that strain AN1937 was very sensitive to the bactericidal action of streptonigrin at the time of subculture from iron-rich nutrient broth but became increasingly resistant during growth in minimal medium as the intracellular iron pool was progressively depleted. Sensitivity to streptonigrin was regained when citrate was added, due to immediate uptake of iron existing in the medium as ferric-citrate.

Identical results were obtained for three other mutant

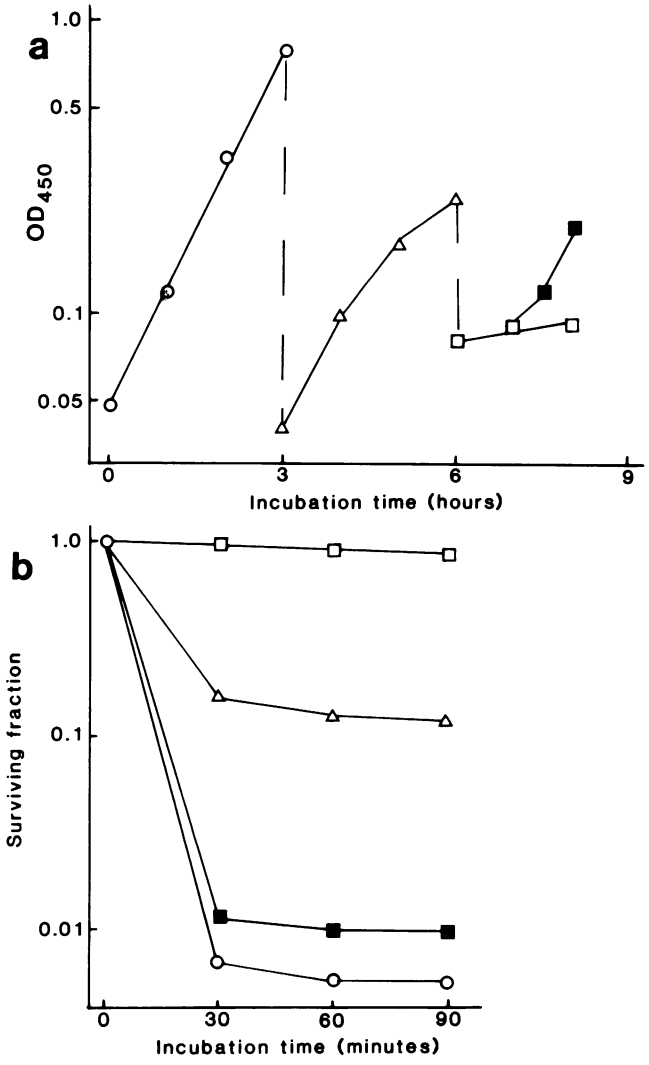

FIG. 1. Streptonigrin sensitivity and growth-dependent depletion of intracellular iron pools. (a) Nutrient broth-grown cells of *E. coli* AN1937 (*entA*) were washed, suspended in M9 medium, and incubated with aeration at 37°C (○). Bacterial growth was monitored by measurement of optical density at 450 nm (OD_{450}), and cultures were diluted with fresh medium at 3 (△) and 6 (□) h. Sodium citrate (10 mM) was added to a portion of the culture (■) after cessation of growth. (b) Samples were removed for determination of streptonigrin sensitivity as described in Materials and Methods at 0 (○), 3 (△), and 6 (□) h of incubation and at 1 h after the addition of sodium citrate (■).

strains defective in high-affinity iron transport systems (Fig. 2). AN263 synthesizes enterochelin but is unable to utilize it because of a mutation (*fepA*) in the ferric-enterochelin receptor protein gene (11, 21), and strains LG1418 and LG1419 are derivatives of AN1937 harboring ColV-K30 *iuc* (deficient in aerobactin biosynthesis) and *iut* (defective ferric-aerobactin receptor protein) plasmids, respectively (29).

Streptonigrin sensitivity during siderophore-mediated iron uptake. Strain W3110 secretes enterochelin (Ent^+). Strain LG1315 is the *entA* strain AN1937 carrying plasmid ColV-K30, which specifies aerobactin biosynthesis (Iu^+). Both strains grew at the same constant rate (doubling time, 40 min) for at least 10 cycles of subculture into fresh minimal medium as described in the legend to Fig. 1. Samples were removed at intervals for determination of streptonigrin sensitivity (Fig. 2); strain W3110 remained sensitive to streptonigrin during growth after a shift from nutrient to minimal medium, but by contrast, growth of strain LG1315 at the maximal rate was accompanied by rapid loss of streptonigrin sensitivity. These data suggest that, while enterochelin releases its iron into the intracellular pool, iron assimilated by aerobactin somehow remains inaccessible to streptonigrin.

TABLE 2. Effect of iron chelators on streptonigrin sensitivity of a strain able to synthesize both aerobactin and enterochelin

Strain	Additions to M9 medium (μM)	Survival (%)[a]
W3110 (Ent^+)[b]		0.85
LG1315 (Iu^+)[b]		62.1
KH576 (Ent^+ Iu^+)		58.9
KH576	Dipyridyl (10)	55.4
	Dipyridyl (50)	6.7
	Dipyridyl (200)[c]	0.9
KH576	Transferrin (1.3)	58.1
	Transferrin (6.5)	45.7
KH576	Lactoferrin (1.3)	59.4
	Lactoferrin (6.5)	53.6

[a] Percent survival (40 μM streptonigrin, 1-h incubation, 37°C) of samples taken 9 h after subculture from nutrient broth into minimal medium (and diluted subsequently at three hourly intervals as described for experiments shown in Fig. 1 and 2).
[b] Data from Fig. 2 (9-h samples).
[c] Doubling time was approximately 55 min in the presence of 200 μM dipyridyl (compared with 40 min for all other cultures).

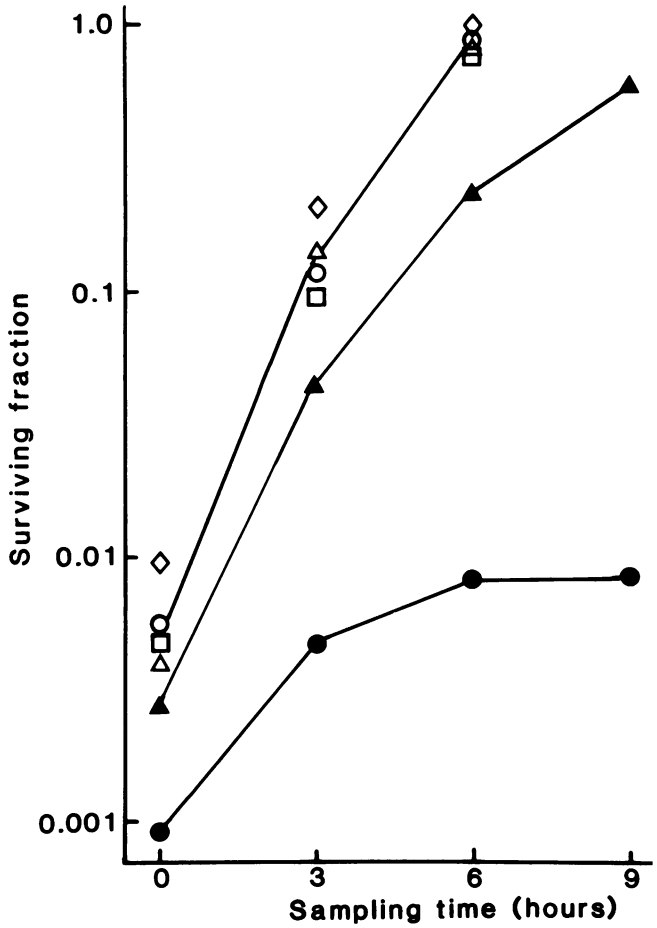

FIG. 2. Changes in streptonigrin sensitivity of bacterial strains during siderophore-promoted growth in iron-limiting conditions. Nutrient broth-grown cells were washed, suspended in M9 medium, and incubated with aeration at 37°C. Cultures were diluted in fresh minimal medium to an optical density at 450 nm of approximately 0.1 at three hourly intervals. Samples were taken at the times indicated for determination of streptonigrin killing during 1 h of incubation at 37°C as described in Materials and Methods. Strains tested were AN1937 (○; data from Fig. 1), AN263 (△), LG1418 (□), LG1419 (◇), W3110 (●), and LG1315 (▲).

Streptonigrin sensitivity of a strain able to synthesize both enterochelin and aerobactin. Strain KH576 synthesizes both enterochelin and aerobactin and so was able to grow at a constant rate throughout many cycles of subculture in minimal medium as described in Fig. 1. After 9 h of active growth, the streptonigrin sensitivity of this strain (Table 2) was only marginally greater than that of LG1315 (which makes aerobactin alone), suggesting that in KH576 iron is taken up predominantly by the aerobactin system. However, in conditions of iron limitation imposed by addition of the iron-chelating agent α,α'-dipyridyl to the medium, cells were markedly more sensitive to streptonigrin, indicating induction of the enterochelin system and its consequent greater involvement in iron uptake into these cells, particularly at higher concentrations of the chelator. Interestingly, addition of purified human transferrin or lactoferrin did not result in a similar enhancement in streptonigrin sensitivity, suggesting that the presence of these glycoproteins, even at only partial iron saturation, may not significantly induce the enterochelin system in cells that can synthesize both types of siderophore.

Bacterial growth at low siderophore concentrations. Strain LG1418 can synthesize neither aerobactin (*iuc*) nor enterochelin (*entA*), but carrying *fepA iut*, it can take up both siderophores supplied exogenously. With a culture of LG1418 incubated in minimal medium to deplete intracellular iron pools (as described for Fig. 1 and 2), it was shown that concentrations of exogenous aerobactin in excess of 5 nM were adequate to stimulate growth at maximum rate (mass doubling time, approximately 40 min), and that even a concentration as low as 0.5 nM promoted slow but significant growth (Fig. 3a). By contrast, the minimum exogenous enterochelin concentration for optimum growth was over 500 nM, and virtually no bacterial growth was observed below 50 nM (Fig. 3b). Fresh human serum (10% [vol/vol]) added to portions of culture containing the lowest concentration of either siderophore adequate to support normal growth resulted in severe inhibition of growth in the presence of enterochelin but did not affect aerobactin-promoted growth.

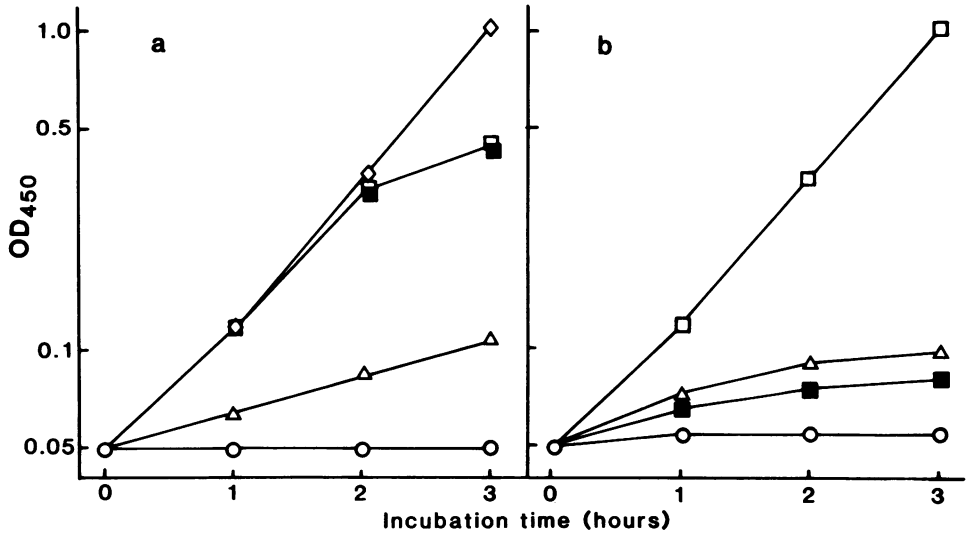

FIG. 3. Siderophore concentration optima for growth of iron-starved strain LG1418. Strain LG1418 (*entA iuc*) was preincubated in M9 medium until cessation of cell division, as described for Fig. 1 and 2. (a) Aerobactin was added to 10-ml portions of culture at concentrations of 0.5 (△), 5.0 (□, ■), and 50 (◇) nM. Higher concentrations of aerobactin (500 or 5,000 nM) gave the same rate of growth as that shown for 50 nM. In a control culture (○), no siderophore was added. (b) Enterochelin was added to 10-ml portions of culture at concentrations of 50 (○), 500 (△), or 5,000 (□, ■) nM. No growth was observed at lower concentrations (5.0 or 0.5 nM) of enterochelin (data not included). Bacterial growth was monitored by measurement of optical density at 450 nm during incubation with aeration at 37°C. Solid symbols represent cultures to which human serum (10% [vol/vol]) was added. Samples of each culture were removed 2 h after addition of siderophores for estimation of streptonigrin sensitivity as shown in Table 3.

Samples were taken from each of the cultures to determine their streptonigrin sensitivity (Table 3). LG1418 cells growing on exogenously supplied enterochelin became markedly sensitive to streptonigrin; even at a ferric-enterochelin concentration that was rate limiting for growth, only 4% of the population survived treatment with the drug. On the other hand, cells supplied with aerobactin remained streptonigrin resistant during growth. These data confirm the previous suggestion that iron sequestered by aerobactin is channeled as required, rather than released into an intracellular pool.

Iron uptake at low siderophore concentrations. Figure 4 shows the total uptake of radioactive iron into nongrowing cells of strain LG1418 during a 1-h incubation period in the presence of the same range of siderophore concentrations as used in the experiment shown in Fig. 3. Note that dilution of aerobactin over a 10,000-fold concentration range resulted in only a fivefold overall reduction in assimilation of ^{55}Fe; on the other hand, enterochelin was more effective than aerobactin at high concentrations, but dilution to levels that were suboptimal for growth (Fig. 3) resulted in a corresponding exponential reduction in the efficiency of iron uptake into bacterial cells. This represents the uptake of a single mole of iron per mole of enterochelin in the incubation mixtures at these low concentrations, while at equivalent low concentrations of aerobactin each siderophore molecule acts as a shuttle, delivering several ferric ions to a bacterial cell even in the absence of growth.

DISCUSSION

The association constant of ferric-aerobactin is many orders of magnitude lower than that of ferric-enterochelin (approximately 10^{23} and 10^{52}, respectively [19]). While it should, of course, be noted that such values are derived for completely deprotonated ligands so that they have little direct relevance to physiological conditions (13), nevertheless it is clear that aerobactin is thermodynamically inferior to enterochelin and indeed even to transferrin (the association constant of ferric-transferrin is approximately 10^{30} [26]). It is interesting to speculate, therefore, how aerobactin is able to confer significant selective advantage for growth in vivo upon bacterial cells which can synthesize the two siderophores. Exchange of iron between transferrin and aerobactin probably proceeds through the initial formation of a ternary complex in which iron is bound to both compounds (15). In fact the rate of removal of iron from transferrin to aerobactin is significantly higher than to other hydroxamate chelators with apparently superior affinities for iron, implying that the chemical structure of the competing ligand rather than merely its affinity for ferric ions plays an important role in the acquisition of iron. In this paper we present evidence that, in addition to particular structural features as yet undefined, aerobactin possesses physiological and regulatory properties which enhance its efficiency as a siderophore in environmental conditions of extreme iron stringency. Indeed, the fact that aerobactin is virtually never found to be the sole siderophore synthesized by isolates of *E. coli* (unpublished data) may be due in large part to the fact that its receptor is also recognized by a range of bacteriocins

TABLE 3. Streptonigrin sensitivity of strain LG1418 grown with exogenously supplied siderophores

Siderophore concn (nM)	Survival (%)[a] during growth on:	
	Aerobactin	Enterochelin
0.5	100	100
5.0	100	85
50	98	22
500	90	4.0
5,000	75	0.2

[a] Percent survival (40 μM streptonigrin, 1-h incubation, 37°C) of samples taken 2 h after addition of siderophores at the concentrations indicated.

(7, 10), so constituting a major selective disadvantage in natural (mixed) bacterial populations.

First, our data indicate that aerobactin is in some way able to compartmentalize iron so that activation of the bactericidal drug streptonigrin is prevented. It is somewhat puzzling, therefore, that Braun et al. (5) were able to isolate aerobactin-defective mutants by selecting for streptonigrin resistance, although it should be noted that under normal culture conditions part of the population is streptonigrin sensitive, suggesting that iron is taken up in excess of immediate growth requirements. Nevertheless, it is tempting to speculate that our observations reflect the more efficient utilization of the iron assimilated by aerobactin, in that it may be channeled directly for bacterial growth rather than as with enterochelin and citrate contributed to an intracellular pool from which its rate of withdrawal for growth is probably concentration dependent. Even the potential growth advantage of an apparently greater efficiency of iron uptake by high enterochelin concentrations may thus be dissipated because assimilated iron is released to an intracellular pool.

Second, aerobactin is more effective than enterochelin even at very low concentrations of siderophore, a property that is likely to be particularly advantageous in the dynamic and fluid environments of the bloodstream and the urinary tract, in which any extracellular bacterial product will be continually diluted. The substantial difference between the two siderophores may be due to the fact that while enterochelin is enzymatically degraded during intracellular release of ferric ions and therefore wasted (24), iron is more readily released from aerobactin, and the siderophore may subsequently be resecreted (3). There is certainly a major intrinsic difference in the efficiencies with which the two compounds transport iron even into nongrowing bacterial cells, and this is particularly striking at very low siderophore concentrations. Each molecule of enterochelin probably delivers only a single ferric ion to receptive bacteria, while aerobactin appears to shuttle iron to cells, even in the absence of active metabolism. Konopka et al. (15) have demonstrated that aerobactin is more effective than enterochelin at equal molarity in delivering iron from transferrin to bacterial cells in human serum. It is suggested that this may be due to the fact that enterochelin binds to serum proteins so that its activity as a siderophore is effectively reduced. Consistent with this is our observation that the presence of serum in the media used to determine limiting siderophore concentrations for growth of LG1418 cells markedly reduced the effectiveness of enterochelin but had no significant influence on aerobactin-mediated iron assimilation. Thus the relative inefficiency of enterochelin is likely to be exacerbated within an infected animal body by siderophore inactivation due to binding to serum proteins.

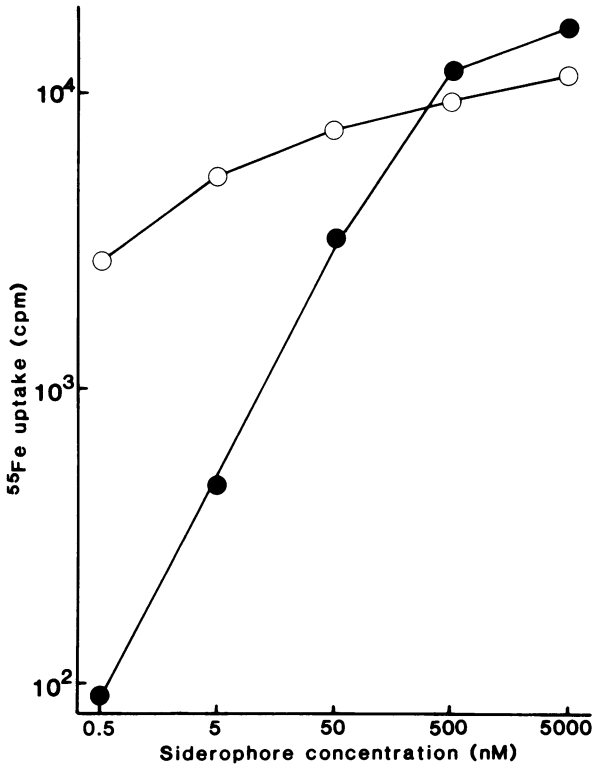

FIG. 4. Effects of siderophore concentration on uptake of radioactive iron by iron-starved strain LG1418. Strain LG1418 (*entA iuc*) was preincubated in M9 medium until cessation of growth as described for Fig. 1 and 2. Cells were harvested by centrifugation, washed, and suspended in low-iron (0.4 μM) medium lacking glucose and essential amino acids but containing 100 μM sodium nitrilotriacetate and the siderophore aerobactin (○) or enterochelin (●) at the concentrations indicated. Carrier-free $^{55}FeCl_3$ (1 μCi/ml) was added, and suspensions were incubated with aeration for 120 min at 37°C. Cells from 5 ml of suspension were washed thoroughly on membrane filters with low-iron medium containing 100 mM sodium citrate. Filters were air dried, and the amount of ^{55}Fe label retained was counted in a Packard Tricarb liquid scintillation counter; points are the averages of duplicate samples.

Finally, the genetic determinants of aerobactin biosynthesis and uptake are expressed more readily than those of enterochelin in response to iron limitation. Williams showed several years ago (28) that the operation of the ColV-K30 plasmid-encoded iron uptake system had a marked sparing effect on the expression of the outer membrane proteins (including the ferric-enterochelin receptor) characteristically induced in conditions of iron stress. The clear implication of this is that synthesis of adequate levels of aerobactin to maintain maximal growth is possible even when other iron-regulated gene expression is repressed. Furthermore, in the present paper we suggest that the relative streptonigrin resistance of a strain that is able to synthesize both aerobactin and enterochelin growing in the presence of transferrin and lactoferrin at only partial iron saturation indicates that aerobactin is preferentially active in conditions of iron stress, possibly due to differential expression of the aerobactin and enterochelin systems. Indeed, only in the very extreme stress imposed by the presence of α,α′-dipyridyl at a concentration of 50 μM or greater was significant activity of the enterochelin system apparent. This may, in part at least, reflect differences in genetic and regulatory complexity. The arobactin cluster of five genes is 7.5 kilobases long (6) and is probably arranged as a single operon (2), while the enterochelin system comprises as many as ten genes (of which seven are required for siderophore biosynthesis) scattered over about 20 kilobases of the chromosome and organized in several transcriptional units (16, 20). It has been suggested that iron uptake systems in enteric bacteria have common regulatory components; for example, mutations in the *fur* (ferric uptake regulation) gene cause constitutive expression of both the enterochelin and aerobactin systems (4, 5, 12). However, one interpretation of our data, that the two systems may have markedly different sensitivities to iron stress, suggests the action of a rather more complex regulatory mechanism than has been proposed previously.

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