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THE INDUCIBLE CITRATE-DEPENDENT IRON TRANSPORT SYSTEM IN *ESCHERICHIA COLI* K12

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SUMMARY

A citrate-dependent system of iron uptake was found in wild-type cells of *Escherichia coli* K12, and in mutants which were unable, for a variety of reasons, to make use of the enterochelin system of iron transport. A mutant strain which did not show a growth response to citrate, was shown to lack the citrate-dependent iron uptake system. Identification of this citrate-dependent system was facilitated in the presence of nitrilotriacetate, which abolished a low-affinity iron uptake observed in the absence of added chelators.

In growing cells, induction of the citrate-dependent system occurred within 20 min of exposure to citrate, and required new protein synthesis. The citrate-dependent system has a lower initial rate of iron uptake and a slightly lower affinity for iron (K_m 0.2 μM) than the enterochelin-mediated system (K_m 0.1 μM). In cells with both systems operating optimally, the overall initial rate of iron uptake was equal to the sum of the uptakes contributed by the two individual systems. The inducible citrate-dependent system is thus entirely separate from the repressible enterochelin-mediated system, and the two systems can operate simultaneously in wild-type cells.

INTRODUCTION

When growing under iron-deficient conditions, wild strains of *Escherichia coli* excrete the iron-chelating ligand, enterochelin[1], into the medium, and subsequently transport the ferric–enterochelin complex very efficiently back into the cell. Whilst this system has been fairly extensively studied[2–4], the specific effect of citrate on iron uptake has not previously been generally recognised, although this ligand was known to stimulate growth of *E. coli* mutants deficient in iron assimilation[2,4–6] and had been used in the isolation of such mutants[4].

Mutants of *E. coli* which are blocked in the common pathway of aromatic biosynthesis (for example *aroB*$^-$) are unable to synthesize enterochelin, and hence fail to grow under iron-deficient conditions, unless supplemented with enterochelin, or its metabolic precursor 2,3-dihydroxybenzoate[5,7,8]. Young *et al.*[5] first reported the ability of sodium citrate to replace dihydroxybenzoate as a growth factor for such mutants in conditions of iron deficiency. Subsequently Somerville[9] used a similar

aromatic auxotroph strain of *E. coli* to show that mutants which had lost the ability to grow on medium containing citrate were still able to respond to dihydroxybenzoate. These mutants carried a deletion in the tryptophan region of the genome. More recently Cox *et al.*[2] have briefly mentioned an inducible citrate-stimulated iron uptake in a strain of *E. coli* K12 unable to transport ferric–enterochelin (*fep*$^-$).

When assaying iron uptake under conditions similar to those reported earlier[2] we have frequently experienced difficulty in distinguishing between citrate-stimulated iron uptake and a low-affinity iron uptake observed in the absence of any specific iron-chelating ligands. Recently we have described the successful use of nitrilotriacetate to inhibit this low-affinity uptake, when measuring ferric–enterochelin transport[4]. In the present study, using a series of mutants unable to produce enterochelin, we have extended the use of nitrilotriacetate to the study of the citrate-dependent iron uptake system in *E. coli* K12.

MATERIALS AND METHODS

Chemicals

Chemicals used were of the highest purity commercially available and batches with the lowest iron content were chosen. They were not further purified. $^{55}FeCl_3$ in 0.1 M HCl was obtained from the Radiochemical Centre, Amersham, Bucks., Great Britain. Enterochelin was isolated from culture supernatants as described previously[3]. All water was doubly glass-distilled before use. Sterile stocks of citrate and nitrilotriacetate were prepared by adjusting solutions of the corresponding free acid to pH 6.9 with an equimolar mixture of NaOH and KOH before autoclaving.

Bacterial strains

All the *E. coli* strains used, with the exception of AN299 have been described previously[2,4]. Strain AN299 (derived from strain AN92 (ref. 4)) is a mutant which was isolated during a search for mutants defective in the citrate-dependent system of iron transport (Woodrow, Langman, Young and Gibson, unpublished results) and was obtained from I. G. Young. This strain is shown in the present paper to be incapable of citrate-mediated iron uptake.

Media

The iron-deficient media used for growth of cells and for the measurement of iron uptake, have been described previously, as has the procedure for preparation of the iron-free glassware used throughout this work[4]. Uptake medium used in K_m determinations was extracted with chloroform in the presence of 8-hydroxyquinoline as described by Waring and Werkman[10], in order to remove the bulk of the residual endogenous iron. Chloroform extractions were continued until no further ultraviolet absorption, attributable to 8-hydroxyquinoline, was detected in the washings. Dissolved chloroform was then removed by boiling the medium for 10 min.

Growth of cells

All strains were maintained on nutrient agar slopes containing 1 mM sodium citrate, except in the case of strain AN299, where the citrate was omitted. The growth conditions for strain AN92 and its derivatives, strains AN260, AN272 and AN299,

were as described by Langman *et al.*[4]. For the growth of strain AB1515, and its derivative strain AN103, the basic growth medium was supplemented as described by Cox *et al.*[2]. In addition, mutants blocked in enterochelin biosynthesis were supplemented with 5 μM 2,3-dihydroxybenzoic acid or, when it was intended to induce the citrate-dependent system, with 1 mM citrate.

Growth tests with strain AN103

The cells were grown in 15-ml batches in 50-ml Erlenmeyer flasks and the medium was supplemented with sterile citrate to the required concentration. Cultures for inoculum were grown to early stationary phase in the presence of 1 mM citrate and then washed twice in unsupplemented growth medium. After inoculation with approximately $2 \cdot 10^5$ cells per ml, the cultures were shaken in a water bath at 37 °C and sampled at intervals. Cell density was measured as the absorbance at 660 nm in a Gilford 300 spectrophotometer.

Iron uptake measurements

The basic method of measuring iron uptake has been described previously[4]. Uptakes were routinely measured in medium containing all necessary growth supplements except tryptophan, unless specifically stated that uptake into growing cells was measured. All uptake data were corrected using controls in which uptake medium alone replaced the cell suspension. Cell densities were measured at 660 nm and converted to mg dry wt/ml using a standard curve. Cells were routinely used at a density of about $3 \cdot 10^8$/ml and uptake results expressed in patoms Fe^{3+}/mg dry wt cells. During longer term experiments an appropriate correction for growth was applied to all data. Initial rates of iron uptake were calculated from four or more samples taken during the first two minutes following addition of $^{55}Fe^{3+}$. Since uptake was linear with time over this period the data was analysed by a "least squares" method of regression analysis. Details of individual experiments will be described in the text.

RESULTS

Growth response to citrate in strain AN103

Mutant strain AN103 (*entA*$^-$) is unable to synthesize dihydroxybenzoate[2], and thus cannot use its ferric–enterochelin transport system to obtain the iron necessary for growth, unless supplied with dihydroxybenzoate or enterochelin. Nevertheless, in the absence of either of these two supplements, the mutant grows well if supplemented with citrate.

Fig. 1 shows the effect of citrate concentration on the growth of this strain. A maximal growth rate was achieved with 1 mM citrate. After 37 h incubation, when all cultures grown with 12 μM citrate or more had reached stationary phase, the final growth yields increased with increasing citrate concentration. No measurable growth occurred over the 37-h period in cultures supplemented with 8 μM citrate or less, or in a control culture from which citrate was omitted. Thus it would appear that the citrate-dependent iron transport system fails to operate below a critical citrate concentration of around 10 μM, possibly as a result of failure to maintain sufficient iron in the medium in a utilisable form. As the proportion of citrate to iron at neutral pH falls below a critical ratio of about 20:1 an increasing proportion of the iron is

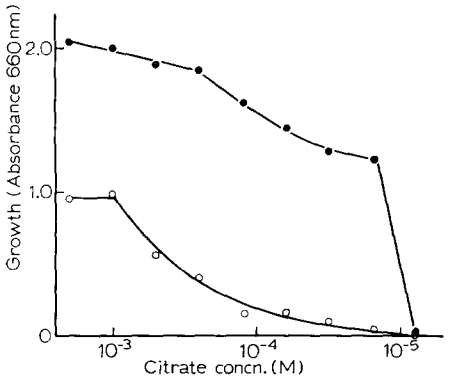

Fig. 1. Effect of citrate on the growth of strain AN103 (*entA*—) in iron-deficient medium. Growth was measured after 16 h (○—○) and 37 h (●—●) at 37 °C.

present as a highly polymerised species[11,12] which is unlikely to be utilised by the citrate-dependent uptake system.

Effect of nitrilotriacetate on iron-uptake measurements

Precipitation of Fe(III) as the insoluble hydroxide, or as a polynuclear complex with phosphate, is a difficulty commonly experienced during iron-uptake studies employing microorganisms suspended in buffered media at physiological pH. We have partially overcome this problem by manipulation of the balance of cations, in a phosphate-buffered uptake medium. When stabilizing chelators are omitted from this medium, low concentrations of Fe(III) are maintained in a form(s) which is not retained on a membrane filter (average pore size 0.45 μm) at neutral pH. When iron-starved cells were suspended in this medium a low-affinity iron uptake was observed, which could easily be confused with the citrate-dependent iron uptake (see Fig. 3a).

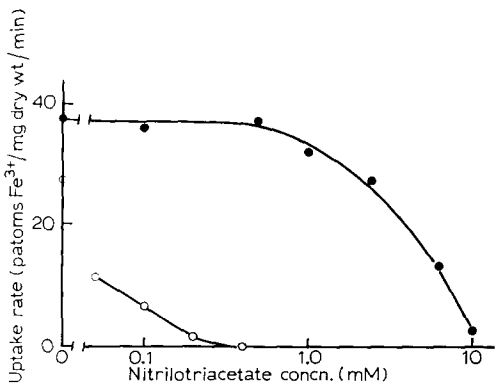

Fig. 2. The effect of nitrilotriacetate on initial iron uptake rates in the presence (●—●) and absence (○—○) of citrate in *E. coli* strain AN92 (*aroB*—), grown with 1 mM citrate. The uptake medium contained 1 μM $^{55}Fe^{3+}$, nitrilotriacetate in amounts shown and citrate, where present, at 1 mM.

However, this citrate-independent uptake proved to be considerably more sensitive than was the citrate-dependent uptake to inhibition by the competing iron chelator, nitrilotriacetate. Thus, in a medium containing 1 μM Fe^{3+}, addition of 400 μM nitrilotriacetate resulted in complete abolition of the low-affinity uptake, whereas the citrate-dependent uptake was unaffected by 500 μM nitrilotriacetate (Fig. 2). When the uptake medium was supplemented with 1 mM citrate, nitrilotriacetate concentrations in excess of 10 mM were now necessary to abolish iron uptake completely in citrate-induced cells. Although the cells used in this work were strain AN92 (*aroB*$^-$), the results described were generally applicable to all the mutant strains to be discussed. Consequently, 100 μM nitrilotriacetate was routinely added to the uptake medium to facilitate positive identification of the citrate-dependent iron-uptake system.

Citrate-dependent uptake in mutants defective in iron transport

Fig. 3 illustrates the inducible nature of the citrate-dependent uptake system in strain AN92 (*aroB*$^-$). This strain, which is unable to produce enterochelin unless supplied with shikimate or dihydroxybenzoate, has been previously shown to possess a normal ferric–enterochelin transport system[4]. Cells grown in the presence of 1 mM citrate were able to take up iron from uptake medium containing 100 μM nitrilotriacetate, if 1 mM citrate was also added. Uninduced cells, grown in the presence of 5 μM dihydroxybenzoate instead of citrate, failed to show any enhancement of iron uptake in the presence of citrate, but both induced and uninduced cells took up iron rapidly in the presence of enterochelin. Similar results were obtained using mutant strain AN103.

Strain AN260, a mutant derived from AN92, is unable to transport ferric–enterochelin[4] and has been designated *fep*$^-$. Such *fep*$^-$ strains are unable to grow in

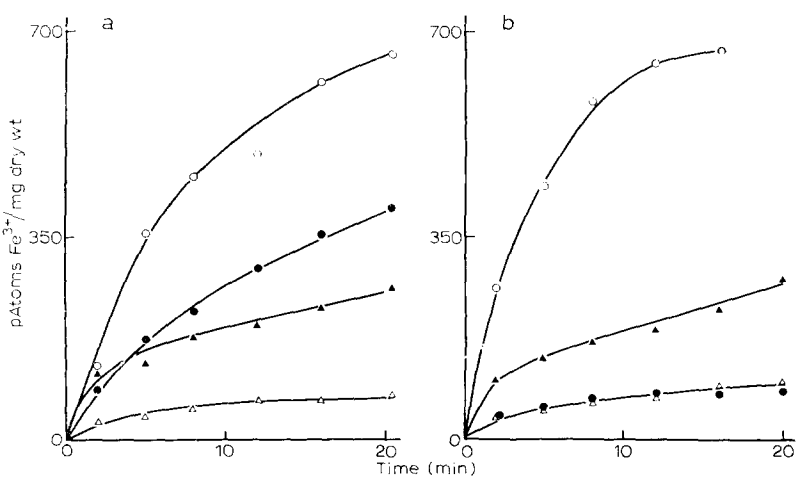

Fig. 3. Iron uptake by cells of strain AN92 grown in the presence of 1 mM citrate (a) or 5 μM 2,3-dihydroxybenzoate (b). The uptake medium contained 1 μM $^{55}Fe^{3+}$ and either 100 μM nitrilotriacetate (△—△); 100 μM nitrilotriacetate + 1 mM citrate (●—●); 100 μM nitrilotriacetate + 1 μM enterochelin (○—○); or no added chelators (▲—▲).

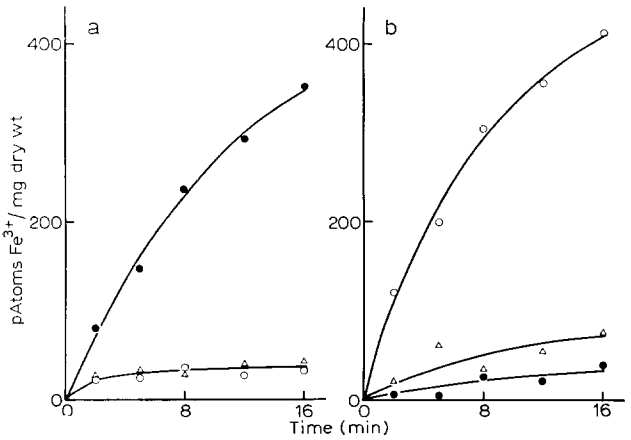

Fig. 4. Iron uptake by two iron-transport-defective strains of *E. coli* K12 grown in the presence of 1 mM citrate. (a) strain AN260 (*fep*⁻); (b) strain AN299. Symbols as in Fig. 3.

iron-deficient medium when supplied with dihydroxybenzoate as a growth factor, but grow well when the medium is supplemented with citrate. Citrate-grown cells of strain AN260 possessed a normal citrate-dependent iron uptake, but were entirely blocked in ferric–enterochelin transport (Fig. 4a). Similarly strain AN272, a mutant derived from strain AN92, but lacking a functional ferric-enterochelin esterase (*fesB*⁻) also possessed a normal, inducible, citrate-dependent iron uptake (not shown). These cells were previously shown to be impaired in their ability to accumulate iron *via* the ferric–enterochelin system[4]. In contrast, strain AN299, a further mutant derived from strain AN92, was able to grow in the presence of dihydroxybenzoate, but failed to respond to citrate under iron-deficient conditions. These cells grew poorly in unsupplemented medium (doubling time about 2 h) and were further inhibited by addition of 1 mM citrate (doubling time about 4 h). Stimulation of iron uptake by citrate could not be demonstrated in strain AN299 whether the cells were grown in the presence, or absence of citrate (Fig. 4b), but the cells possessed a normal ferric–enterochelin uptake system. It would appear therefore, that a mutation which

TABLE I

IRON UPTAKE IN STRAIN AB1515 (WILD TYPE)

Uptakes were measured in the presence of 1 μM $^{55}Fe^{3+}$ at the following ligand concentrations; nitrilotriacetate, 100 μM; enterochelin, 1 μM; citrate, 1 mM.

Uptake in presence of	*Initial rate of iron uptake (patoms/mg dry wt per min)*	
	Grown with 1 mM citrate	*Grown minus citrate*
Nitrilotriacetate	79	103
Nitrilotriacetate + enterochelin	103	128
Nitrilotriacetate + citrate	133	107

directly affects iron transport *via* the enterochelin-mediated system has no affect on the citrate-dependent system and *vice versa*. In order to conclusively demonstrate that the citrate-dependent iron transport system was normally present in wild-type cells, strain AB1515, an *E. coli* K12 strain unaffected in enterochelin-mediated iron transport, was tested for citrate-dependent iron transport. In this strain 100 μM nitrilotriacetate failed to inhibit iron uptake in the absence of added citrate or enterochelin (Table I). It would appear that, in the 10 min warming-up period prior to the assay the cells produced sufficient enterochelin to almost saturate the transport system, since addition of 1 μM enterochelin resulted in only a small enhancement of iron uptake. Citrate-dependent stimulation of iron uptake occurred only in cells grown in the presence of citrate (Table I). Thus the induction of a citrate-dependent iron uptake clearly takes place in wild type *E. coli* K12, growing aerobically in the presence of citrate, under conditions of iron deficiency.

Induction of the citrate-dependent iron uptake system

The time course of the induction process in strain AN103, was followed by comparing iron uptake in the presence and absence of citrate (Fig. 5). In growing cells a marked enhancement of iron uptake was observed after 20 min of incubation in the presence of citrate. However, identical cells pre-incubated with chloramphenicol failed to induce a citrate-dependent uptake. In these cells uptake in the presence of citrate was lower than in the control throughout the 40 min duration of the experiment. Thus induction of the citrate-dependent iron uptake system requires new protein synthesis. When cells were denied the essential amino acid tryptophan, a greatly reduced citrate-dependent uptake was eventually observed after about 35 min of exposure to citrate, presumably as a result of a residual level of protein synthesis.

Induction of the citrate-dependent system was inhibited in cells of strain AN103 which were supplied with dihydroxybenzoate, and were thus able to synthesize entero-

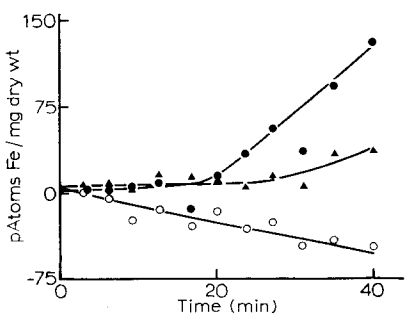

Fig. 5. Induction of the citrate-dependent uptake system by cells of strain AN103 previously grown in the presence of 5 μM 2,3-dihydroxybenzoate. Cells were preincubated for 15 min in uptake medium lacking dihydroxybenzoate, but otherwise containing either full growth supplements (●—●), full growth supplements + 20 μg/ml chloramphenicol (○—○) or all growth supplements except tryptophan (▲—▲). At zero time the cells were mixed with an equal volume of medium supplemented with 2 μM $^{55}Fe^{3+}$, 200 μM nitrilotriacetate and 2 mM citrate and uptake measurements were commenced. All data was corrected for uptake occurring in control cells in the absence of citrate. Note that the apparently negative values for iron uptake in the chloramphenicol- treated cells arose as a result of a slightly higher rate of iron uptake in the control cells than in the cells given citrate.

TABLE II

EFFECT OF 2,3-DIHYDROXYBENZOATE CONCENTRATION DURING GROWTH, ON INDUCTION OF THE CITRATE-DEPENDENT UPTAKE SYSTEM IN STRAIN AN103

Uptakes were measured under the conditions described for Table I.

Growth supplement		*Initial rate of iron uptake (patoms/mg dry wt per min)*		
Citrate (mM)	*2,3-dihydroxybenzoate (μM)*	*Enterochelin system*	*Citrate system*	*Low-affinity uptake*
	5	139	< 1	1
1	—	92	62	5
1	5	58	27	5
1	50	50	< 1	9

chelin during growth (Table II). When the availability of endogenous iron was enhanced in this way, the iron-uptake capacities of the cells were correspondingly decreased, as noted previously by Wang and Newton[13], but the citrate-dependent system proved to be the more sensitive of the two uptake systems in this regard. Since the highest rate of ferric–enterochelin uptake occurred when the citrate-dependent system was not induced, it would appear that iron entering *via* the citrate-dependent system can repress the enterochelin-mediated system. Cells grown in the presence of 50 μM dihydroxybenzoate completely failed to induce the citrate-dependent system. The enterochelin-mediated uptake was inhibited to a somewhat lesser extent. Clearly this is not a true reflection of the regulatory effects of iron on these two transport systems in wild-type cells, since a citrate-dependent stimulation of iron uptake was detected in strain AB1515, although these cells have an unimpaired capacity for enterochelin synthesis (Table I).

Kinetics of iron uptake in induced cells of strain AN103

In order to study the enterochelin- and citrate-dependent iron-uptake systems in identical cells, citrate-induced cells were used throughout these experiments, although higher initial rates of iron uptake *via* the enterochelin-mediated system were obtained in cells lacking the citrate system (see Table II).

Since the true concentrations of ferric citrate and ferric–enterochelin could not be assessed accurately in the presence of a competing chelator, kinetic parameters were measured at fixed ligand concentration (2 μM enterochelin or 1 mM citrate) in the absence of nitrilotriacetate. In exploratory experiments, doubling the citrate concentration to 2 mM had no effect on the maximal rate of citrate-dependent uptake.

Both iron-uptake systems were found to obey simple Michaelis–Menten saturation kinetics, as shown in Fig. 6. The ferric–enterochelin uptake system, with an apparent $K_m = 0.1$ μM Fe^{3+}, had a slightly higher affinity for iron than the citrate-dependent system, for which the apparent K_m was 0.2 μM Fe^{3+} (Fig. 6). The V for the enterochelin- and citrate-dependent systems were calculated to be 85 and 66 patoms Fe^{3+}/mg dry wt per min, respectively.

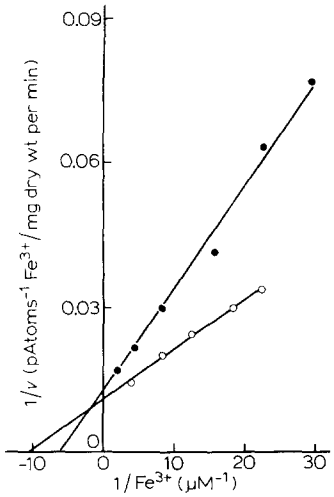

Fig. 6. Kinetics of iron uptake in strain AN103 grown in the presence of 1 mM citrate. Uptake rates were measured either in the presence of 2 μM enterochelin (○—○) or 1 mM citrate (●—●) and the Michaelis constants calculated by a "least squares" method of regression analysis.

Summation of iron uptake in induced cells of AN103

Since it is apparent that both the enterochelin- and citrate-dependent systems of iron transport are present in citrate-induced cells, it was of interest to determine

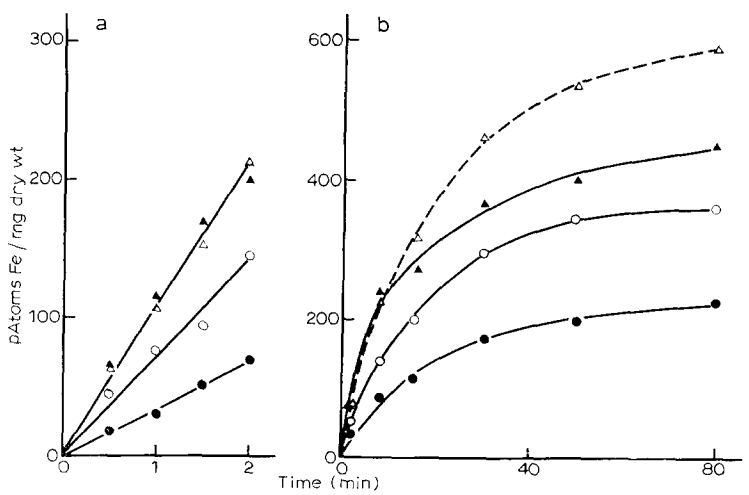

Fig. 7. Independent operation of the two iron transport systems in strain AN103 grown in the presence of 1 mM citrate. Uptake medium contained 400 μM nitrilotriacetate and either 1 μM $^{55}Fe^{3+}$ + 1 mM citrate (●—●), 1 μM $^{55}Fe^{3+}$ + 1 μM enterochelin (○---○) or 3 μM Fe^{3+} + 1 mM citrate + 1 μM enterochelin (▲—▲). The sum of the enterochelin- and citrate-dependent uptakes (△---△) was calculated by addition of values determined for the individual systems. In control experiments with the individual systems, no increase in iron uptake rates occurred on raising the iron concentration from 1 μM to 3 μM (and the enterochelin concentration, where present, to 3 μM).

whether or not the two systems interacted in any way when operating simultaneously, under optimal conditions. This was investigated in strain AN103 in both short-term and long-term experiments (Fig. 7). It was found that initially the individual uptake rates for the two systems were additive (Fig. 7a), but that this relationship lapsed after about 10 min and the measured total uptake then began to plateau out more rapidly than the calculated sum of the two individual uptakes (Fig. 7b). In several experiments the total measured uptake consistently achieved a higher maximal level than either of the individual uptakes, even when measured in growing cells. It would appear therefore, that neither transport system alone is capable of satisfying the maximal iron uptake and storage capacity of cells grown in the presence of citrate, under optimal laboratory conditions.

DISCUSSION

The results presented here suggest that, in addition to the enterochelin-mediated iron transport system, at least two further systems are available for the uptake of iron by *E. coli*. Thus, in the absence of any highly specific ligand, mutants defective in enterochelin-mediated iron transport accumulate iron *via* a low-affinity system. This observation confirms earlier reports that such mutants show growth responses to ferrous and ferric salts[4–6]. Wang and Newton[13] have suggested that iron may diffuse passively into such mutant cells and accumulate by incorporation into proteins. Obviously Fe(III), when supplied as a chelate with nitrilotriacetate is unable to enter *via* this process.

There is no accurate data in the literature concerning the stability of ferric complexes with citrate at neutral pH. In particular, no mention is made of a ferric dicitrate complex which may have been the predominant species present in our studies[12]. The published stability data for Fe(III)–citrate complexes[14,15], which were obtained at much lower pH values, predict that nitrilotriacetate[16,17] should be a far stronger inhibitor of the citrate-dependent iron uptake than was observed; assuming that the inhibition resulted from simple competition, for iron, between the two ligands. This discrepancy may be due to either, (a) published Fe(III)–citrate stability data being inapplicable at pH 7, or (b) the existence of a more complicated situation where citrate may interact with a component of the transport system, resulting in a complex which can more effectively compete with nitrilotriacetate for iron. Recent observations (Frost, G., unpublished data) suggest that the published data are indeed insufficient to account for iron chelation by citrate at pH 7.

The citrate-dependent iron transport system is clearly genetically and functionally distinct from the enterochelin-mediated system.

Furthermore the two systems are apparently able to function simultaneously in wild-type cells of *E. coli* K12. Wang and Newton[6,13] previously reported that citrate stimulated both growth and iron uptake in an *E. coli* B/r mutant (Chr 2) unable to synthesize enterochelin, but did not postulate the existence of two separate iron transport systems. Since growth in the presence of citrate was not necessary to elicit the uptake response in these cells, it may be that the citrate-dependent system is constitutively present in *E. coli* B, or that the strain described had mutated to constitutivity. Previous work[9] had suggested that this response to citrate was specifically associated with the *ton B* region of the chromosome, adjacent to the *trp* operon.

The mutation in strain Chr 2 did not map in this area, although several other iron-transport-defective mutants described by these workers were associated with *tonB*$^-$ characteristics[6,13,18] (resistance to coliphages T1 and ϕ80). In the light of our experience, the conditions used by Wang and Newton, to measure iron uptake, would yield data representing the sum of uptake contributions from two independent transport processes, for the Fe(III)–enterochelin and Fe(III)–citrate complexes, respectively.

Although evidence for enterochelin-mediated iron transport has been found in all species of enteric bacteria so far examined[1,19] the citrate-dependent system is less widely distributed. *E. coli* strains B/r[6,13] and K12 and *Aerobacter aerogenes*[5] are stimulated by citrate, but *E. coli* W[5] and *Salmonella typhimurium*[19] both apparently lack this system. A similar citrate-dependent iron uptake system has also recently been reported in *Neurospora crassa*[20], an organism whose primary siderochrome is not enterochelin but coprogen, a hydroxamate.

The citrate-dependent system appears to function as an auxiliary iron-scavenging mechanism. Possession of such a mechanism, specifically induced when iron is available in the medium as a soluble chelate, would clearly result in economy of metabolic effort within the cell. Synthesis and transport of the organism's own siderochrome, together with associated protein synthesis, would therefore be repressed, when iron was readily obtainable by less metabolically extravagant means. Citrate is known to repress dihydroxybenzoate biosynthesis in *A. aerogenes*[5], and we have presented evidence here to indicate that ferric–enterochelin uptake is reduced in citrate-induced cells of *E. coli* K12.

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