

## Iron Uptake and Iron Limited Growth of *Escherichia coli* K-12

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**Abstract.** Cells of *Escherichia coli* K-12 could grow aerobically at an iron concentration as low as 0.05  $\mu\text{M}$  without any of the known iron ionophores present. The growth rate increased between 0.05 and 2  $\mu\text{M}$  iron. Supplementation with the iron ligands ferrichrome and citrate resulted in optimal growth already at 0.05  $\mu\text{M}$  iron. Under certain conditions iron uptake preceded growth of cells by more than an hour. During logarithmic growth the rate of iron uptake matched the growth rate. The radioactive tracer method revealed a cellular iron content of 4 nmol/mg dry weight.

After consumption of the iron in the medium cells continued to grow with high rate for 1–2 generations. The iron uptake activity was increased during iron starvation.

**Key words:** Low affinity iron uptake – Ferrichrome- and citrate-dependent iron transport – Iron limited growth – Iron content of *Escherichia coli* K-12

In aerobic environments and at neutral pH, and therefore under most biological conditions, iron occurs as highly insoluble ferric hydroxide polymer. The iron supply is therefore a crucial nutritional factor for most cells. Iron is also the limiting nutrient for bacteria invading humans, animals and plants (Bullen et al. 1974; Kloepper et al. 1980a, b; Weinberg 1978). For many bacteria it was demonstrated that they excrete compounds of low molecular weight that form iron chelates with an extremely high stability. In cells of *Escherichia coli* whose iron supply has been studied most extensively, five independent iron transport systems have been identified. Enterochelin (enterobactin) is synthesized, excreted and taken up as ferric enterochelin. The iron is released and enterochelin is hydrolyzed (Rosenberg and Young 1974). During growth in the presence of citrate an iron transport system is induced (Frost and Rosenberg 1973; Hussein et al. 1981; Woodrow et al. 1978). This system is peculiar in that the inducing compound, ferric citrate, does not have to enter the cytoplasm. Also for ferrichrome, a siderophore produced by the fungus *Ustilago sphaerogena*, a highly specific active transport system exists in *E. coli* (Hartmann and Braun 1980; Kadner et al. 1980; Leong and Neilands 1976). Ferrichrome is taken up into the cells, the

iron is reduced in the membrane, the ligand is inactivated by acetylation of one of the binding sites of ferric iron, and released into the medium (Schneider et al. 1981). Proteins have been identified in the outer membrane which are components of these transport systems (Braun et al. 1976; Wagegg and Braun 1981). In virulent strains of *E. coli*, harbouring the ColV plasmid, a new transport system has been detected (Williams and Warner 1980; Stuart et al. 1980) which uses aerobactin as iron ligand (Braun 1981; Warner et al. 1981). Besides these uptake systems a less understood low affinity iron uptake functions which can be repressed by iron chelators such as nitrilotriacetate and 2,2'-dipyridyl (Frost and Rosenberg 1973).

With regard to the regulation of the iron supply it is known that enterochelin and the corresponding transport system are only formed at iron limiting growth conditions (Brot and Goodwin 1968; Rosenberg and Young 1974). The citrate-dependent iron transport system requires citrate and iron for induction (Hussein et al. 1981). The ferrichrome transport system at aerobic conditions seems to be expressed constitutively (Braun et al. 1976; Hantke 1981). Besides the outer membrane proteins which could be related to iron transport systems, two additional proteins were synthesized in response to iron limiting growth conditions (Braun et al. 1976).

Since regulation of synthesis of the iron transport systems was up to now mostly related to the iron supplied in the medium we determined the iron content in the cells to obtain the real iron concentration effective in regulation. The publication of our data was also prompted by results showing that cells of *E. coli* accumulate large amounts of iron via the ferric enterochelin transport system (McIntosh and Earhart 1977). In contrast, we found for the ferrichrome and citrate-dependent iron uptake adaptation of the rate of iron transport to that of growth. In particular, we took care that the iron supplied in the medium was not converted to the insoluble polymer that could adsorb to the surface of the cells and thus give a false value for cellular iron.

### Materials and Methods

*Escherichia coli* K-12 AB2847 *aroB tsx malT* and its tonB derivative BR158 were used (Hancock et al. 1977). Tryptone yeast extract, M9 and CR growth media were prepared as described previously (Hartmann et al. 1979; Hartmann and Braun 1980). Glassware was thoroughly rinsed with 10 mM EDTA solution and then with twice distilled water. Iron was extracted from buffers and glucose by passage through a

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column of "Chelex 100" (Bio-Rad Laboratories, München, FRG). Solutions of ferric chloride were prepared in 0.01 or 0.1 M HCl. The iron complexes of nitrilotriacetate, citrate and ferrichrome were made by incubation of the compounds at room temperature for 3 h prior to use. Growth was started from single colonies on tryptone yeast extract agar plates. Cells from overnight cultures in minimal media specified in the legends to the figures were washed twice before they were used. The culture density of 1.0 at 578 nm corresponded to  $1 \times 10^9$  cells/ml (0.38 mg dry weight). The iron content of the cells was calculated from the specific radioactivity of iron supplied in the medium. The uptake was measured by filtering 1 ml samples at various times as outlined previously (Hartmann and Braun 1980). Growth and iron uptake were determined simultaneously.

## Results

### Solubility and Use of Iron in Different Growth Media

Solubility of iron differs in various minimal salt media and depends among other things on the  $K^+/Na^+$  ratio (Langman et al. 1972). When trace amounts of radioactive ( $^{55}Fe^{3+}$ ) and 0.5  $\mu M$  non-radioactive iron in 0.1 M HCl were added to CR medium containing only  $K^+$  phosphate salts, increasing amounts of radioactivity remained on filters when the medium was passed through nitrocellulose filters (pore size 0.45  $\mu m$ ) at 2 h intervals. Accordingly, *E. coli* AB2847 *aroB* which cannot synthesize enterochelin and depends entirely on the "low affinity" iron uptake system, did not grow in this medium. In contrast, M9 medium supported growth and iron stayed soluble during the incubation time. This medium contains  $K^+$  and  $Na^+$  phosphate salts in a 4:1 molar ratio. When the iron was extracted to a remaining concentration of 0.05  $\mu M$ , the M9 medium still supported growth (Fig. 1). The generation time of 150 min was reduced to 90 min by addition of 2  $\mu M$   $Fe^{3+}$  dissolved in 0.1 M HCl. Nitrilotriacetate at a concentration of 100  $\mu M$  inhibited growth showing that under these conditions iron was taken up via the "low affinity system".

The low affinity uptake system also operated when cells were grown in tryptone yeast extract medium which contained 37  $\mu M$  iron. To test the radioactive tracer method for the determination of the cellular iron content we added 1  $\mu Ci$  (0.08 nmol) of  $^{55}Fe^{3+}$  in 0.1 M HCl to 20 ml of culture. Iron uptake and growth proceeded with the same rate (data not shown). The cellular iron content remained constant at 4 nmol/mg dry weight, and agreed with the spectrophotometrically determined value (Rouf 1964). Under these conditions the iron supply was sufficient since the high affinity transport systems were largely repressed (Braun et al. 1976; Hantke 1981).

### Citrate-Dependent Iron Uptake

Addition of 1 mM citrate to iron-extracted M9 medium resulted in equal growth rates at iron concentrations between 0.05  $\mu M$  and 2  $\mu M$  in the medium. However, when the iron uptake was measured simultaneously with the growth rate iron uptake preceded growth (Fig. 2). The cellular iron content increased (Fig. 3). During exponential growth, iron uptake and growth were balanced. The iron content stayed constant. After the iron in the medium was used up the intracellular iron content declined (Fig. 3) but the cells

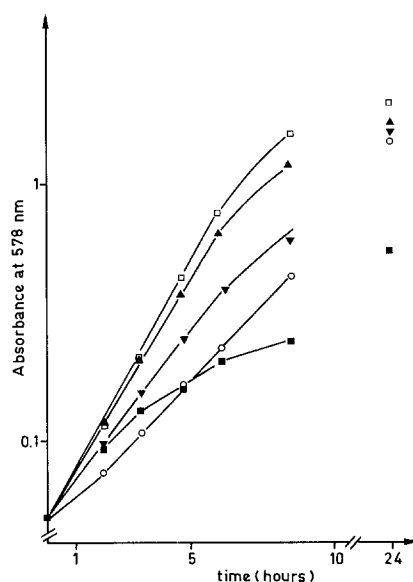


Fig. 1. Growth of *Escherichia coli* K-12 AB2847 in iron extracted M9 medium without added chelator. Different iron concentrations were used by addition of  $FeCl_3$  solution (in 0.1 M HCl). (O) 0.05  $\mu M$   $Fe^{3+}$  (without iron added), (▼) 0.15  $\mu M$   $Fe^{3+}$ , (▲) 0.5  $\mu M$   $Fe^{3+}$ , (□) 2.0  $\mu M$   $Fe^{3+}$  and (■) 2.0  $\mu M$   $Fe^{3+}$  plus 100  $\mu M$  nitrilotriacetate. Cells from a stationary phase culture in iron-extracted M9 medium supplemented with 2  $\mu M$   $Fe^{3+}$ , without citrate were used

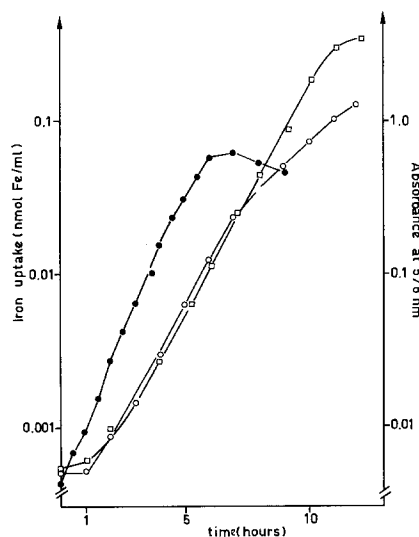
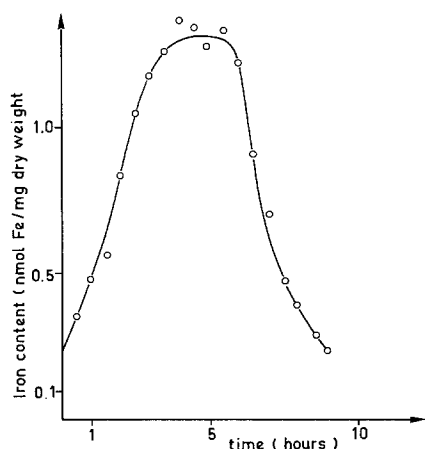


Fig. 2. Growth (O) and iron uptake (●) of *Escherichia coli* K-12 AB2847 in iron extracted M9 medium containing 1 mM citrate and 0.5  $\mu M$  ferric chloride,  $^{55}Fe^{3+}$  (10  $\mu Ci$ ; 0.8 nmol). Growth (□) of a culture with 1 mM citrate and 2  $\mu M$   $Fe^{3+}$ . Cells from an overnight culture in iron extracted M9 medium supplemented with 2  $\mu M$   $Fe^{3+}$  and 10 mM citrate were used

continued to grow for one to two generations (Fig. 2). With increasing iron concentrations in the M9 medium (0.2–1.75  $\mu M$ ) the cellular iron content rose up to 2.5 nmol/mg dry weight.

With the carbon sources xylose and ribose which led to slower growth rates, it was also demonstrated that growth and iron uptake proceeded during logarithmic growth with equal rates (Table 1). Furthermore, an *tonB* mutant which was devoid of any high affinity iron uptake grew at the beginning faster than it transported iron, due to an internal iron pool from the pregrowth, but then growth ceased.



**Fig. 3.** Change of the iron content of *Escherichia coli* K-12 AB2847 in iron extracted M9 medium containing 1 mM citrate and  $0.05 \mu\text{M } ^{55}\text{Fe}^{3+}$ . The iron content was calculated from growth and iron uptake shown under Fig. 2. The figures given do not reflect accurately the iron content of the cells since the iron content of the inoculum was not taken into account

**Table 1.** Relationship between rates of growth and of iron uptake

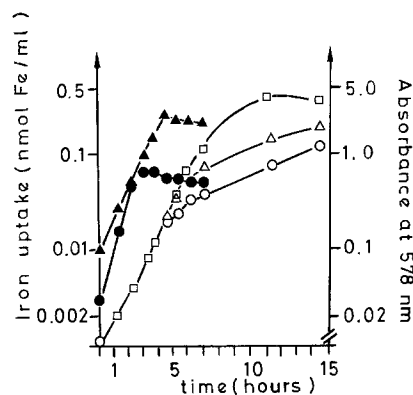
Strain	Growth conditions	Generation time (min)	Doubling time of cell-bound iron (min)
AB2847	1 mM citrate		
	+ 0.4% glucose	54	54
	or 0.4% xylose	74	66
	or 0.4% ribose	108	112
AB2847	0.1 $\mu\text{M}$ ferrichrome + 0.4% glucose	54	55
BR158	2 $\mu\text{M}$ DHB + 0.4% glucose	90	175

Growth and iron uptake were measured in the same culture supplemented with  $0.1 \mu\text{M Fe}^{3+}$  and  $0.8 \text{ nmol } ^{55}\text{Fe}^{3+}$  ( $10 \mu\text{Ci}$ ). DHB = 2,3-dihydroxybenzoic acid

### Ferrichrome-Dependent Iron Uptake

Ferrichrome provided sufficient amounts of iron at an iron concentration in the medium as low as  $0.05 \mu\text{M}$  to support growth until the iron in the medium was exhausted (Fig. 4). The status of the intracellular iron pool was apparently similar to the one transported via citrate since growth also ceased 1–2 generations after the medium iron had been used up.

The question arose whether cells approaching iron deficiency show a higher transport capacity than iron proficient cells. From the culture used in the experiment reported in Fig. 4 samples were taken at the time of iron supply, at the beginning of the decrease of the intracellular iron content, and 40, 80 and 120 min thereafter. The results listed in Table 2 show that the transport activity increased during iron starvation but only by a factor of two. Furthermore, immediate acceleration of growth could only be obtained when ferrichrome ( $1 \mu\text{M}$ ) was added at the beginning of iron limited growth (data not shown). When administered at later times growth started after considerable lag periods. For example, when cells of *E. coli* AB2847 were incubated in M9 medium containing  $0.1 \text{ mM}$  nitrilotriacetate, growth proceeded very



**Fig. 4.** Growth (O,  $\Delta$ ) and iron uptake (●,  $\blacktriangle$ ) of *Escherichia coli* K-12 AB2847 in iron extracted M9 medium to which  $2 \mu\text{M}$  deferrri-ferrichrome,  $10 \mu\text{Ci } ^{55}\text{Fe}^{3+}$  ( $0.8 \text{ nmol}$ ), and  $0.05 \mu\text{M}$  (O, ●),  $0.2 \mu\text{M Fe}^{3+}$  ( $\Delta$ ,  $\blacktriangle$ ), or  $2.0 \mu\text{M Fe}^{3+}$  ( $\square$ ) were added. Cells from an overnight culture in iron extracted M9 medium supplemented with  $2 \mu\text{M Fe}^{3+}$  and  $10 \text{ mM}$  citrate were used

**Table 2.** Rate of iron uptake via ferrichrome at iron limiting growth conditions

Time of iron uptake assay	transport rate $\text{pmol Fe}^{3+}/\text{min} \times \text{mg cell dry weight}$
1 h before exhaustion of the medium iron	40
onset of iron starvation	48
after 40 min of iron starvation	100
after 80 min of iron starvation	99
after 120 min of iron starvation	84

Samples were taken from the culture used in the experiment reported in Fig. 4 ( $0.2 \mu\text{M}$  ferrichrome)

slowly due to iron limitation. When  $1 \mu\text{M}$  of ferrichrome was added after 16 h, iron uptake started immediately with a rate of  $6 \text{ pmol}/\text{min} \times \text{mg dry weight}$ . Growth was only resumed after 3 h.

### Discussion

Iron as nutrient is peculiar because it functions only catalytically. It is "metabolized" by incorporation into proteins. From there it can be mobilized again. It was therefore of interest to find out how long a cell can grow on an iron-free medium before growth decreases. This questions have a bearing on the problem whether there exists an iron pool from where iron can be fed into iron-requiring processes. The major technical problem for such studies comes from the ubiquitous occurrence of iron in all media and glassware, its very low solubility (free ferric ion concentration in equilibrium with the ferric hydroxide polymer at pH 7 is  $10^{-12} \mu\text{M}$ ), and the very low amounts needed to support growth.

The approach we used was to provide iron to the medium as a complex with ferrichrome, citrate or nitrilotriacetate, or to add iron as soluble ferric chloride in  $0.1 \text{ M HCl}$  to M9 medium where we determined that it remained soluble. The values obtained with the radioactive tracer method used in this study depended on the concentration of the non-

radioactive iron in the medium and in the cells. We relied on published values for the remaining concentration of iron in extracted minimal medium (0.05  $\mu\text{M}$ ) (Davis et al. 1971; Poyer and McCay 1971) because we observed that addition of 0.05  $\mu\text{M}$  iron to extracted M9 medium already stimulated growth of *E. coli* AB 2847 without additional iron ligand (enterochelin, desferri-ferrichrome or citrate). The values for intracellular iron obtained with the tracer method also agreed with the spectrophotometrically determined content (Rouf 1964). Under our conditions we found, in contrast to McIntosh and Earhart (1977), that no large iron reserves accumulated in the cells which could be used under iron starvation. Cell growth and iron uptake appeared balanced. With citrate and ferrichrome as iron ligands, the intracellular iron content did not exceed 4 nmol/mg cell dry weight. McIntosh and Earhart (1977) found, in experiments with enterochelin, cell-associated iron as high as 110 nmol/mg dry weight. Either the enterochelin system functions much more effectively than the ferrichrome and citrate-dependent iron transport systems, or some iron became adsorbed to the cells and was not really taken up. The latter interpretation seems possible since  $\text{FeCl}_3$  was apparently added to the growth media without taking precautions to prevent its polymerisation.

We were able to demonstrate iron-limited growth rates in iron-extracted M9 medium when the low affinity iron uptake system was operating. If the chelators ferrichrome and citrate were present the uptake of iron even at very small concentrations in the medium were no longer growth-limiting. However, an iron limited growth could be shown 1–2 h after complete exhaustion of the iron in the medium (Fig. 2). It is of interest that iron uptake with ferrichrome and citrate preceded growth (Fig. 3, 4), in the extreme case of iron limitation, for 3 h. In the latter case about 1 nmol iron/mg cell dry weight was taken up before cells resumed growth. Below this iron concentration the inducible iron transport systems may be turned on. Our data argue against a large iron pool since cells could grow only 1–2 generations after exhaustion of the iron in the medium. On the other hand there seems to be some storage of iron, since it is difficult to envision that all iron requiring processes tolerate a two- to four-fold dilution while still supporting full growth. Furthermore, the iron entered cells with a high rate even under conditions cells did not grow, but the chelating ligands were not accumulated in the cells (Hartmann and Braun 1980; Hussein et al. 1981). It is therefore likely that the iron is first incorporated into storage compounds before it is used in iron requiring metabolic processes. The storage compounds could participate in the regulation of iron uptake (Ernst et al. 1978; Hantke 1981).

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