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Anaerobic Iron Uptake by *Escherichia coli*

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Assimilation and uptake of iron in anaerobic cultures of *Escherichia coli* were supported by iron supplied as ferrienterobactin, ferrichrome, and ferrous ascorbate; however, as in the aerobic cultures, ferrichrome A was a poor iron source. Albomycin inhibited both aerobically and anaerobically grown cells. The siderophore outer membrane receptor proteins FepA and FhuA were produced under anaerobic iron-deficient conditions. Anaerobic transport of ferrienterobactin and ferrichrome was inhibited by KCN and dinitrophenol. The K_m for ferrienterobactin uptake in anaerobically grown cells was 0.8 μM, and the V_{max} was 38 pmol/min per mg, compared with 0.1 μM and 80 pmol/min per mg, respectively, in aerobically grown cells.

Although the natural habitat of *Escherichia coli* is the highly anaerobic animal gut, almost all studies of iron transport in this organism have been done with aerobic cultures. It is well established that aerobically grown *E. coli* synthesizes a specific ferric chelating compound, enterobactin (enterochelin), which serves to transport iron into the cells (2, 14). Ferrichrome and other siderophores from diverse organisms can also function to supply iron to *E. coli* (2, 5, 11, 12) via specific outer membrane siderophore receptor proteins (5, 7, 12).

It is often assumed that the greater solubility of ferrous ion compared with ferric ion offers an obvious solution to the problem of iron assimilation in anaerobic organisms. However, it is very unlikely that the highly hydrated ferrous ion can pass freely through the cellular membrane. Specific high affinity transport systems have been described for both magnesium and manganous ions in *E. coli* (6, 17), although both ions are more soluble than ferrous ions.

Although it has been established that various organisms can transport ferrous ion by specific uptake mechanisms (1), Hussein et al. (5) have shown that ferrienterobactin and ferrichrome uptake systems are present in anaerobically grown *E. coli*. However, their uptake assays were performed

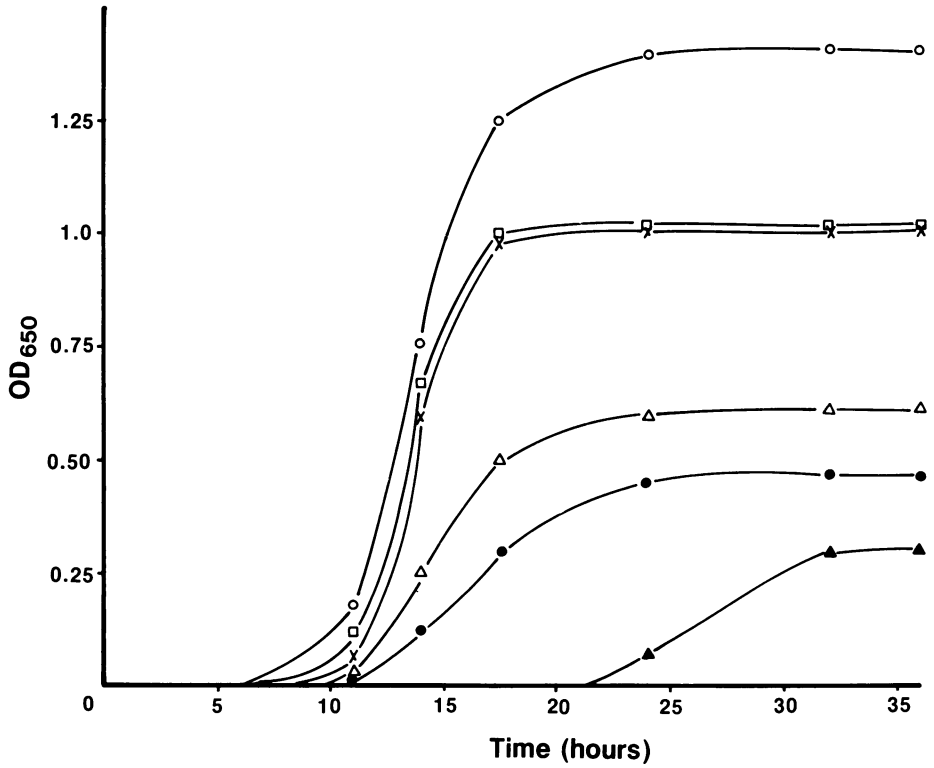

FIG. 1. *E. coli* B grown anaerobically with various iron sources. Siderophores were added to a final concentration of 5 μM. Symbols: ○, ferrienterobactin; □, ferrichrome; ×, ferrous ascorbate; △, $FeSO_4$; ●, ferrichrome A; ▲, no iron. OD_{650}, Optical density at 650 nm.

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aerobically, and their results are in contrast to those of Pugsley and Reeves (15), who found no anaerobic ferrienterobactin uptake. We provide evidence in this paper that anaerobic *E. coli* synthesized and utilized its aerobic iron transport systems.

E. coli B (prototroph), obtained from E. Boeker of this department, was maintained on L broth slants and grown on an iron-deficient M9 salts medium (10). The salts solution was made iron deficient by passage of a 10× solution through a Chelex-100 column (Bio-Rad Laboratories). The pH was adjusted to 7.0 with 5 M NaOH–5 M KOH (3:1) and diluted with doubly glass-distilled water. After sterilization, sterile $MgSO_4$ and $CaCl_2$ were added by the method of Miller (10). Sterile glucose was added to a final concentration of 0.5%. For anaerobic growth, 75 mM KNO_3, 1 μM Na_2MoO_4, and 1 μM Na_2SeO_4 were added. All glassware was soaked in 25 mM EDTA for at least 6 h and then rinsed thoroughly with doubly distilled water.

Enterobactin was purified by the method of Young (18), and the ferric chelate was prepared by the method of Klebba et al. (7). A 2 mM suspension in ethanol was kept at −20°C before use. Ferrichrome and ferrichrome A were purified in this laboratory. Purity of all siderophores was checked by paper electrophoresis, using pyridine acetate buffer (pH 6). Lyophilized albomycin was suspended in 10 mM phosphate buffer, pH 7. All siderophores were filter sterilized with 0.22-μm-pore Gelman filters.

Iron-starved *E. coli* B was inoculated into Chelex-treated M9 salts medium with the ferrisiderophore to be tested. The inoculum was 2×10^3 cells per ml, and the siderophore concentration was 5 μM. Nitrilotriacetate (100 μM) was added to the iron-deficient medium, and growth, as measured by turbidity at 650 nm, was monitored by using an anaerobic jar at 30°C with filled, capped tubes.

Ferrienterobactin, ferrichrome, and ferrous ascorbate were all effective in satisfying the iron requirements for anaerobic growth of *E. coli*, whereas ferrous sulfate and ferrichrome A were much less effective (Fig. 1). Levels of growth with ferrichrome A were similar to those found under aerobic conditions. Analysis of growth of *E. coli* also showed that of two *Pseudomonas* siderophores, pyoverdine and pyoverdine A, the former could satisfy iron requirements under both aerobic and anaerobic conditions, but pyoverdine A could not support growth under either condition (data not shown). Spent supernatants from cultures with no added iron gave a positive Arnow reaction for catechols, suggesting that anaerobic cells maintain the ability to synthesize enterobactin-type iron-complexing agents. Cells grown either aerobically or anaerobically wtih 10 μM iron added to the medium gave a negative Arnow reaction.

The antibiotic analog of ferrichrome, albomycin, has been shown to compete for the ferrichrome uptake system (4). We found that anaerobic growth was as sensitive to albomycin inhibition as was aerobic growth, and numerous resistant colonies were observed within the inhibition zone, in agreement with the results of Neüsch and Knüsel (13).

Functional outer membrane receptors are required for uptake and utilization of iron from ferric siderophore complexes (12). Expression of receptor proteins is increased aerobically under iron deprivation conditions (3, 12). The rapid isolation of outer membranes was performed by the method of Hantke (3), except that the cells were grown in Chelex-treated M9 salts containing 100 μM nitrilotriacetate. Polyacrylamide gel electrophoresis was done by using the system of Lugtenberg et al. (9). Samples were boiled for 5 min before electrophoresis. We found that in outer membrane preparations from anaerobically grown cells, the 81,000-dalton (ferrienterobactin receptor, FepA), 78,000-dalton (ferrichrome receptor, FhuA), and functionally unidentified 74,000-dalton proteins were all present, but they were not found under iron-sufficient growth conditions. Thus, repression of ferrisiderophore receptor systems was caused by iron availability, but not by anaerobic growth conditions.

Aerobic and anaerobic iron uptake are compared in Fig. 2. The method of Rosenberg (16) was used for transport assays, except that for anaerobic-transport assays, sterile 75 mM KNO_3, 1 μM Na_2MoO_4, and 1 μM Na_2SeO_4 were added to the uptake medium. Anaerobic-transport assays were performed in capped, filled flasks that were purged with nitrogen. Samples (0.5 ml) were filtered through 0.45-μm-pore filters that were presoaked in 0.9% NaCl containing 40 μM ferric-EDTA and then distilled water. The filter papers were washed twice with 5 ml of 0.9% NaCl before scintillation counting. The effect of 4 mM KCN or 0.5 mM dinitrophenol on anaerobic ferric enterobactin transport was determined by incubating nitrogen-purged cells for 20 min with the inhibitor before starting the assay.

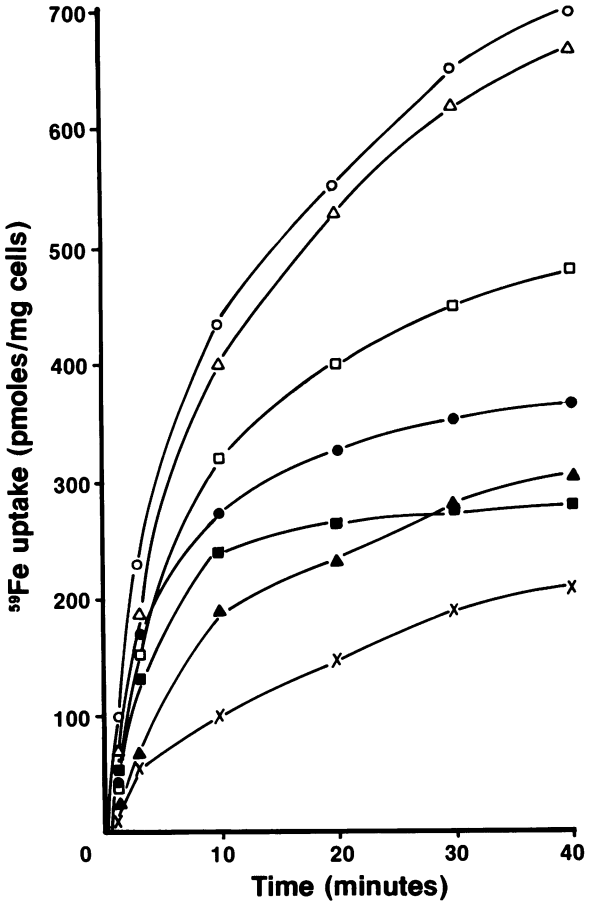

FIG. 2. Iron transport in *E. coli* B under aerobic and anaerobic conditions. Cells were grown in M9 minimal medium. Anaerobic-transport assays were performed with nitrogen-purged medium containing KNO_3, Na_2SeO_4, and Na_2MoO_4. Enterobactin (○,●), ferrichrome (△,▲), ferric citrate (□,■), and ferrous ascorbate (×) were added to 0.8 μM; open symbols indicate aerobic conditions, and closed symbols indicate anaerobic conditions.

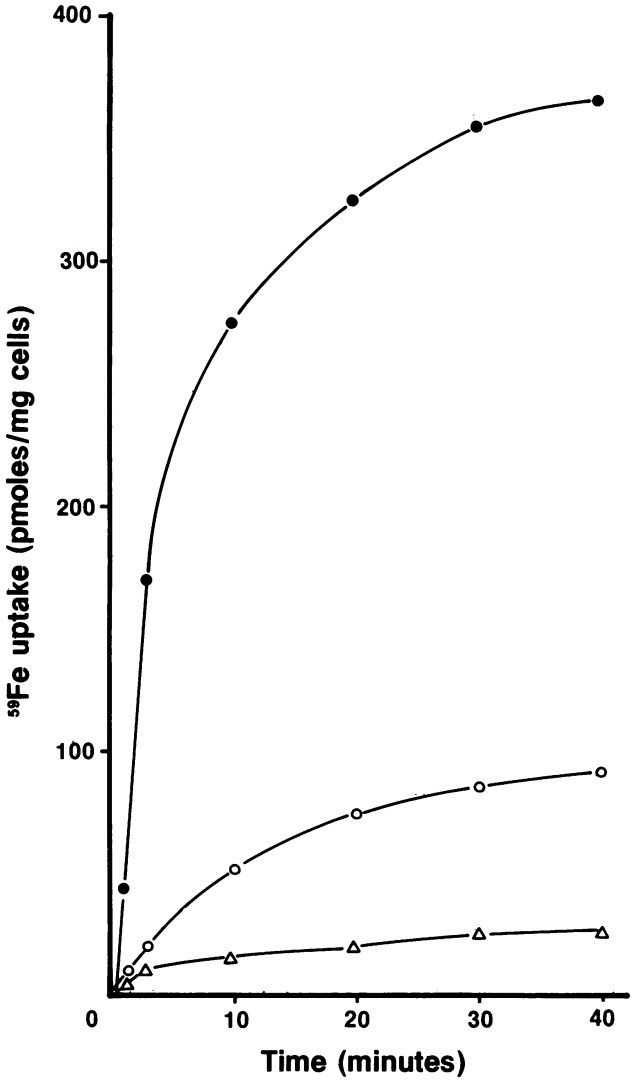

FIG. 3. Inhibition of anaerobic ferrienterobactin transport by KCN and DNP. Transport assays were performed as previously described except that nitrogen-purged *E. coli* cells were preincubated for 20 min with either 4 mM KCN or 0.5 mM DNP. Symbols: ●, control; ○, KCN; △, DNP.

Under anaerobic conditions, both ferrienterobactin and ferrichrome were taken up to a significant extent, although only about 50% of that occurred under aerobic conditions. However, the initial rates of ferrienterobactin uptake were very similar under both conditions. Cells grown anaerobically in the presence of citrate, which is known to induce a ferric citrate uptake system aerobically (2, 5), transported iron from citrate at a rate comparable to that of ferrienterobactin during the first 3 min, but at a significantly diminishing rate at longer intervals. Ferric uptake from citrate in noninduced cells was low (data not shown). The K_m for ferrienterobactin in anaerobically grown cells was 0.8 μM and the V_{max} was 38 pmol/min per mg, compared with the reported values of 0.1 μM and 80 pmol/min per mg (3), respectively, in cells grown aerobically but under otherwise identical conditions.

Anaerobic lactose transport in *E. coli* is energy dependent and totally inhibited by 20 mM KCN with nitrate in the uptake medium (8). We therefore tested the effect of 4 mM KCN and 0.5 mM dinitrophenol on anaerobic ferrienterobactin uptake (Fig. 3). Significant inhibition was observed with both compounds.

Because of the specificity of siderophores for ferric ion, it is generally believed that iron transport by siderophores is restricted to aerobic conditions. Although it has been suggested that anaerobic *E. coli* may be able to assimilate siderophore iron (3, 5), Pugsley and Reeves found no siderophore iron transport by anaerobically grown cells (15). In our experiments, the organism was grown and maintained under anaerobic conditions throughout all assays. Our growth data clearly demonstrated that siderophore iron was available to anaerobically grown cells. In agreement with Neüsch and Knüsel (13), we found that anaerobic cells were inhibited by albomycin, which is known to compete for siderophore transport systems. We also found that three membrane proteins known to be involved in siderophore transport were produced anaerobically and that the production was repressed by the addition of iron to the medium. It is also significant that the ferric ion of ferrichrome was as efficient a source of the metal as was ferrous ascorbate.

The above results supported the conclusion that anaerobic *E. coli* maintained mechanisms to utilize siderophore iron. The conclusion was supported by our transport data that showed that ferrienterobactin and ferrichrome were both actively taken up by anaerobically grown cells, and transport was inhibited by dinitrophenol and cyanide. Although the rate and extent of uptake were less than those in aerobically grown organisms, they were sufficient to supply the iron requirements for cell growth and reproduction.

It is no longer a justifiable assumption that anaerobic microorganisms need only deal with ferrous ion. It may be an ecological advantage for anaerobic organisms to maintain the ability to sequester iron from siderophores. In soil, for example, siderophores produced in the top aerobic layers may percolate down into anaerobic layers. A similar situation may prevail in gastrointestinal tracts. It would be interesting to determine whether obligate anaerobes also have the ability to utilize iron from siderophores produced by aerobic organisms.

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