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Iron Acquisition Systems of *Listeria monocytogenes*

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The uptake of iron by *Listeria monocytogenes* was studied. The microorganism was found to bind both $^{59}Fe(II)$ and [$^{59}Fe^{3+}$]citrate. In contrast, *L. monocytogenes* was unable to acquire iron from [$^{59}Fe^{3+}$] ferroxamine or [$^{59}Fe^{3+}$]EDTA or as $^{59}FeCl_3$. The data suggest that iron is acquired principally as iron(II) and that a citrate-inducible iron uptake system is also operative.

Listeria monocytogenes is a small, gram-positive opportunistic pathogen. Iron has been shown to be important for the growth of this organism during experimental infection (13). Most bacteria acquire iron through the secretion of high-affinity chelating agents, known as siderophores. The processes of recognition and transport have been shown to be highly specific (6), and the molecular aspects of microbial iron assimilation have been reviewed (2, 4). Siderophores have not been detected, however, in organisms such as *Yersinia* spp. (14) and *Legionella* spp. (11). In our studies of *L. monocytogenes*, we were unable to detect any iron-binding compounds released into culture supernatant fluids, but we did identify an iron reductant which rapidly reduced iron from transferrin (5).

We feel that iron transport by *L. monocytogenes* should be succinctly characterized as two separate but concurrent processes. The first of these is the acquisition phase. This includes both the mobilization of iron from the environment and the subsequent interaction of the metal with the cell surface. The second phase involves the transport of iron through the cell wall and cell membrane, followed by the release of the metal into the cytoplasm. In this study, we addressed the process of acquisition which concerns the interaction of iron with the cell surface. We wanted to determine whether *L. monocytogenes* acquires both ferric and ferrous iron, whether an iron-binding site exists on the surface of the cell, and whether a surface iron reductase is operative. This work was performed by T. J. Adams in partial fulfillment of the requirements of the degree Bachelor of Science.

Bacterial strain and growth conditions. *L. monocytogenes* EDG was used in all studies. The organism was maintained on brain heart infusion agar slants (Difco Laboratories) at 25°C. All glassware was acid washed. Starter cultures were grown overnight at 37°C in defined media (15) and inoculated into defined medium which had been treated with Chelex 100 (Bio-Rad Laboratories) to remove residual iron. The cells were grown at 37°C at 200 rpm and harvested at mid-exponential-growth phase by centrifugation. They were then washed twice in Chelex-treated 25 mM Tris hydrochloride (pH 7.4) and suspended in buffer to 10^{10} cells per ml.

Radioactive iron uptake. Radioiron uptake experiments were carried out in duplicate by a modification of the method of Rosenberg (12). Radioiron, as $^{59}FeCl_3$, was obtained from Amersham Corp. at an activity of 6 to 25 mCi/mg of Fe. $^{59}Fe^{2+}$ and $^{59}Fe^{3+}$ were prepared by adding $^{59}FeCl_3$ to freshly prepared $Fe(NH_4)_2(SO_4)_2$ in 0.1 mM HCl and to freshly prepared $FeCl_3$ in Chelex-treated 25 mM Tris hydrochloride buffer (pH 7.4), respectively. The final nonradioactive iron(II) and iron(III) concentrations in all experiments were 750 nM. After the addition of ^{59}Fe to the washed cells, 1-ml portions were removed at 0.5, 5, 15, 30, and 45 min,

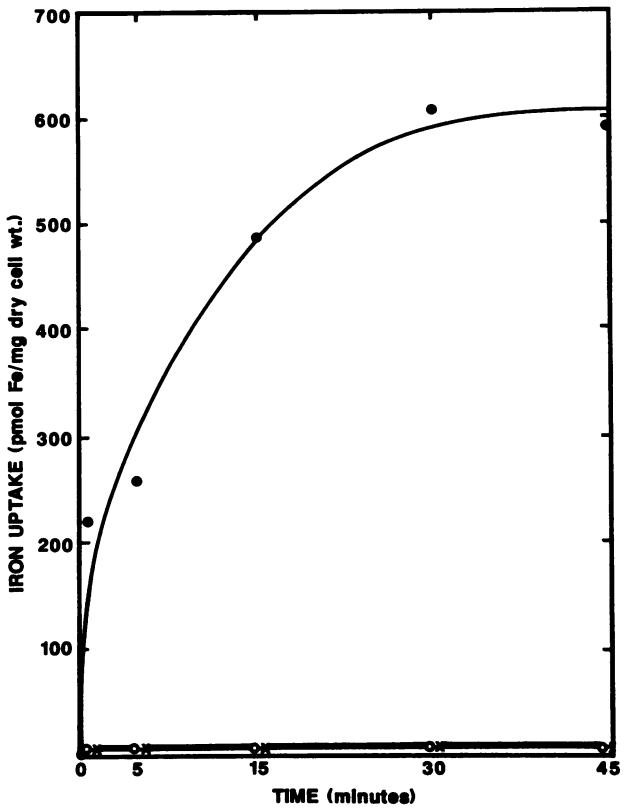

FIG. 1. Uptake of ^{59}Fe by *L. monocytogenes* EDG. Washed cell suspensions were placed in capped 50-ml polystyrene vials and gently bubbled with nitrogen for 10 min prior to addition of radioiron and throughout the experiment. In one experiment, BPS was added to a final concentration of 37.5 μM prior to the addition of radioiron. Each point represents the mean of duplicate experiments. Iron was added to the cells as $^{59}Fe^{2+}$ (●), $^{59}Fe^{2+}$ plus BPS (○), and $^{59}FeCl_3$ (×).

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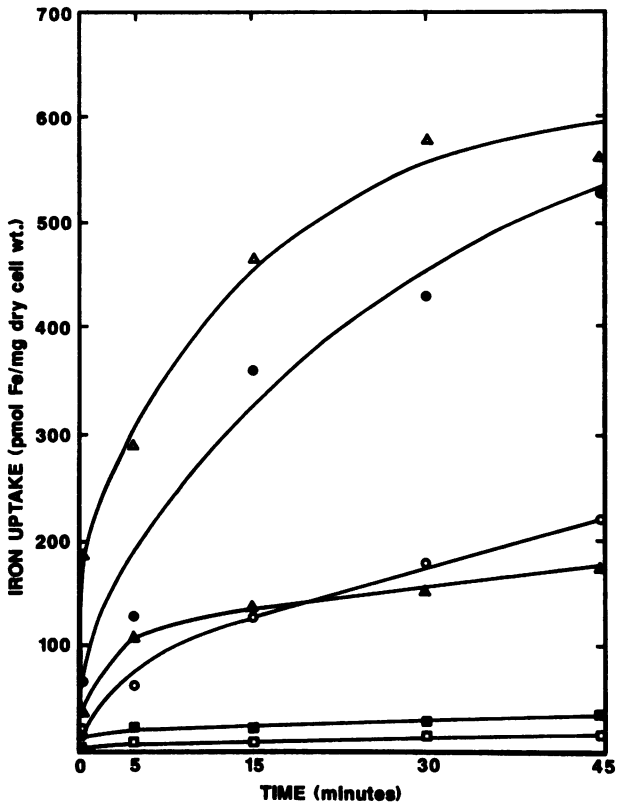

FIG. 2. Uptake of [$^{59}Fe^{3+}$]citrate in citrate-induced cells (●), [$^{59}Fe^{2+}$] in citrate-induced cells (△), [$^{59}Fe^{3+}$]citrate in non-citrate-induced cells (○), [$^{59}Fe^{3+}$]citrate in non-citrate-induced cells plus BPS (▲), [$^{59}Fe^{3+}$]citrate in citrate-induced cells with citrate inhibition (■), and [$^{59}Fe^{3+}$]citrate in non-citrate-induced cells with citrate inhibition (□) by *L. monocytogenes*. The citrate-iron ratio in all experiments was 20:1. Citrate-induced cells were grown in Chelex-treated media supplemented with sodium citrate to 0.1 mM. Citrate inhibition was carried out by preincubation of washed cells with 1 mM sodium citrate for 1 h prior to initiation of uptake studies. Final BPS concentration was 37.5 μM. The conditions for iron uptake were as described in the legend to Fig. 1.

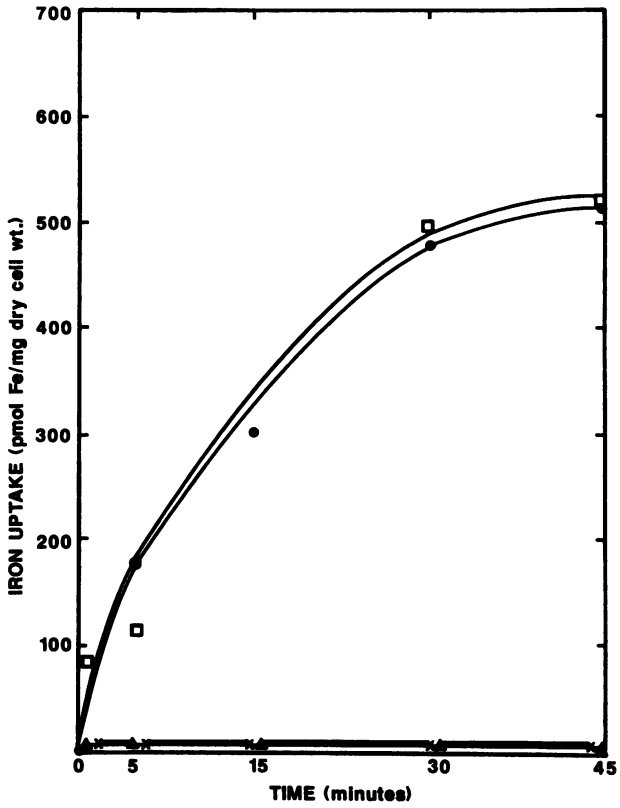

FIG. 3. Uptake of $^{59}Fe^{2+}$ plus KCN (□), $^{59}Fe^{2+}$ plus DNP (●), [$^{59}Fe^{3+}$]ferroxamine (▲), and [$^{59}Fe^{3+}$]EDTA (×) by *L. monocytogenes*. DNP and KCN were added to final concentrations of 0.5 mM and 4 mM, respectively, 20 min prior to initiation of uptake studies. Desferrioxamine and EDTA were at final concentrations of 37.5 μM. Iron uptake was carried out as described in the legend to Fig. 1.

filtered through a 0.45-μm-pore-size nitrocellulose membrane (Schleicher & Schuell, Inc.), and washed three times with Tris hydrochloride buffer. The filters were counted with a Packard 5360 gamma counter and the net ^{59}Fe uptake was calculated.

Iron uptake by *L. monocytogenes*. In this study, we define iron uptake as the binding of iron to the cell surface; these two terms are used interchangeably throughout the study. Figure 1 shows that 600 pmol of $^{59}Fe^{2+}$ per mg of dry cell weight was bound by *L. monocytogenes* within 30 min, while unligated $^{59}Fe^{3+}$ was not taken up by the microorganism. When bathophenanthroline sulfonate (BPS), a ferrous-ion chromophore (3), was added to the uptake media, there was essentially a 100% inhibition of iron binding.

Uptake of iron in citrate-induced cells. Figure 2 shows the uptake of [$^{59}Fe^{3+}$]citrate by *L. monocytogenes*. In iron-poor media, the uptake of [$^{59}Fe^{3+}$]citrate was approximately one-third that of ferrous iron. When the medium was supplemented with citrate, there was an induced uptake of [$^{59}Fe^{3+}$]citrate of approximately 200%. To determine whether this was a function of the metal or the ligand, we repeated the experiment, except that the cells (which had been grown in both citrate-supplemented and nonsupplemented media) were preincubated with citrate. This resulted in an inhibition of iron binding, which suggested that citrate was recognized by the cell and acted as a carrier of the metal. We found that there was essentially no difference in $^{59}Fe^{2+}$ uptake in Chelex-treated media and citrate-supplemented media, which indicated that ferrous-iron uptake was not influenced by citrate induction. To test whether the cells had a surface iron reductase, we included BPS with [$^{59}Fe^{3+}$]citrate, since ferrozine, also a ferrous-ion chromophore, inhibits the reductive acquisition of [$^{55}Fe^{3+}$]citrate in *Saccharomyces cerevisiae* (9). We found that BPS had no effect on the acquisition of [$^{59}Fe^{3+}$]citrate, indicating that the metal was not being reduced at the bacterial surface.

Uptake of [$^{59}Fe^{3+}$]chelates and effect of metabolic inhibitors. Figure 3 shows the effect of metabolic inhibitors on $^{59}Fe^{2+}$ uptake and the acquisition of [$^{59}Fe^{3+}$]ferroxamine and [$^{59}Fe^{3+}$]EDTA by *L. monocytogenes*. Iron acquisition was found not to be affected by either KCN or 2,4-dinitrophenol (DNP), which inhibit respiration and uncouple oxidative phosphorylation, respectively. These two compounds were individually found to totally inhibit the growth of *L. monocytogenes* (unpublished observations). We also determined that *L. monocytogenes* was unable to acquire $^{59}Fe^{3+}$ when chelated by either desferrioxamine or EDTA.

We feel that the simplest interpretation of the data is as follows. *L. monocytogenes* possesses at least two iron acquisition systems, one being citrate inducible for the

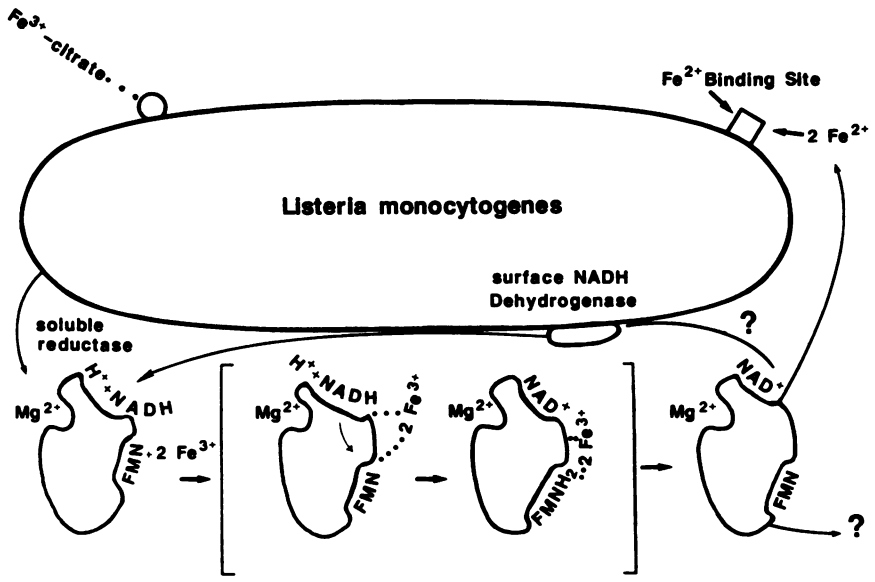

FIG. 4. Proposed model for the acquisition of ferrous and ferric iron by *L. monocytogenes*. FMN, flavin mononucleotide. This model presupposes that the extracellular reductase would donate two electrons from NADH to FMN, the latter of which would then reduce two atoms of ferric iron via two sequential one-electron reductions. If recycling of the reductase occurs, it is likely the regeneration of NAD^+ would involve a surface NADH dehydrogenase.

uptake of [$^{59}Fe^{3+}$]citrate and the other involving an iron(II)-binding site on the cell surface. The data show that the binding of iron occurred as a saturation phenomenon, which suggests binding via a surface receptor. Had this process involved passive diffusion, then one could predict linear kinetics of uptake. BPS did not interfere with the uptake of [$^{59}Fe^{3+}$]citrate, indicating that iron was in the oxidized form and suggesting that a surface iron reductase was not operative. The finding that neither KCN nor DNP inhibited the binding of iron(II) to the surface of *L. monocytogenes* suggests that this process did not require energy. This is in contrast to the report that both KCN and DNP were found to significantly inhibit anaerobic uptake of ferrienterobactin in *Escherichia coli* (10).

In previous studies, we were unable to identify any type of siderophore produced by this microorganism but did identify a powerful iron-reducing activity (5). The data suggest that most likely the major means of iron acquisition by *L. monocytogenes* is that of a reductive mobilization of iron, with the acquisition of free iron(II) via binding to a surface receptor and transport occurring by facilitated diffusion. Our studies have shown that the extracellular reductant is a low-molecular-weight protein which requires NADH, flavine mononucleotide, and Mg^{2+} as cofactors (R. E. Cowart, K. Fillmore, J. Kardatzke, and E. Barchini, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D-179, p. 101). A model of the iron acquisition process based on these findings is shown in Figure 4.

Lodge and Emery (10) and Hantke (8) reported that *E. coli* transports ferrous iron, while Bagg and Neilands (1) reported that iron(II), by acting as a cofactor for the Fur protein, is required for the regulation of aerobactin expression by *E. coli*. Thus, there is accumulating evidence that microorganisms that produce siderophores and possess a high-affinity transport system utilize ferrous iron. Evans et al. (7) reported that *Streptococcus mutans* does not produce siderophores and suggested that it transports only ferrous iron. They further suggested the presence of a surface iron reductase which is similar to that proposed for *S. cerevisiae* (9), a result in contrast to our findings with *L. monocytogenes*. Additional studies are needed to further characterize the mobilization, binding, and transport of reduced iron and its role in growth of both siderophore- and non-siderophore-dependent microorganisms. If iron reductases are found to be essential for both groups of organisms, then the proper inhibitor may reduce the enzymatic activity to the extent that iron acquisition is abolished and growth is alleviated. This would give serious hope to development of nonantibiotic means for the treatment of infectious diseases.

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