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Iron Availability Affects Entry of *Listeria monocytogenes* into the Enterocytelike Cell Line Caco-2

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The influence of iron on the entry of *Listeria monocytogenes* into Caco-2 cells was studied. Iron availability was found to modify the surface hydrophobicity and protein profile of *L. monocytogenes*, with the result that cell invasion strongly increased upon bacterial growth in iron-rich medium. The enhanced invasive capability of iron-overloaded *L. monocytogenes* cells correlates to the higher-level expression of the *inlAB* virulence genes, which were positively iron regulated at the transcriptional level.

Iron availability in vivo is an important environmental stimulus for pathogenic bacteria for coordinate regulation of gene expression (reviewed in reference 22). During many infections, the withholding of iron by the host's transferrins constitutes an important factor of nonspecific mammalian defense mechanisms (14, 38). Thus, the ability to acquire iron is essential for in vivo survival, and microorganisms have evolved several strategies to mobilize iron from the environment or host compounds. Many bacteria secrete low-molecular-weight iron chelators, termed siderophores; others acquire iron from host transferrins by binding with membrane proteins acting as receptors or by enzymatic reduction to the soluble ferrous form (25).

Host iron metabolism has been supposed to play a relevant part in the onset and progress of infections by the facultative intracellular pathogen *Listeria monocytogenes*, which is capable of causing severe diseases in humans and animals (33). Three different iron mobilization systems have been hypothesized for *L. monocytogenes*. These include the reduction of iron by a ferric reductase (9, 10), the direct binding of ferric citrate by a citrate-inducible receptor (1), and the acquisition of iron by a cell surface transferrin-binding protein (19). Besides iron acquisition, other strategies allow *L. monocytogenes* to survive and replicate in host tissues. In particular, this microorganism is able to invade, survive within, and replicate within epithelial cells, hepatocytes, and phagocytic cells, such as macrophages and monocytes (4, 27, 29, 30). Internalin, an 88-kDa protein encoded by the chromosomal *inlA* gene, is responsible for bacterial entry into epithelial cells (16, 28). Once the bacterium is inside the phagosome, the exotoxin listeriolysin O, a 58-kDa protein encoded by the *hly* gene, mediates the lysis of the phagolysosomal membrane, allowing the invading bacterium free access to the host cytoplasm (7, 23). Then, a 90-kDa protein encoded by the *actA* gene induces actin polymerization, leading to bacterial movement and eventually to bacterial spreading to adjacent cells (24; reference 27 and references therein).

During infection, reduced iron availability constitutes an important environmental stimulus for bacteria for coordinate regulation of virulence genes (22, 35). It is well documented that listeriolysin O production is enhanced by reduced iron concentrations (8, 10, 17), whereas little is known about the influence of iron on the expression of other virulence factors of *L. monocytogenes*. Hence, in order to gain further insight into the role of iron in the pathogenesis of listerial infections, we investigated the invasive capability of, the surface hydrophobicity and protein profile of, and the expression of *inlAB* and *hly* genes in *L. monocytogenes* grown under different conditions of iron availability.

Growth and hemolytic activity of *L. monocytogenes* under conditions of increasing iron deficiency. The clinical isolate of *L. monocytogenes* L37 (6) used in this study is a hemolytic wild-type strain capable of invading and multiplying in Caco-2 cells similarly to *L. monocytogenes* ATCC 7644 (unpublished data). Preliminary experiments showed that growth of *L. monocytogenes* L37 in brain heart infusion (BHI) (Merck) was correlated with the level of assimilable iron. Iron limitation ($Fe^{3+} < 1$ μM) was achieved by culturing bacteria in Chelex 100-treated BHI (1) or in BHI pretreated with 5 mg of bovine apolactoferrin (Lf) (Sigma Chemical Co.) per ml according to the procedure described by Valenti et al. (37). Extreme iron stress was achieved by adding 100 μM ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA) to the treated media. Iron excess was achieved by culturing bacteria in Chelex 100-treated or Lf-treated medium supplemented with 100 μM ferric citrate.

Maximal growth rates of *L. monocytogenes* L37 were achieved in iron-replete media (Chelex 100- or Lf-treated BHI plus 100 μM ferric citrate), whereas addition of the iron chelator EDDHA caused more reduction of bacterial growth than did the use of Chelex 100- or Lf-treated media. Although some differences of growth kinetics were observed, the biomass yields at the end of the exponential phase (9 to 12 h) in the Chelex 100- and Lf-treated media were comparable; yields were highest for iron-replete media, next highest for Chelex 100- or Lf-treated media, and lowest for EDDHA-supplemented media. Interestingly, the ability of *L. monocytogenes* to multiply efficiently under low-iron conditions (Chelex 100- and Lf-treated media) was maintained for more than five passages. The addition of EDDHA caused a progressive growth reduction up to the fourth passage, at which point there was no longer any appreciable increase in optical density with 24-h subculturing (data not shown).

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In order to assess whether iron availability in our treated media affected listeriolysin O production, we determined the hemolytic activity in culture supernatants of *L. monocytogenes* L37 grown for 24 h at 37°C in iron-deprived media with or without ferric citrate. The hemolytic activity of *L. monocytogenes* L37 was assayed according to the method of Dominguez Rodriguez et al. (12) and expressed in minimal hemolysis units, corresponding to the reciprocal of the highest dilution at which complete hemolysis was detected.

As other investigators have found with different strains (8, 9, 17), we confirmed that hemolytic activity in our isolate is dependent on iron restriction. A titer of 16 minimal hemolysis units was detected in supernatants from *L. monocytogenes* L37 grown under iron excess conditions (treated media supplemented with 100 μM ferric citrate), whereas a titer of ≥64 minimal hemolysis units was observed when bacteria were grown under conditions of iron restriction (Chelex 100-treated media and Lf-treated media with or without 100 μM EDDHA).

Effect of iron on surface hydrophobicity and protein profile of *L. monocytogenes*. Differences between iron-deficient and iron-sufficient cultures were also observed at the level of cell surface hydrophobicity. The hydrophobic interaction was quantitatively estimated according to the method of Tylewska et al. (36). Columns filled with 0.6 ml of phenyl-Sepharose CL-4B (Pharmacia Biotech) were equilibrated with 8 ml of 1 M $(NH_4)_2SO_4$, pH 7.0. The bacterial suspensions (approximately 2×10^{10} cells in 100 μl of the above-mentioned buffer) were applied onto the gel and eluted with 1 M $(NH_4)_2SO_4$. The retention of the cells reflected a hydrophobic interaction and was expressed as the percentage of bacteria retained in triplicate experiments. In control experiments columns were equilibrated and eluted with distilled water.

L. monocytogenes L37 cells grown with an excess of ferric ions (in Chelex 100-treated medium containing 100 μM ferric citrate) showed an increased affinity for the hydrophobic matrix, with a mean value of 90% ± 6% of bacteria adhering to the gel. When *L. monocytogenes* L37 was grown in the Chelex 100-treated medium supplemented with 100 μM EDDHA, 35% ± 3% of organisms were retained in the column, indicating that the hydrophobicity of iron-restricted bacteria is reduced.

Iron availability is also known to affect the microbial surface protein profile (26). Therefore, we analyzed the surface proteins of *L. monocytogenes* L37 grown under conditions of increasing iron restriction. Surface proteins were prepared according to the procedure described by Tabouret et al. (34). Bacteria grown for 18 h at 37°C under agitation in 50 ml of Chelex 100-treated medium supplemented or not with 100 μM ferric citrate or 100 μM EDDHA were harvested by centrifugation at 6,000 × *g* for 20 min at 4°C, washed twice in phosphate-buffered saline (PBS), and resuspended in 250 μl of PBS containing 1% (wt/vol) sodium dodecyl sulfate (SDS). After 5 min of shaking at room temperature, cellular bodies were removed by centrifugation (15,000 × *g* for 10 min) and the supernatant, containing the SDS-extracted surface proteins, was analyzed by SDS–10% polyacrylamide gel electrophoresis under denaturing conditions as described by Laemmli (20).

There are marked differences in the surface protein profiles of *L. monocytogenes* cells grown under conditions of increasing iron starvation (Fig. 1). These differences are confined to proteins with apparent molecular masses of >45 kDa. The most evident changes were observed for a protein of approximately 64 kDa which was expressed under conditions of extreme iron starvation (in EDDHA-supplemented medium) and for proteins of approximately 45, 50, 52, 72, and 105 kDa, whose expression decreased proportionally to the iron depletion. Although at present we cannot assign a specific function to these iron-regulated proteins, it appears that a progressive reduction of iron availability in the growth environment leads to a remarkable alteration in the surface protein composition of *L. monocytogenes* L37.

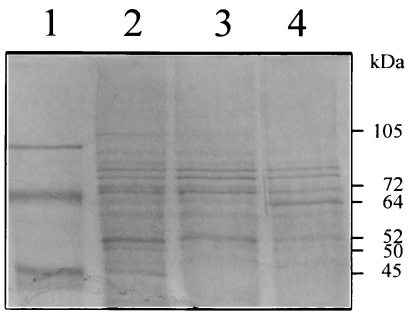

FIG. 1. Comparison of the surface proteins of *L. monocytogenes* L37 grown under conditions of increasing iron deficiency. Lane 1, molecular mass standards, including rabbit muscle phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), and hen egg white ovalbumin (45.0 kDa); lane 2, *L. monocytogenes* L37 grown in Chelex 100-treated medium supplemented with 100 μM ferric citrate; lane 3, *L. monocytogenes* L37 grown in Chelex 100-treated medium; lane 4, *L. monocytogenes* L37 grown in Chelex 100-treated medium supplemented with 100 μM EDDHA. The protein concentration was estimated according to the method of Bradford (3), and samples of 30 μg were loaded in each lane. The gel was stained with Coomassie brilliant blue. The positions of relevant iron-regulated proteins of approximately 45, 50, 52, 64, 72, and 105 kDa are shown on the right.

Adhesive and invasive properties of *L. monocytogenes* grown under conditions of increasing iron deficiency. The iron-induced changes of hydrophobicity and surface proteins of *L. monocytogenes* L37 prompted us to examine the adhesion and the invasion efficiency of bacteria grown under different conditions of iron availability. Caco-2 cells (from a human colon carcinoma cell line) were cultured in minimum essential medium (Eagle's MEM) to obtain semiconfluent monolayers (2×10^5 cells per well) as described elsewhere (6). For adherence assays, semiconfluent monolayers in Eagle's MEM without antibiotics were infected for 1 h at 4°C at a multiplicity of infection (MOI) of 100 exponentially grown bacteria per cell. After infection, cell monolayers were carefully washed five times with Eagle's MEM to remove unattached bacteria, lysed by the addition of cold 0.1% Triton X-100, and plated on listeria-selective agar base (Unipath) to determine the number of viable bound bacteria by performing colony counts (expressed in CFU per milliliter). The entry of *L. monocytogenes* into Caco-2 cells was tested according to the method of Conte et al. (6). After infection for 1 h at 37°C at an MOI of approximately 100 exponentially grown bacteria per cell, the monolayers were washed five times with Eagle's MEM and 1 ml of fresh medium containing 5 μg of gentamicin per ml was added to each well. After 1 h of incubation at 37°C, the cells were lysed by the addition of cold 0.1% Triton X-100 and plated to determine the number of viable intracellular bacteria. For the intracellular-growth assays, Caco-2 cells were infected for 1 h at 37°C at a lower MOI (approximately 10 CFU per cell) and incubated in gentamicin-containing medium for an additional period of 8 h at 37°C (23). At various intervals (2, 4, 6, and 8 h), cell monolayers were washed with Eagle's MEM and lysed with Triton X-100 for CFU counts.

Results reported in Table 1 show that the ability of *L. monocytogenes* L37 to adhere to and invade Caco-2 cells varied depending on the iron status of the bacteria; both adherence and invasion were increased by growth in iron-rich media. Although poorly adherent, *L. monocytogenes* L37 cells adhered

TABLE 1. Efficiency of adherence to and entry of *Listeria monocytogenes* L37 into Caco-2 cells[a]

Growth conditions	Adhesion (%)[b]	Entry (%)[c]
Chelex-treated BHI		
With ferric citrate (100 μM)	0.76 ± 0.31	4.30 ± 0.52
Alone	0.03 ± 0.02	0.34 ± 0.08
With EDDHA (100 μM)	0.02 ± 0.02	0.04 ± 0.01
Lf-treated BHI		
With ferric citrate (100 μM)	1.12 ± 0.43	9.42 ± 1.58
Alone	0.05 ± 0.03	0.75 ± 0.20
With EDDHA (100 μM)	0.04 ± 0.03	0.12 ± 0.01

[a] Adhesion and entry are expressed as the percentages of the inoculated CFU which adhered to or were internalized into Caco-2 cells, respectively. Data are presented as means ± standard deviations of at least five experiments.

[b] Semiconfluent cell monolayers were infected for 1 h at 4°C with exponentially grown bacteria at an MOI of 100 CFU per cell. After washings, cells were lysed and the number of adherent bacteria was determined.

[c] Semiconfluent cell monolayers were infected for 1 h at 37°C with exponentially grown bacteria at an MOI of 100 CFU per cell. After washings, cells were incubated for an additional 1 h at 37°C with fresh culture medium containing 5 μg of gentamicin per ml. Cells were then lysed, and the number of intracellular bacteria was determined.

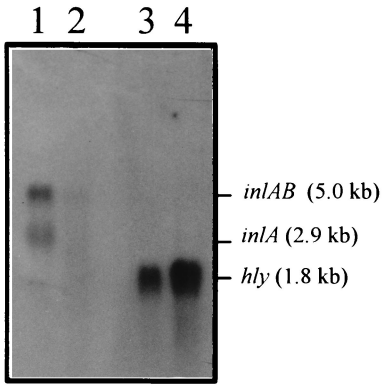

FIG. 2. Northern analysis and transcriptional regulation of the *inlAB* and *hly* genes. Total RNA was isolated from *L. monocytogenes* L37 cells grown under conditions of iron sufficiency (Chelex 100-treated medium supplemented with 100 μM ferric citrate [lanes 1 and 3]) and iron deficiency (Chelex 100-treated medium supplemented with 100 μM EDDHA [lanes 2 and 4]). The A_{600} of the bacterial cultures was approximately 0.8 under both conditions. Total RNA samples (10 μg for each lane) were hybridized with a 5,210-bp *Kpn*I probe encompassing the *inlAB* open reading frame (lanes 1 and 2) and with a 1,593-bp PCR-generated *hly* gene probe (lanes 3 and 4). Prior to blotting, the quality and the relative amounts of RNA samples loaded in each lane were checked by visualization of rRNAs by ethidium bromide staining. DNA probes were labeled with [α-^{32}P]dATP as described in the text. Relevant mRNAs are shown on the right.

better to Caco-2 cells when grown under conditions of iron excess, whereas adhesion was nearly nonexistent after iron limitation. A comparable trend was observed for invasion efficiency. The percentage of internalized bacteria was enhanced by a factor of >10 when *L. monocytogenes* was grown in iron-supplemented media compared with iron-deprived media. Indeed, iron-stressed bacteria, while invading cultured epithelial cells with a lower efficiency, exhibited higher intracellular growth rates, reaching approximately the same numbers in Caco-2 cells as iron-rich organisms 8 h after the onset of the infection (data not shown). The enhanced intracellular multiplication rate of iron-deficient bacteria might be ascribed to overproduction of listeriolysin O, which would cause more efficient escape of iron-stressed organisms from intracellular vesicles compared with that of iron-replete bacteria.

Northern (RNA) blot analysis of *inlAB* and *hly* transcripts in *L. monocytogenes* cells grown under low- and high-iron conditions. Among the virulence determinants of *L. monocytogenes*, the internalin protein, the product of the *inlA* gene, is responsible for the invasion of epithelial cells (16). To investigate whether iron affects the invasive phenotype of *L. monocytogenes* L37 by regulating the expression of the *inlA* gene at the transcriptional level, Northern blot analyses were performed with total cellular RNA from *L. monocytogenes* L37 following late logarithmic growth in low- and high-iron media. Total RNA was isolated by a modification of the hot-phenol extraction method (31). Bacteria were grown to an A_{600} of ≃0.8 with vigorous aeration at 37°C in 8 ml of Chelex 100-treated BHI supplemented with either 100 μM ferric citrate or 100 μM EDDHA. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, and the pellet was suspended in 500 μl of lysis buffer containing 20 mM sodium acetate (pH 5.5), 5 mg of lysozyme per ml, and 1 mM EDTA (pH 8.0). The suspension was kept in ice for 8 min and then supplemented with 500 μl of a solution containing 20 mM sodium acetate (pH 5.5), 20 mM ribonucleoside vanadyl complexes (Sigma Chemical Co.), 1% SDS, and 1 mM EDTA (pH 8). After an additional 5-min incubation in ice, cells were extracted for 5 min at 65°C with 1 ml of prewarmed phenol saturated with 20 mM sodium acetate (pH 5.5). After centrifugation, the aqueous phase was extracted with an equal volume of chloroform-isoamyl alcohol (24/1, vol/vol) and the nucleic acids were precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 6) and 3 volumes of absolute ethanol. The precipitated material was treated for 15 min at 37°C with 25 U of DNase I (RNase free; Boehringer) and 25 U of RNasin (Boehringer), then phenol extracted twice, ethanol precipitated, and redissolved in diethyl pyrocarbonate-treated water. The RNA concentration was estimated by optical density measurements at 260 and 280 nm. Aliquots of 10 μg of total RNA were denatured at 65°C for 15 min in the presence of 2 M formaldehyde and 50% formamide and then were electrophoresed for 4 h at 4 V/cm on a 1% agarose gel containing 2 M formaldehyde in MOPS (morpholine propanesulfonic acid) buffer (29). The RNA was transferred onto nylon filters (Hybond-N; Amersham Corp.) as described by Sambrook et al. (31) and heat fixed. DNA probes were labeled with [α-^{32}P]dATP (3,000 Ci/nmol; Amersham Corp.) with a primer extension kit (Boehringer). Hybridization was performed for 12 h at 65°C in a solution containing 1% SDS, 6× SSC (6× SSC is 0.9 M NaCl, 0.09 M sodium citrate [pH 8.0]), 5× Denhardt's solution, and 50 μg of denatured salmon sperm DNA per ml. The filters were washed twice for 15 min in 2× SSC at 65°C, once for 30 min in 2× SSC–0.1% SDS at 65°C, once for 30 min in 0.1× SSC–0.1% SDS at 65°C, and once for 5 min in 0.1× SSC at room temperature. The membranes were analyzed with a Betascope model 603 blot analyzer (Betagen) and were then exposed to Kodak XAR film.

Hybridization of RNA extracted from iron-rich cells with a 5,210-bp *inlAB* probe generated by *Kpn*I digestion of plasmid pPE2 (16) led to the detection of two bands of approximately 5 and 2.9 kb, while barely visible signals were obtained with RNA from cells grown in the low-iron medium (Chelex 100-treated medium supplemented with 100 μM EDDHA), indicating a stringent positive control of *inlAB* transcription by iron (Fig. 2, lanes 1 and 2). On the basis of quantitative estimations of mRNAs with the blot analyzer, it appeared that the level of the longer (*inlAB*) transcript exceeded that of the shorter (*inlA*) transcript by a factor of 1.6, and both mRNAs were

reduced by a factor of >10 under conditions of iron deficiency. In consideration of the key role played by internalin in the process of entry of *L. monocytogenes* into the host cell, it can be argued that increased transcription of the internalin gene might account for the higher invasion efficiency observed with bacteria grown under conditions of iron excess compared with that of iron-deprived bacteria. Further investigation is required to ascertain whether the iron-dependent increase of the *inl* mRNAs is due to the activation of transcription arising from the *inlAB* promoter or to increased mRNA stability.

It was also interesting that the positive modulation of *inlAB* expression by iron was in contrast to the iron repression of *hly* gene expression. This gene encodes listeriolysin O, which mediates the lysis of bacterium-containing vacuoles inside the host cell. To demonstrate the negative regulation of *hly* transcription by iron in *L. monocytogenes* L37, we probed the same RNA preparation with a PCR-generated *hly* gene probe. Two oligonucleotides annealing to bp 127 to 150 of the noncoding strand and to bp 1699 to 1719 of the coding strand of the published *hly* sequence (23) were used in amplification reactions. PCR was carried out by using 100 ng of genomic DNA of *L. monocytogenes* L37 in a 100-μl reaction mixture containing 1× PCR buffer (Perkin Elmer), 1.5 μM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1 μM each primer, and 2.5 U of *Taq* DNA polymerase (Perkin Elmer). Thirty cycles were performed in a Perkin Elmer 480 thermal cycler, each cycle comprising 30 s at 95°C, 1 min at 60°C, and 45 s at 74°C.

In line with functional evidence of iron-repressible hemolytic activity, there was a fivefold increase in *hly* transcription in response to iron deprivation (Fig. 2, lanes 3 and 4). However, the 1.8-kb *hly* mRNA was still detectable in iron-replete cultures of *L. monocytogenes* L37, indicating that iron in excess was not sufficient to totally repress transcription of this gene. The increased transcription of the *hly* gene under low-iron conditions also constitutes an important control for the validation of our results on *inlAB* expression and leads us to conclude that iron affects expression of these genes in opposite ways.

For many bacterial parasites it has been established that iron availability affects the synthesis of metabolites involved in the pathogenetic process (5, 14, 22, 35), but little is known about the influence of iron on the expression of *L. monocytogenes* virulence genes (8). For this bacterium some stress conditions, e.g., temperature, nutrient limitation, and stationary phase, have been shown to trigger the expression of relevant pathogenetic determinants through finely tuned transcriptional control (13, 21, 32). In this report we show that iron also influences the expression of the main virulence proteins connected with invasion (internalin) and escape from vacuoles (listeriolysin O): high-iron conditions elicited cell invasion and transcription of the *inlAB* cluster, while low-iron conditions enhanced intracellular growth and *hly* expression. On the basis of current models for iron-regulated gene expression in both gram-positive and gram-negative bacteria, it is tempting to speculate that a functional homolog of the *Corynebacterium diphtheriae* DtxR (35) or of the gram-negative Fur (22) repressor proteins might directly or indirectly control transcription of the *hly* gene in *L. monocytogenes*. Nevertheless, sequence homology searches did not allow us to recognize Fur- or DtxR-like consensus sequences within the promoter regions of the *hly* gene (11) or of its transcriptional activator, *pfrA* (15).

The differences in iron modulation of *inlAB* and *hly* may also reflect differences in the temporal expression of these genes. It appears advantageous for the bacterium to express *inlAB* when it is in the extracellular environment and needs to attach to and invade a host cell; expression of *hly* at the same time could potentially damage a host cell. Similarly, escape from the vesicle requires *hly* but not *inlAB*. Thus, it appears advantageous for the bacterium to express these two genes, which are required at different stages of invasion, under different environmental conditions. Whether these observations are valid for the in vivo situation is still an open question. Relatively large amounts of assimilable iron are present in the luminal phase of the gut in healthy individuals (2, 18), and dietary iron overload is known to increase susceptibility to several parasitic infections (14, 38). It can therefore be speculated that an iron-replete environment at the level of the intestinal mucosa may contribute to the expression of a virulence factor, such as internalin, which is essential for the entry of *L. monocytogenes* into enterocytes, while an iron-deficient intracellular environment might account for the increased expression of listeriolysin O, which is needed for the delivery of bacteria into the cytoplasm. This is a fine model for coordinate iron-dependent regulation of virulence genes in *L. monocytogenes.*

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