INFECTION AND IMMUNITY, July 1997, p. 2778–2785 Vol. 65, No. 7
0019-9567/97/$04.00+0

Utilization of Iron-Catecholamine Complexes Involving Ferric Reductase Activity in *Listeria monocytogenes*

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Received 23 December 1996/Returned for modification 25 March 1997/Accepted 13 April 1997

Listeria monocytogenes **is a ubiquitous potentially pathogenic organism requiring iron for growth and virulence. Although it does not produce siderophores, *L. monocytogenes* is able to obtain iron by using either exogenous siderophores produced by various microorganisms or natural catechol compounds widespread in the environment. In the presence of tropolone, an iron-chelating agent, growth of *L. monocytogenes* is completely inhibited. However, the growth inhibition can be relieved by the addition of dopamine or norepinephrine under their different isomeric forms, while the catecholamine derivatives 4-hydroxy-3-methoxyphenylglycol and normetanephrine did not relieve the inhibitory effect of tropolone. Preincubation of *L. monocytogenes* with chlorpromazine and yohimbine did not antagonize the growth-promoting effect of catecholamines in iron-complexed medium. In addition, norepinephrine stimulated the growth-promoting effect induced by human transferrin in iron-limited medium. Furthermore, dopamine and norepinephrine allowed ^{55}Fe uptake by iron-deprived bacterial cells. The uptake of iron was energy dependent, as indicated by inhibition of ^{55}Fe uptake at 0°C as well as by preincubating the bacteria with KCN. Inhibition of ^{55}Fe uptake by *L. monocytogenes* was also observed in the presence of Pt(II). Moreover, when assessed by a whole-cell ferric reductase assay, reductase activity of *L. monocytogenes* was inhibited by Pt(II). These data demonstrate that dopamine and norepinephrine can function as siderophore-like compounds in *L. monocytogenes* owing to their *ortho*-diphenol function and that catecholamine-mediated iron acquisition does not involve specific catecholamine receptors but acts through a cell-bound ferrireductase activity.**

Iron is an essential element for bacterial survival and growth. This metal enters as a cofactor in the composition of a number of cellular enzymes such as catalase, cytochromes, and peroxidases. Bacteria have developed different systems for iron acquisition (48). The main mechanism consists of the synthesis of Fe(III) chelators, named siderophores, which are low-molecular-weight molecules with high iron affinity secreted by many bacteria (4, 22, 35; for a review, see reference 37) and fungi (24, 26, 49) under iron-starved conditions. Phytosiderophore systems have also been described for plants (9, 38). After binding extracellular Fe(III), the iron-siderophore complex is either taken up by the cell via specific transport systems or reduced to release Fe(II) (9). Nevertheless, some fungi such as *Saccharomyces cerevisiae* (39) or bacteria such as *Listeria monocytogenes*, *Neisseria* spp., and *Legionella* spp. do not seem to produce any siderophores (8, 20, 42). In *L. monocytogenes*, iron acquisition is mediated by two different systems, one being inducible ferric citrate uptake (1) and the other involving a surface bound reductase as described by Deneer et al. (13) and/or an extracellular reductant as described by Barchini and Cowart and Cowart and Foster (3, 8). A ferric reductase activity has also been reported for several other bacteria, e.g., *Streptococcus mutans* (18), *Legionella* spp. (27), fungi, e.g., *Ustilago sphaerogena* (15), *Candida albicans* (36), and *S. cerevisiae* (16), and many plant species (17).

L. monocytogenes is an opportunistic pathogen responsible for severe infections in humans, the most important of which are meningitis or meningoencephalitis, septicemia, and, in the case of pregnant women, intrauterine infections of the fetus which may result in abortion, stillbirth, or neonatal infection (43). The major route of *L. monocytogenes* infection is by ingestion of contaminated foodstuffs (19, 21). The pathogen, a facultative intracellular parasite, disseminates by cell-to-cell spread and rapidly propagates in blood circulation and cerebrospinal fluid (6). It is well known that *Listeria* requires iron to support growth during experimental infection (46). However, it is also known that although there is plenty of iron present in the body fluids of humans and animals, the amount of free iron which might be readily available to bacteria is extremely low (10^{-18} M). Most of the body's iron is found intracellularly, in ferritin, hemosiderin, and heme, while the remaining extracellular iron is bound to high-affinity iron-binding proteins, transferrin in body fluids, and lactoferrin in secretions and milk (2).

Upon entrance into a host, following survival through the stomach and until reaching the brain, bacteria encounter a myriad of neurohormones such as catecholamines. Various effects of catecholamines on bacteria have been already described. Catecholamines have been reported to modulate growth of the gram-negative bacteria *Escherichia coli*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa* (32). Norepinephrine has also been reported to relieve *Enterobacteria* and *Pseudomonas* growth-inhibiting action of bovine and porcine sera but not of human or chicken sera (29). In addition, catecholamines also exert a protective effect against oxidative damage in the opportunistic pathogenic yeast *Cryptococcus neoformans* (40).

We previously reported that of a series of 10 iron-chelating agents tested, tropolone and 8-hydroxyquinoline were the most

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effective in inhibiting the growth of *L. monocytogenes* (47) and that this growth inhibitory effect was completely reversed by ferric citrate (7). In addition, we showed that *L. monocytogenes* was able to grow in tropolone or 8-hydroxyquinoline iron-restricted medium in the presence of various siderophores or some natural catechols or catecholamines (44).

The purpose of the present work was to investigate the actual role of norepinephrine and dopamine in *L. monocytogenes* iron acquisition and to assess the biological relevance of that specific function by testing the norepinephrine effect in the presence of transferrin. Furthermore, we also examined whether the ability of the catecholamines to promote bacterial growth occurs through a receptor-mediated process or requires a ferric reductase activity.

MATERIALS AND METHODS

Bacterial strain, media, and chemicals. *L. monocytogenes* B38 (serovar 4b), isolated from cheese, was used in this study. This strain was virulent for mice, as shown by the 50% lethal dose (10^5 CFU) (7). The organism was maintained by storage at −80°C in brain heart infusion broth (bioMérieux, Marcy-l'Etoile, France) supplemented with 20% glycerol. Before each experiment, one platinum loopful was scratched from the frozen surface of the stock culture and bacteria were grown at 37°C overnight on a slant of tryptic soy agar containing 0.6% yeast extract (TSYA; bioMérieux). Tryptic soy broth with 0.6% yeast extract (TSYB; bioMérieux) was used for monitoring in vitro growth of bacteria. Alternatively, bacteria were grown in defined Welshimer medium (WM) modified as described by Premaratne et al. (41) consisting of the following (in a volume of 1.0 liter of deferrated water [see below]): KH_2PO_4, 3.28 g; Na_2HPO_4, 15.48 g; $MgSO_4$, 0.41 g; glucose, 10 g; L-isoleucine, 0.1 g; L-leucine, 0.1 g; L-valine, 0.1 g; L-arginine, 0.1 g; L-glutamine, 0.6 g; L-histidine, 0.1 g; L-methionine, 0.1 g; L-cysteine, 0.1 g; L-tryptophan, 0.1 g; riboflavin, 1 mg; thiamine, 1 mg; D-biotin, 100 mg; DL-6,8-thioctic acid, 1 μg. All glassware was washed with 1 N HCl and rinsed three times with deionized water before use.

Iron was removed from tryptic soy broth by shaking the medium with 10% Chelex 100 resin (Bio-Rad, Yvry-sur-Seine, France) for 3 h at 200 rpm. The resin was removed by decantation, and the medium was filter sterilized. Because the Chelex resin is also able to remove other divalent cations, including Ca^{2+} and Mg^{2+}, we added $MgSO_4$ (25 mg/liter) and $CaCl_2$ (50 mg/liter) to the treated medium (subsequently referred to as TSBm). The iron concentration (132 μg/liter) of the deferrated medium was determined by atomic absorption spectroscopy.

Deferrated water was obtained by mixing double-distilled water with 2.5% Chelex 100 for 24 h under agitation (200 rpm). The resin was removed by decantation.

All solutions were prepared with deferrated water and sterilized by filtration through 0.45-μm-pore-size filters (Dutscher, Brumath, France). $PtCl_2$ stock solution was prepared as a saturated solution in 1 N HCl. The concentration of dissolved Pt(II) was then measured by atomic emission spectroscopy. Solution of 4-hydroxy-3-methoxyphenylglycol (MHPG)-piperazine was prepared in degassed phosphate-buffered saline (pH 7.2) and stored under nitrogen atmosphere.

All products were obtained from Sigma (St. Quentin-Fallavier, France), except L-arginine, which was purchased from Merck (Darmstadt, Germany).

Bioassay and growth measurement. Bioassays were performed by the disk diffusion test as described previously (44). Briefly, cell suspensions were prepared by suspending overnight culture in deferrated water and adjusting the suspension to an optical density at 650 nm (OD_{650}) of 0.07 (Lumetron, Photovolt Co., New York, N.Y.) (1×10^8 to 3×10^8 CFU/ml). One hundred microliters of that suspension was plated on TSYA medium supplemented with tropolone at the minimal inhibitory concentration (20 μM) or with 8-hydroxyquinoline (minimal inhibitory concentration, 10 μM) poured into petri dishes. Sterile filter paper disks (6.0-mm diameter) were loaded with 25 μl of different sterile-filtered catecholamines and added to inoculated tropolone-TSYA medium or 8-hydroxyquinoline–TSYA medium. The stimulatory effect of catecholamines on *L. monocytogenes* growth was determined after 24 h of incubation at 37°C by measuring the bacterial growth diameter around catecholamine-impregnated disks. Dopamine and racemic (±)-norepinephrine were tested under their iron-free forms.

Growth curve measurement was carried out with 20 μM tropolone-supplemented TSYB. The broth medium was supplemented with dopamine or norepinephrine at various concentrations with or without addition of their antagonist molecules, chlorpromazine and yohimbine, respectively. Cells were preincubated with antagonist 10 min before the addition of the catecholamine. Normetanephrine, MHPG-piperazine salt, (+)-norepinephrine, and (−)-norepinephrine were also tested for their ability to reverse the inhibitory effect of tropolone. Differences between norepinephrine and MHPG or normetanephrine result from a catechol-*o*-methylation or oxidative deamination of catecholamines, respectively. Cell suspensions were prepared as described above and inoculated at a final concentration of 1.0×10^6 CFU/ml. The seeded medium was dispensed into a 96-well microplate, and growth was monitored by directly measuring every hour the OD_{620} of each well of the microplate, incubated at 37°C and shaken for 5 s before measurement (microreader HT2; Anthos Labtec, Vienna, Austria). For experiments with holo-transferrin and apo-transferrin, TSBm was used in place of TSYB.

Radiolabelled iron uptake. Radiolabelled iron uptake experiments were carried out in triplicate in capped 50-ml polypropylene vials. $^{55}FeCl_3$ was obtained from Amersham France (Les Ulis, France) at a minimal activity of 5 mCi/mg of Fe. The labelled iron, supplied as ferric chloride in 0.1 M HCl, was diluted in 0.1 M HCl to a final concentration of 36.4 μM (stock solution). ^{55}Fe-catecholamine complexes were prepared in WM 24 h before uptake experiments and stored at room temperature. Radiolabelled iron complexes were obtained at a ratio of 30:1 by mixing 5.5 μM norepinephrine or 5.5 μM dopamine with 0.18 μM $^{55}FeCl_3$. Cells cultivated overnight at 37°C in TSYA were harvested, washed twice in sterile deferrated water, inoculated into 200 ml of WM, and incubated at 30°C under agitation (200 rpm). This step was crucial to deplete iron from the bacterial inoculum grown on TSYA. After 22 to 24 h of growth, the cells were harvested by centrifugation, washed twice in deferrated water, resuspended into 200 ml of fresh WM, and incubated at 30°C under agitation for 6 h. The cells were induced during the two successive cultures by supplementing WM with 0.75 μM catecholamine. Following the second incubation, the cells were harvested, washed twice, suspended in 37°C-prewarmed WM to a density of about 10^9 CFU/ml, and supplemented with radiolabelled iron-catecholamine complexes. Afterwards, samples (1.0 ml) were withdrawn at intervals (5, 10, 20, and 30 min), filtered through a 0.45-μm-pore-size nitrocellulose filter (Millipore, Molsheim, France), and washed 10 times with 1.0 ml of WM. Filters were dried for 1 h at 50°C and then counted in a Beckman LS 6000 SC beta scintillation counter after adding scintillation liquid (Fluoransafe XEScintran; Polylabo, Illkirch, France).

In one set of experiments, incubation was maintained at 0°C instead of 37°C to assess the effect of temperature on iron uptake. When specified, KCN or $PtCl_2$ was added to a final concentration of 9 mM or 46 μM, respectively, 30 min prior to initiation of the uptake experiments. For competition studies with $^{55}FeCl_3$, unlabelled $FeCl_3$ (18 μM) was added to the uptake medium 10 min after the beginning of the reaction.

Control samples without cells were included to deduce nonspecific binding of radiolabelled iron to the filters. All binding assays were corrected for background, and the net ^{55}Fe uptake was calculated.

Whole-cell ferric reductase assay. The reduction of ferric to ferrous iron was measured by trapping Fe^{2+} with ferrozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine] as described by Dailey and Lascelles (10) and modified by a procedure developed for yeast cells by Dancis et al. (11). Bacterial culture was prepared by the same procedure as that used for iron uptake. Cells were harvested by centrifugation, washed twice in deferrated water, and resuspended in deferrated water to a final concentration of 5.0×10^9 to 1.0×10^{10} CFU/ml. To assess the effect of Pt(II) on reductase activity, various concentrations of $PtCl_2$ were added to prewarmed cell suspensions 30 min before beginning the assay. The reaction mixture contained in a flask 5 ml of bacterial suspension treated or not treated with $PtCl_2$ and the following reagents: $MgCl_2$ (10 mM), NADH (50 μM), flavin mononucleotide (3 μM), ferrozine (2 mM), ferric ammonium citrate (100 μg ml^{-1} final concentration). After the addition of reagents, the flasks were incubated at 37°C, and 1.0-ml aliquots were removed at intervals over 30 min and centrifuged for 10 min at 10,000 × *g*. The supernatant was carefully collected, and its change in OD_{562} was monitored in a Shimadzu double-beam spectrophotometer. The reference cuvette contained all of the reaction components except bacterial cells. The amount of Fe(II)-ferrozine produced was calculated by use of a molar extinction coefficient of 27,900 (45). CFU were determined on TSYA medium after incubation at 37°C for 24 h.

RESULTS

Effect of catecholamines on tropolone-inhibited *L. monocytogenes* growth. As shown by bioassays (Table 1), dopamine and norepinephrine antagonized the growth inhibitory effect of tropolone and supported growth of *L. monocytogenes* in a dose-dependent manner. The size of the growth zone around the disks was proportional to the catecholamine load, with an increase in the growth zone diameter from 10.0 to 17.3 mm for dopamine and from 11.3 to 17.1 mm for norepinephrine resulting when the catecholamine load was increased from 0.09 to 1.5 μmol. The lowest effective amount of dopamine or norepinephrine was 0.09 μmol. The growth-promoting activity appeared the same for norepinephrine as for dopamine. The same results were obtained when tropolone was replaced by 8-hydroxyquinoline.

The effect of catecholamines on the growth curve of *L. monocytogenes* was monitored in tropolone iron-restricted TSYB. The addition of dopamine and norepinephrine in

TABLE 1. Growth stimulation of *L. monocytogenes* by dopamine and norepinephrine in tropolone iron-complexed medium[a]

Ligand	Disk load (μmol)	Growth zone diam (mm) on tropolone
Dopamine	1.50	17.3
	0.75	15.3
	0.37	13.5
	0.18	11.9
	0.09	10.0
	0.03	—[b]
Norepinephrine	1.50	17.1
	0.75	15.1
	0.37	14.8
	0.18	13.3
	0.09	11.3
	0.03	—

[a] Paper disks were loaded with different concentrations of each catecholamine and deposited onto TSYA medium containing 20 μM tropolone seeded with 10^6 bacteria. Growth zone diameters were checked after incubation at 37°C for 24 h. Data are averages of triplicate experiments.

[b] —, no growth zone.

TSYB free of iron chelator had no effect on the *L. monocytogenes* growth curve (Fig. 1A and C). The stimulatory growth effect induced by dopamine or norepinephrine in iron-sequestered medium was concentration dependent (Fig. 1B and D). The addition of 0.5 and 5 mM dopamine or norepinephrine restored nearly completely the bacterial growth to the same rate as that of the control, while 0.05 mM of catecholamine was totally ineffective. To determine if *L. monocytogenes* possessed dopamine or norepinephrine receptors which could be involved in iron binding, we used chlorpromazine (14 μM) and yohimbine (256 μM) as antagonists of dopamine and norepinephrine receptors, respectively. The addition of chlorpromazine alone in TSYB had no significant effect on *L. monocytogenes* growth (Fig. 2A). In contrast, the addition of yohimbine alone with TSYB resulted in a significant decrease in comparison with the control growth curve in TSYB, due to a slight bacteriostatic effect of the compound. This inhibitory effect was not increased by simultaneous addition of norepinephrine (Fig. 2B). The same displacement of the growth curve was observed in the presence of tropolone. When bacteria were incubated in the presence of tropolone and catecholamines

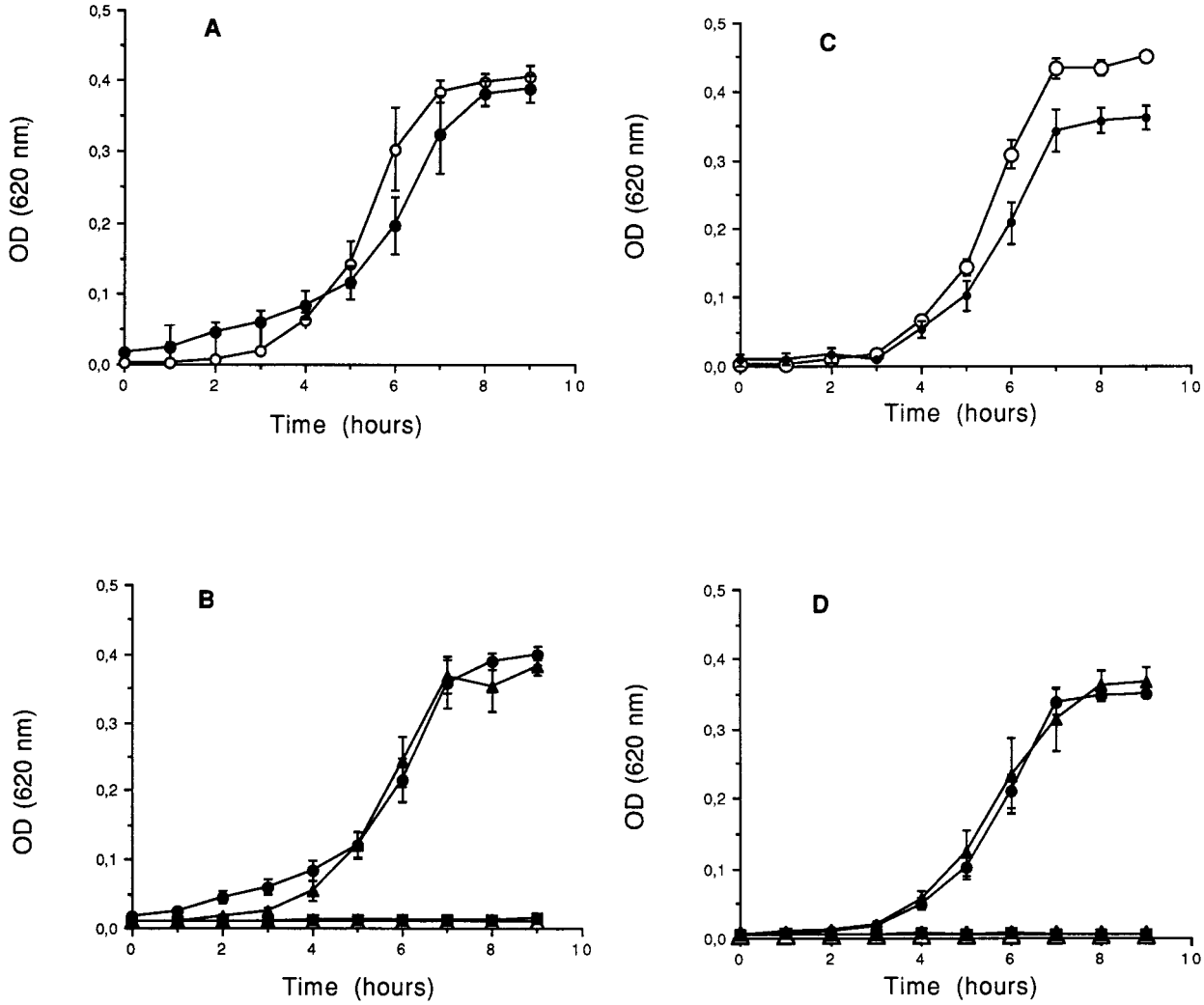

FIG. 1. Effect of dopamine (A and B) and norepinephrine (C and D) on growth of *L. monocytogenes* in TSYB (A and C) and in tropolone iron-complexed TSYB (B and D). Incubation was carried out at 37°C, and growth was monitored by recording changes in the OD_{620}. The tropolone concentration was 20 μM. Symbols: ■, 0.05 mM catecholamine; ▲, 0.5 mM catecholamine; ●, 5.0 mM catecholamine; ○, TSYB; △, TSYB plus tropolone. Data are averages of triplicate experiments. Bars indicate standard deviations.

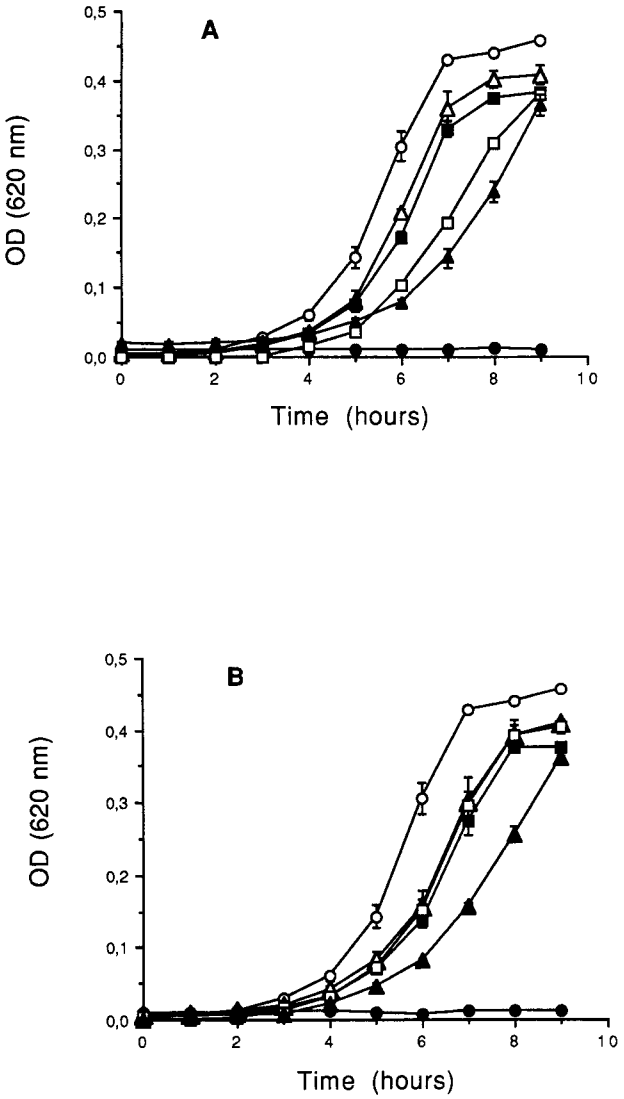

FIG. 2. Effect of chlorpromazine (14 μM) and yohimbine (256 μM) on growth-promoting activity of dopamine (0.5 mM) (A) or norepinephrine (0.5 mM) (B), respectively, towards *L. monocytogenes* in tropolone (20 μM) iron-complexed TSYB. Symbols: ○, TSYB; △, TSYB plus antagonist; ■, TSYB plus catecholamine plus antagonist; ●, TSYB plus tropolone; □, TSYB plus tropolone plus catecholamine; ▲, TSYB plus tropolone plus catecholamine plus antagonist. Incubation was carried out at 37°C, and growth was monitored by recording changes in the OD_{620}. Data are averages of triplicate experiments. Bars indicate standard deviations.

with or without the addition of yohimbine or chlorpromazine, no significant difference was observed (Fig. 2). Furthermore, under the same conditions, (+)-norepinephrine or (−)-norepinephrine exhibited the same growth curves as the racemic (±)-norepinephrine (Fig. 3). These results suggest that no catecholamine receptors similar to eukaryotic ones seem to be involved in iron binding. In the presence of tropolone, the addition of MHPG or normetanephrine did not restore any growth of *L. monocytogenes* compared with the growth-promoting effect of norepinephrine (Fig. 3), while addition of MHPG or normetanephrine in TSYB free of iron chelator had no effect on *L. monocytogenes* growth (data not shown).

Effect of norepinephrine on transferrin-promoted *L. monocytogenes* growth. In iron deprived tryptic soy broth (TSBm), *L. monocytogenes* growth was promoted by iron-loaded transferrin in a dose-dependent manner but not by apo-transferrin (Fig. 4B). When norepinephrine (0.5 mM) was added to the medium, the growth-promoting effect of transferrin was stimulated, resulting in a twofold increase in OD after 8 h of culture, in comparison with the growth curve displayed without catecholamine (Fig. 4A). In contrast, the addition of norepinephrine alone to the culture broth stimulated only slightly the growth of *L. monocytogenes* during the first 10 h. Finally, the stimulatory effect of norepinephrine was dose dependent between 0.05 and 0.5 M, and the OD reached at the end of the exponential growth phase with the higher norepinephrine concentration tested was typically identical to that shown by the control growth curve with 0.05 M $FeCl_3$ (Fig. 4A).

Iron uptake and effect of metabolic inhibitors. ^{55}Fe uptake mediated by catecholamines followed the same kinetics with norepinephrine as with dopamine, as shown by the linear response curve obtained during the first 30 min of the experiment (Fig. 5). About 33 and 25 pmol of iron per 10^9 CFU was incorporated after 30 min in the presence of norepinephrine and dopamine, respectively.

The addition to cells of unlabelled Fe-norepinephrine or unlabelled Fe-dopamine complexes, 10 min after the start of the uptake, immediately resulted in an arrest of the uptake of radiolabelled iron. Iron acquisition was strongly reduced by 9 mM KCN, an inhibitor of the electron transport chain, or by incubating the cells at 0°C, providing evidence that catecholamine-mediated iron uptake was an active process. Moreover, preincubation of the cells for 30 min with 46 μM $PtCl_2$, a blocker of ferric reductase (16), totally inhibited iron uptake.

Whole-cell ferric reductase assay. It has been previously shown that ferrozine, a colorimetric chelator of ferrous iron, could be used to monitor the extracellular reduction of ferric iron by *Listeria* spp. (12). The ferric reductase activity was assessed in the early exponential phase of bacteria grown in aerated low-iron medium. Accumulation of the red Fe(II)-ferrozine complex in the supernatant was monitored by measuring the OD_{562}. As shown in Fig. 6, reduction of Fe(III) was nearly immediate and increased linearly until 30 min. In contrast, ferric reduction was less pronounced when cells were

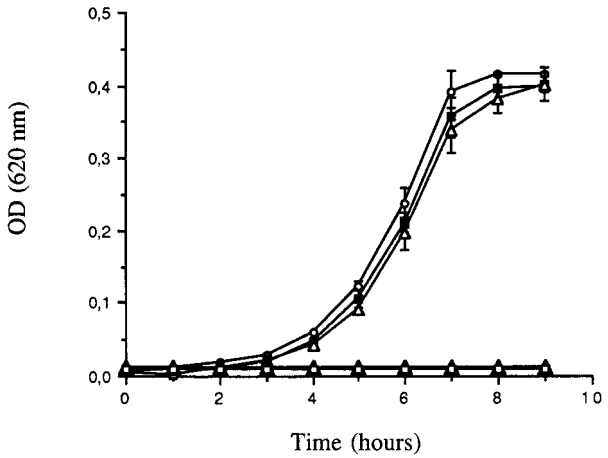

FIG. 3. Effect of stereoisomers and metabolic derivatives of norepinephrine on growth of *L. monocytogenes* in iron-complexed TSYB. Incubation was carried out at 37°C, and growth was monitored by recording changes in the OD_{620}. The tropolone concentration was 20 μM. Symbols: ○, (±)-norepinephrine; ■, (+)-norepinephrine; △ (−)-norepinephrine; ▲, normetanephrine; □, MHPG. Data are averages of triplicate experiments. Bars indicate standard deviations.

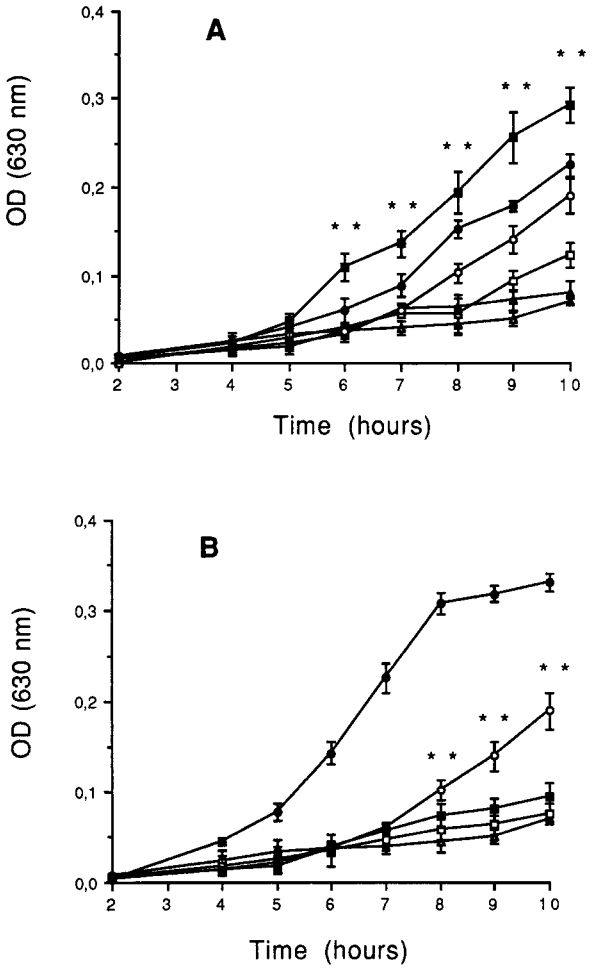

FIG. 4. (A) Effect of norepinephrine on growth of *L. monocytogenes* in iron-depleted medium (TSBm) supplemented with human holo-transferrin (hTf; 2 mg/ml). Symbols: △, TSBm; ▲, TSBm; plus 0.05 mM norepinephrine; □, TSBm plus 0.5 mM norepinephrine; ○, TSBm plus hTf; ●, TSBm plus hTf plus 0.05 mM norepinephrine; ■, TSBm plus hTf plus 0.5 mM norepinephrine. **, $P < 0.01$, hTf significantly different from hTf plus 0.5 mM norepinephrine by the Fisher test. (B) Growth of *L. monocytogenes* in TSBm with human apo-transferrin at 2 mg/ml (■), TSBm plus hTf at 0.2 mg/ml (□), TSBm plus hTf at 2 mg/ml (○), TSBm plus 50 μM $FeCl_3$ (●), TSBm (△). Incubation was carried out at 37°C, and growth was monitored by recording changes in the OD_{630}. Data are averages of triplicate experiments. Bars indicate standard deviations. **, $P < 0.01$, hTf at 2 mg/ml significantly different from hTf at 0.2 mg/ml by the Fisher test.

preincubated with Pt(II). The lowest concentration of Pt(II) tested (0.23 μM) was poorly efficient, beyond 5 min of incubation, in inducing a significant decrease of ferric reductase activity, while a higher concentration of Pt(II) (0.46 to 0.84 μM) severely inhibited ferric reductase activity. Exposure of the bacteria to 0.86 μM Pt(II) for a minimum of 2 h caused no loss in cell viability. These results showed that Pt(II) inhibited *L. monocytogenes* ferric reductase activity in a concentration-dependent manner.

In the same manner, ferric reductase activity was determined on cell-free culture supernatant and on a washed cell suspension from a 6-h TSYB culture of *L. monocytogenes*. As shown in Table 2, no significant iron reduction was detected in the supernatant, whereas washed cells displayed a ferric reductase activity which resulted in the yield of 110 fmol of Fe(II)-ferrozine complex per 10^6 CFU until 30 min.

DISCUSSION

It is well known that iron is required by *Listeria* in relatively large amounts to support growth during experimental infection (46). However, iron can be sequestered under various forms depending on the environmental conditions, either as insoluble ferric hydroxides and oxyhydroxides in an aerobic environment, or as a ferric transferrin complex in human serum, or bound to ferritin and heme compounds intracellularly (37). Although *L. monocytogenes* does not seem to produce siderophores, it is widespread in the environment. This ubiquity is consistent with the capacity of *L. monocytogenes* to use a vari-

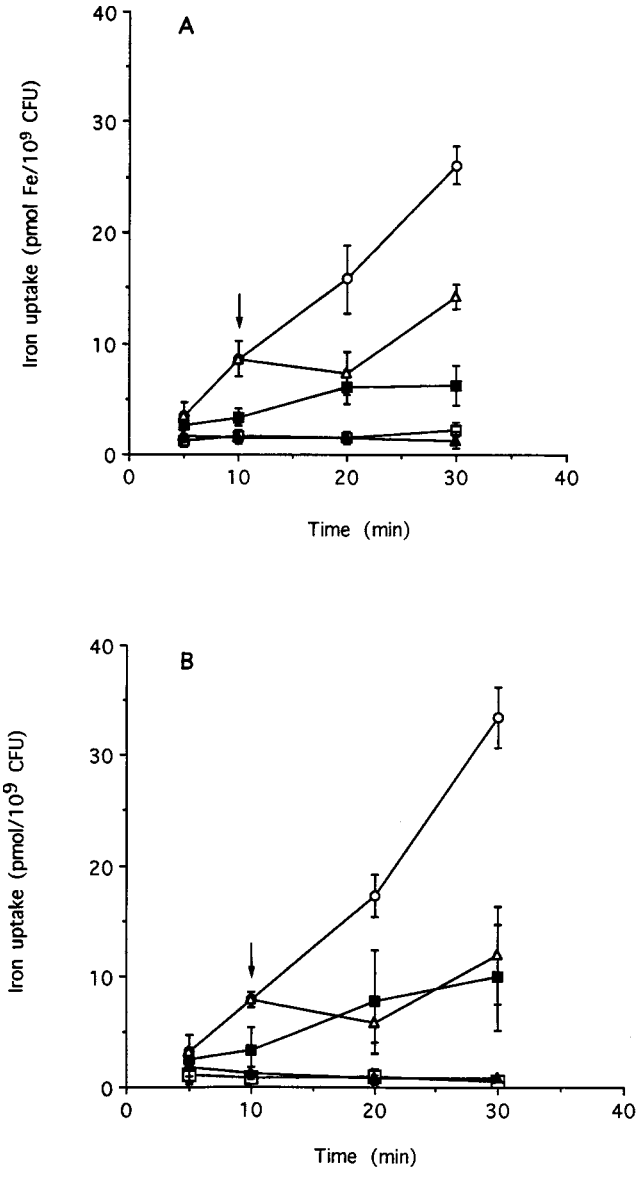

FIG. 5. Catecholamine-induced $^{55}Fe(III)$ uptake by *L. monocytogenes*. Iron-starved WM-grown cells were incubated at 37°C in the presence of a ^{55}Fe-dopamine complex (A) or a ^{55}Fe-norepinephrine complex (B) (0.18 μM iron). Symbols: ○, uptake under standard conditions; △, uptake under standard conditions with addition of unlabelled Fe(III) (18 μM) at 10 min (arrow); ■, uptake in the presence of 9 mM KCN; ▲, uptake with cells preincubated with 46 μM $PtCl_2$; □, uptake at 0°C. The catecholamine/iron ratio was 30:1 in all experiments. Cells were preinduced in iron-free medium supplemented with 0.75 μM catecholamine. Data are averages of triplicate experiments. Bars indicate standard deviations.

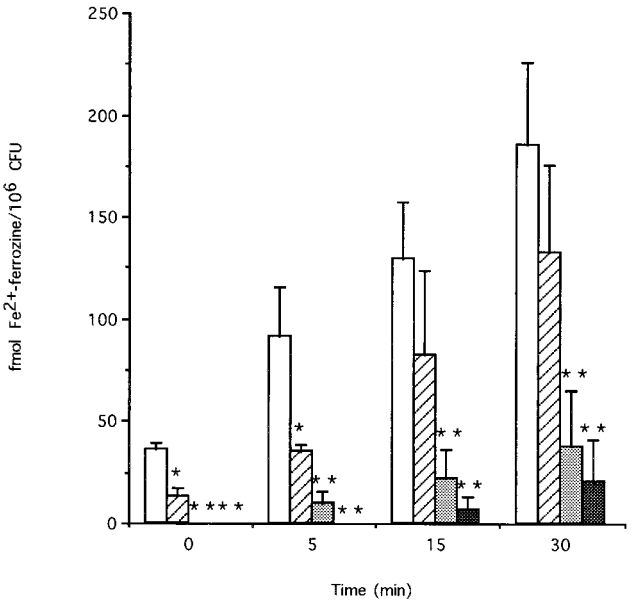

FIG. 6. Effect of Pt(II) on ferric reductase activity of *L. monocytogenes*. A washed cell suspension of *L. monocytogenes* (10^9 CFU/ml) was incubated with various concentrations of $PtCl_2$ and then assayed for ferric reductase activity at 37°C by the standard whole-cell ferric reductase assay as described in Materials and Methods. Open columns, control; hatched columns, 0.23 μM Pt(II); shaded columns, 0.46 μM Pt(II); closed columns, 0.84 μM Pt(II). Data are averages of four separate experiments. Bars indicate standard deviations. * and **, significantly different from control $P < 0.05$ and $P < 0.01$, respectively, by the Fisher test.

ety of exogenous siderophores such as deferoxamine, coprogen, ferrichrome, pyoverdine, or rhodotorulic acid and natural catechols such as esculetin (7), rutin, or quercetin to acquire iron (44). Recently, Deneer et al. (13) reported that *L. monocytogenes* is able to reduce many types of ferric iron compounds, including iron salts found in natural environments, biological iron chelates, which potentially could be encountered by the bacteria in vivo, and Fe(III)-siderophore complexes such as ferrioxamine B and ferric dihydroxybenzoic acid. They have also shown that the iron of ferric chelates is reduced to the Fe(II) form by a surface-associated ferric reductase. The reduced iron would then become bound to, and transported by, a specific surface-localized Fe(II) receptor. However, as described by Cowart and Foster (8), the ferric reductant is an extracellular component. Recently, Barchini and Cowart (3) suggested that both intra- and extracellular forms may work together in the mobilization of external iron.

We previously demonstrated the ability of catecholamines to promote growth of *L. monocytogenes* in iron-restricted medium (44). However, the mechanism accounting for such an effect was not examined. In the present work, we investigated the role of catecholamines and searched for a possible implication of a dopaminergic or an adrenergic receptor and a ferric reductase activity in iron acquisition by *L. monocytogenes*. Our results indicate that dopamine and norepinephrine are able to help *L. monocytogenes* in overcoming the iron deprivation created by tropolone or 8-hydroxyquinoline, and the growth-promoting effect is of the same order with norepinephrine as it is with dopamine. In contrast, a decreasing order of potency in stimulating growth in iron-replete medium has been observed in *E. coli* and *Y. enterocolitica*, following the order norepinephrine $\gg$ epinephrine $\geq$ dopamine $>$ dopa (32).

The ability of norepinephrine to cause a growth-promoting effect was not due to an energy supply because, under the same conditions, normetanephrine did not promote *L. monocytogenes* growth. Indeed, normetanephrine contains one methyl group instead of a hydroxyl group in norepinephrine and hence should serve as a better energy source for growth if the effect is solely energy dependent. Furthermore, no growth-promoting activity was shown with normetanephrine nor with MHPG, two derivatives which have the same structure as that of norepinephrine except for a catechol-*o*-methylation or oxidative deamination of norepinephrine. These results suggest that the *ortho*-diphenol moiety of the catecholamines plays a crucial role in their growth-promoting effect.

We showed evidence that the ability of catecholamines to promote *L. monocytogenes* growth was not mediated via specific receptors. Indeed, yohimbine and chlorpromazine, which are specific antagonists of adrenergic and dopaminergic receptors, respectively, had no inhibitory effect on the *L. monocytogenes* growth-promoting activity of catecholamines. Furthermore, the additions of (+)- or (−)-norepinephrine show the same growth curves as the racemic catecholamines do. Similar data were reported for *E. coli*, where the addition of either α- or β-adrenergic receptor antagonists failed to block the ability of norepinephrine to significantly increase the growth of *E. coli* (33).

To assess the biological relevance of our findings, we investigated the effect of catecholamines on the growth of *L. monocytogenes* in an iron-depleted medium supplemented with iron-loaded human transferrin. As a matter of fact, transferrin is an iron-binding glycoprotein found in blood which contributes to host defense against microbial infection by withholding iron. However, *L. monocytogenes* has been shown to be able to acquire iron from transferrin (23), although the efficiency level of the process remains undetermined. As expected, *L. monocytogenes* growth was promoted by iron-loaded transferrin as a sole source of iron in the medium, but the growth kinetics was clearly enhanced by the addition of norepinephrine. This observation suggests that catecholamines either remove iron from transferrin or make more effective the process for releasing the transferrin-bound iron to the bacterial pathogen acceptor. Whatever the actual mechanism of the whole stimulatory effect of norepinephrine, our observations are consistent with a likely role of catecholamines in the pathophysiology of listeriosis involving iron acquisition.

It is worthy to note that the concentration of catecholamines used in this study corresponds to a physiological concentration found in blood during development of sepsis (5, 28). The ability of neurohormones to modulate *L. monocytogenes* growth would be of clinical relevance since sepsis and septic shock have been correlated with consistently elevated levels of one or more of

TABLE 2. Ferric reductase activity determined on a cell-free culture supernatant and on a washed cell suspension of *L. monocytogenes*[a]

Substrate	Amt of Fe-ferrozine (fmol)/10^6 CFU[b]		
	0 min	15 min	30 min
Cell suspension	10 ± 1	79 ± 10*	110 ± 14*
Supernatant	8 ± 4	8 ± 4	17 ± 5

[a] A 6-h TSYB culture of *L. monocytogenes* was centrifuged for 10 min at 10,000 × *g*. The pellet was resuspended in phosphate-buffered saline and stored for 1 h at 37°C. Ferric reductase activity was determined on the bacterial pellet and on the supernatant as described in Materials and Methods. The results shown are the average of three determinations.

[b] Values followed by an asterisk are significantly different ($P < 0.01$) from that of the supernatant by the Fisher test.

the catecholamines in both animal and human studies. Likewise, natural catecholamines released into the brain tissue could explain the tropism of *L. monocytogenes* for the central nervous system and the propensity of this bacterium to cause meningoencephalitis and meningitis. Furthermore, high levels of catecholamines resulting from neurophysiological alterations like stress may contribute to infections. Interestingly, Lyte et al. (34) demonstrated that the growth of *E. coli* O157:H7 and the production of Shiga-like toxins were strongly increased in the presence of norepinephrine.

Lenard and Vanderoef (29) demonstrated that norepinephrine reversed the growth inhibitory effect of bovine serum in *E. coli*. They suggested that norepinephrine might act on a specific serum component rather than directly on the bacteria themselves by inactivating a bacteriostatic serum component. Taking into account the ubiquity of *L. monocytogenes* and the possibility for this bacterium to use various exogenous iron-catechol complexes, it is difficult to imagine receptors for each of the numerous ferric ligands available in the environment and ferric siderophores utilizable by *L. monocytogenes*. A far more efficient mechanism might be that the reductant of *L. monocytogenes* would recognize iron rather than the chelate. In our model, we hypothesize that the catechol moiety of catecholamines chelates iron sequestered by tropolone. Afterwards, the resulting catecholamine-iron complex is reduced by a ferric reductase, which results in the release of Fe(II) available for *L. monocytogenes* growth. This is supported by the finding that catecholamines stimulate bacterial growth in iron-sequestered medium and specifically promote iron uptake in iron-deficient cells. Uptake of ^{55}Fe mediated by dopamine and norepinephrine was shown to be an active process, probably involving the proton motive force since it is inhibited by KCN or by incubation at 0°C. ^{55}Fe uptake is really mediated by catecholamines since unlabelled iron complexes competitively inhibit labelled iron uptake.

The second step of our hypothesis is that reduction would facilitate removal of iron from the ligand. An inhibitor of ferric reductase, Pt(II), has been used to investigate the iron acquisition system in *S. cerevisiae* (14, 16). We showed in the present study that Pt(II) is also an inhibitor of ferric reductase activity in *L. monocytogenes*. Preincubation of cells with Pt(II) completely inhibited ^{55}Fe uptake, supporting the evidence that ferric reductase activity is essential for iron uptake. Furthermore, we did not detect ferric reductase activity in the supernatant of cell-free culture. In agreement with Deneer et al. (13), it seems that direct contact between the bacteria and the iron source may be necessary for Fe(III) reduction. Iron reductase enzymes have been shown to be important in the ability of several microorganisms such as *S. mutans* (18), *Agrobacterium tumefaciens* (31), *Azotobacter vinelandii* (25), and *S. cerevisiae* (30) to acquire iron from siderophores.

In conclusion, catecholamines apparently function as siderophore-like compounds for *L. monocytogenes*. This organism seems able to use the capacity of catecholamines to bind iron via their catechol moiety and then to reduce the ferric catecholamine complexes involving a cell-surface bound ferric reductase, releasing ferrous iron available for its utilization.

Involvement of catecholamines could be relevant in animal and human listeriosis. Considering that due to an abundant noradrenergic innervation, a large amount of norepinephrine is secreted throughout the gastrointestinal system, as well as in the central nervous system, and that the majority of listerial infections occur via the oral route, it would therefore seem reasonable to suggest that the neurohormonal environment encountered by the pathogen upon entrance into the host might play a determining role in the pathophysiology of the infection.

ACKNOWLEDGMENTS

Jean-Marie Meyer is gratefully acknowledged for critically reading the manuscript and for providing valuable comments. We thank Martine Marty for supplying the strain *L. monocytogenes* B38, François Gacogne for atomic absorption spectroscopy, and Françoise Bindler for atomic emission spectroscopy.

REFERENCES

1. **Adams, T. J., S. Vartivarian, and R. E. Cowart.** 1990. Iron acquisition systems of *Listeria monocytogenes*. Infect. Immun. **58:**2715–2718.
2. **Aisen, P., and I. Listowsky.** 1980. Iron transport and storage proteins. Annu. Rev. Biochem. **49:**357–393.
3. **Barchini, E., and R. E. Cowart.** 1996. Extracellular iron reductase activity produced by *Listeria monocytogenes*. Arch. Microbiol. **166:**51–57.
4. **Barghouthi, S., R. Young, M. O. J. Olson, J. E. L. Arceneaux, L. W. Clem, and B. R. Byers.** 1989. Amonabactin, a novel tryptophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. J. Bacteriol. **171:**1811–1816.
5. **Benedict, C. R., and D. G. Grahame-Smith.** 1978. Plasma noradrenaline and adrenaline concentrations and dopamine-β-hydrolase activity in patients with shock due to septicaemia, trauma and haemorrhage. Q. J. Med. **185:**1–20.
6. **Cossart, P., and J. Mengaud.** 1989. *Listeria monocytogenes*, a model system for the molecular study of intracellular parasitism. Mol. Biol. Med. **6:**463–474.
7. **Coulanges, V., P. André, and D. J.-M. Vidon.** 1996. Esculetin antagonizes iron-chelating agents and increases the virulence of *Listeria monocytogenes*. Res. Microbiol. **147:**677–685.
8. **Cowart, R. E., and B. G. Foster.** 1985. Differential effects of iron on the growth of *Listeria monocytogenes*: minimum requirements and mechanism of acquisition. J. Infect. Dis. **151:**721–730.
9. **Crowley, D. E., Y. C. Wang, C. P. P. Reid, and P. J. Szaniszlo.** 1991. Mechanisms of iron acquisition from siderophores by microorganisms and plants. Plant Soil **130:**179–198.
10. **Dailey, H., and J. Lascelles.** 1977. Reduction of iron and synthesis of protoheme by *Spirillum itersonii* and other organisms. J. Bacteriol. **129:**815–820.
11. **Dancis, A., R. Klausner, A. Hinnebusch, and J. Barriocanal.** 1990. Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2294–2301.
12. **Deneer, H. G., and I. Boychuk.** 1993. Reduction of ferric iron by *Listeria monocytogenes* and other species of *Listeria*. Can. J. Microbiol. **39:**480–485.
13. **Deneer, H. G., V. Healey, and I. Boychuk.** 1995. Reduction of exogenous ferric iron by a surface-associated ferric reductase of *Listeria* spp. Microbiology **141:**1985–1992.
14. **Dix, D. R., J. T. Bridgham, M. A. Broderius, C. A. Byersdorfer, and D. J. Eide.** 1994. The FET4 gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae*. J. Biol. Chem. **269:**26092–26099.
15. **Ecker, D. J., and T. Emery.** 1983. Iron uptake from ferrichrome A and iron citrate in *Ustilago sphaerogena*. J. Bacteriol. **155:**616–622.
16. **Eide, D., S. Davis-Kaplan, I. Jordan, D. Sipe, and J. Kaplan.** 1992. Regulation of iron uptake in *Saccharomyces cerevisiae*. J. Biol. Chem. **267:**20774–20781.
17. **Emery, T.** 1987. Reductive mechanisms of iron assimilation, p. 235–248. *In* G. Winkelmann, D. Van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Publishers, Weinheim, Germany.
18. **Evans, S. L., J. E. L. Arceneaux, B. R. Byers, M. E. Martin, and H. Aranhas.** 1986. Ferrous iron transport in *Streptococcus mutans*. J. Bacteriol. **168:**1096–1099.
19. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*: a food-borne pathogen. Microbiol. Rev. **55:**476–511.
20. **Genco, C. A., and J. P. Pragnya.** 1996. Iron acquisition in the pathogenic *Neisseria*. Trends Microbiol. **5:**179–184.
21. **Goulet, V., C. Jacquet, V. Vaillant, E. Mouret, C. Lorente, E. Maillot, I. Rebiere, F. Stainer, and J. Rocourt.** 1995. Listeriosis from consumption of raw milk cheese. Lancet **345:**1581–1582.
22. **Grossman, T. H., M. Tuckman, S. Ellestad, and M. S. Osburne.** 1993. Isolation and characterization of *Bacillus subtilis* genes involved in siderophore biosynthesis: relationship between *B. subtilis sfp*^{0} and *Escherichia coli entD* genes. J. Bacteriol. **175:**6203–6211.
23. **Hartford, T., S. O'Brien, P. W. Andrew, D. J. Jones, and I. S. Roberts.** 1993. Utilization of transferrin-bound iron by *Listeria monocytogenes*. FEMS Microbiol. Lett. **108:**311–318.
24. **Huschka, H., H. U. Naegeli, H. Leuenberger-Ryf, W. Keller-Schierlein, and G. Winkelmann.** 1985. Evidence for a common siderophore transport system but different siderophore receptors in *Neurospora crassa*. J. Bacteriol. **162:**715–721.

25. **Huyer, M., and W. J. Page.** 1989. Ferric reductase activity in *Azotobacter vinelandii* and its inhibition by Zn^{2+}. J. Bacteriol. **171:**4031–4037.
26. **Jalal, M. A. F., R. Mocharla, C. L. Barnes, M. B. Hossain, D. R. Powell, D. L. Eng-Wilmot, S. I. Grayson, B. A. Benson, and D. Van der Helm.** 1984. Extracellular siderophores of *Aspergillus ochraceus*. J. Bacteriol. **158:**683–688.
27. **Johnson, W., L. Varner, and M. Poch.** 1991. Acquisition of iron by *Legionella pneumophila*: role of iron reductase. Infect. Immun. **59:**2376–2381.
28. **Jones, S. B., M. V. Westfall, and M. M. Sayeed.** 1988. Plasma catecholamines during *E. coli* bacteremia in conscious rats. Am. J. Physiol. **254:**R470–R477.
29. **Lenard, J., and R. Vanderoef.** 1995. A novel bacteristatic action of bovine and porcine serum that is reversed by norepinephrine. Life Sci. **57:**443–447.
30. **Lesuisse, E., F. Raguzzi, and R. R. Crichton.** 1987. Iron uptake by the yeast *Saccharomyces cerevisiae*: involvement of a reduction step. J. Gen. Microbiol. **133:**3229–3236.
31. **Lodge, J. S., C. G. Gaines, J. E. L. Arceneaux, and B. R. Byers.** 1982. Ferrisiderophore reductase activity in *Agrobacterium tumefaciens*. J. Bacteriol. **149:**771–774.
32. **Lyte, M., and S. Ernst.** 1992. Catecholamine induced growth of gram-negative bacteria. Life Sci. **50:**203–212.
33. **Lyte, M., and S. Ernst.** 1993. Alpha and beta adrenergic receptor involvement in catecholamine-induced growth of gram-negative bacteria. Biochem. Biophys. Res. Commun. **190:**447–452.
34. **Lyte, M., P. B. Arulanandam, and C. D. Frank.** 1996. Production of shiga-like toxins by *Escherichia coli* 0157:H7 can be influenced by the neuroendocrine hormone norepinephrine. J. Lab. Clin. Med. **28:**392–398.
35. **Meyer, J.-M., D. Hohnadel, and F. Hallé.** 1989. Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. J. Gen. Microbiol. **135:**1479–1487.
36. **Morrissey, J. A., P. H. Williams, and A. M. Cashmore.** 1996. *Candida albicans* has a cell-associated ferric reductase activity which is regulated in response to levels of iron and copper. Microbiology **142:**485–492.
37. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:** 715–731.
38. **Neilands, J. B.** 1986. Siderophores in relation to plant growth and disease. Annu. Rev. Plant. Physiol. **37:**187–208.
39. **Neilands, J. B., K. Konopka, B. Schwyn, M. Coy, R. T. Francis, B. H. Paw, and A. Bagg.** 1987. Molecular biology and biochemistry of microbial iron transport, p. 3–30. *In* G. Winkelmann, D. Van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Publishers, Weinheim, Germany.
40. **Polacheck, I., Y. Platt, and J. Aronovitch.** 1990. Catecholamines and virulence of *Cryptococcus neoformans*. Infect. Immun. **58:**2919–2922.
41. **Premaratne, R. J., W. Lin, and E. A. Johnson.** 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. **57:**3046–3048.
42. **Reeves, M. W., L. Pine, J. B. Neilands, and A. Balows.** 1983. Absence of siderophore activity in *Legionella* species grown in iron-deficient media. J. Bacteriol. **154:**324–329.
43. **Seeliger, H. P. R.** 1961. Listeriosis, 2nd ed. Karger, Basel, Switzerland.
44. **Simon, N., V. Coulanges, P. Andre, and D. J.-M. Vidon.** 1995. Utilization of exogenous siderophores and natural catechols by *Listeria monocytogenes*. Appl. Environ. Microbiol. **61:**1643–1645.
45. **Stookey, L. L.** 1970. Ferrozine—a new spectrophotometric reagent for iron. Anal. Chem. **42:**779–781.
46. **Sword, C. P.** 1966. Mechanisms of pathogenesis in *Listeria monocytogenes* infection. Influence of iron. J. Bacteriol. **92:**536–542.
47. **Vidon, D. J.-M., and M. Spreng.** 1992. Effect of some iron-chelating agents on growth of *Listeria monocytogenes*, abstr. 174, p. 340–341. *In* Abstracts of the 11th International Symposium on Problems of Listeriosis (ISOPOL XI), Copenhagen, Denmark.
48. **Weinberg, E. D.** 1995. Acquisition of iron and other nutrients in vivo, p. 81–95. *In* J. A. Roth, C. A. Bolin, K. A. Brogden, F. C. Minion, and M. J. Wannemuehler (ed.), Virulence mechanisms of bacterial pathogens, 2nd ed. American Society for Microbiology, Washington, D.C.
49. **Winkelmann, G.** 1991. Specificity of iron transport in bacteria and fungi, p. 65–105. *In* G. Winkelmann (ed.), Handbook of microbial iron chelates. CRC Press, Inc., Boca Raton, Fla.

Editor: P. J. Sansonetti