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

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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 55Fe uptake by iron-deprived bacterial cells. The uptake of iron was energy dependent, as indicated by inhibition of 55Fe uptake at 0°C as well as by preincubating the bacteria with KCN. Inhibition of 55Fe 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-phytosiderophore 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 sphaerogeta (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 meningocencephalitis, 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 hem, 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
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 (10⁸ 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 loop of bacteria was picked 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 distilled water [see below]): KH₂PO₄, 3.25 g; NaH₂PO₄, 15.48 g; MgSO₄, 0.41 g; glucose, 10 g; l-selenocic, 0.1 g; l-lysine, 0.1 g; l-valine, 0.1 g; l-arginine, 0.1 g; l-glutamie, 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; biotin, 100 mg; Mg₂SO₄·6H₂O, 11 g. 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, Yvy-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 Ca²⁺ and Mg²⁺, we added MgSO₄ (25 mg/liter) and CaCl₂ (50 mg/liter) to the treated medium (subsequently referred to as TSBm). The iron concentration (32 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 0.25-μm pore-size filters (Dutschke, Brummath, France). PCl₃ stock solution was prepared as a saturated solution in 1 N HCl. The concentration of dissolved Pt(II) was then measured by atomic emission spectrophotometry. Solution of 4-hydroxy-3-methoxyphenylglycol (HMPG) was prepared in degassed phosphate-buffered solution (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).

**Whole-cell ferric reductase assay.** The reduction of ferric to ferrous iron was measured by trapping Fe²⁺ with ferrozine [3-(2-pyridyl)-5,6-bis-(4-sulfophenylazo)phenacylamine] and measuring the absorbance of the Fe²⁺-ferrozine complex at 562 nm (22). The method is based on the reduction of iron(III) to iron(II) by cells or cell-free extracts, followed by the formation of a complex with ferrozine and its measurement at 562 nm. The assay was performed in 50 mM potassium phosphate, pH 7.4, containing 10% glycerol and 1 mM DTT.

**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 tropolone-inhibited TSYB. The addition of dopamine and norepinephrine in
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

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Disk load (µmol)</th>
<th>Growth zone diam (mm) on tropolone</th>
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</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>1.50</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>0.37</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>0.18</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>—b</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1.50</td>
<td>17.1</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>15.1</td>
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<td></td>
<td>0.37</td>
<td>14.8</td>
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<td></td>
<td>0.18</td>
<td>13.3</td>
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<td></td>
<td>0.09</td>
<td>11.3</td>
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<td></td>
<td>0.03</td>
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*Paper disks were loaded with different concentrations of each catecholamine and deposited onto TSYA medium containing 20 µM tropolone seeded with 10⁶ bacteria. Growth zone diameters were checked after incubation at 37°C for 24 h. Data are averages of triplicate experiments. —b, no growth zone.*

![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 OD₆₂₀. 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.*
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 FeCl₃ (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 10⁹ 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 PtCl₂, 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 OD₅₆₂. 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
preincubated with Pt(II). The lowest concentration of Pt(II) tested (0.23 mM) 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 mM) severely inhibited ferric reductase activity. Exposure of the bacteria to 0.86 mM 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.
effects 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*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>0 min</th>
<th>15 min</th>
<th>30 min</th>
</tr>
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<tbody>
<tr>
<td>Cell suspension</td>
<td>10 ± 1</td>
<td>79 ± 10*</td>
<td>110 ± 14*</td>
</tr>
<tr>
<td>Supernatant</td>
<td>8 ± 4</td>
<td>8 ± 4</td>
<td>17 ± 5</td>
</tr>
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</table>

* 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.

* 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 Vanderœef (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-cathecol 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 55Fe 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. 55Fe 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 55Fe 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 neurohemoronal environment encountered by the pathogen upon entrance into the host might play a determining role in the pathophysiology of the infection.

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**REFERENCES**