Control of Listeria Superoxide Dismutase by Phosphorylation

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Superoxide dismutases (SODs) are enzymes that protect organisms against superoxides and reactive oxygen species (ROS) produced during their active metabolism. ROS are major mediators of phagocytes microbicidal activity. Here we show that the cytoplasmic Listeria monocytogenes MnSOD is phosphorylated on serine and threonine residues and less active when bacteria reach the stationary phase. We also provide evidence that the most active nonphosphorylated form of MnSOD can be secreted via the SecA2 pathway in culture supernatants and in infected cells, where it becomes phosphorylated. A Δsod deletion mutant is impaired in survival within macrophages and is dramatically attenuated in mice. Together, our results demonstrate that the capacity to counteract ROS is an essential component of L. monocytogenes virulence. This is the first example of a bacterial SOD post-translationally controlled by phosphorylation, suggesting a possible new host innate mechanism to counteract a virulence factor.

Reactive oxygen species (ROS) are continuously produced by multiple enzymes within cells. Whereas a significant amount of ROS is generated in the cytosol of eukaryotic cells, in peroxisomes and at the plasma membrane, oxidative phosphorylation by the mitochondrial respiratory chain is the main cellular source of ROS. Cells beneficially use ROS as antimicrobial agents (1) and regulators of stress signaling pathways, e.g. heat shock response, NF-κB, and p53 activation, phosphatidylinositols 3-kinase/Akt and mitogen-activated protein kinase cascades (2, 3). However, uncontrolled production of ROS is deleterious to cells because they can damage nucleic acids, proteins, and lipids (4). Cellular response to oxidative stress is believed to be a major determinant of lifespan (5, 6). Moreover, accumulation of ROS is associated to human pathology including hyperglycemic-diacs (7), carcinogenesis and tumor progression (8), and neurodegenerative disorders, e.g. Alzheimer's, Parkinson's (9), and prion diseases (10). To prevent oxidative damage, cells synthesize antioxidant systems. Superoxide dismutase (SOD) catalyzes the conversion of superoxide radical anions to hydrogen peroxide, using as cofactors manganese in the mitochondria or copper and zinc, extracellularly and in the cytosol. Mutations in the human copper-zinc SOD (CuZn-SOD) are associated with a dramatic genetic disease, i.e. familial amyotrophic lateral sclerosis (11), highlighting the importance and key role of SOD in life. Bacterial SODs are cytoplasmic, periplasmic, or secreted enzymes. They can bind nickel or iron in addition to manganese, copper, and zinc and are involved in basic processes such as growth, senescence, sporulation, and also virulence (12, 13). The expression of both eukaryotic and bacterial SODs is tightly controlled at the transcriptional and post-transcriptional levels (14–18). To our knowledge, post-translational regulation of bacterial SODs has not been reported.

Listeria monocytogenes is a facultative intracellular pathogen causing a severe food-borne disease in humans and animals (19). Neutrophiles and macrophages are critical cells of host defense against L. monocytogenes (20, 21). Once phagocytosed, L. monocytogenes faces the phagosomal oxidative burst (22) and then escapes from the phagosome because of the secretion of listeriolisin O (23), phosphatidylinositol-specific phospholipase C (24), and other proteins (25). However, L. monocytogenes reacts to this bactericidal aggression has remained elusive. A single sod gene, which encodes a functional manganese-SOD (MnSOD) has been identified (26–28) but it has remained poorly characterized.

Here, we report that L. monocytogenes MnSOD activity is down-regulated by serine/threonine phosphorylation during the stationary phase. We show that the most active, nonphosphorylated form of MnSOD is secreted via the SecA2 pathway in bacterial culture and in infected cells where it is phosphorylated. Inactivation of MnSOD by gene deletion resulted in increased bacterial death within macrophages and dramatic attenuation in mice, demonstrating that the antioxidant potential is a critical factor for L. monocytogenes pathogenesis.
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**Experimental Procedures**

**Bacterial Strains and Growth Conditions**—All *L. monocytogenes* strains were routinely grown in brain heart infusion (BHI) medium (Difco) at 37 °C. When required, chloramphenicol and erythromycin were added at 7 μg/ml and 5 μg/ml, respectively. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Difco) at 37 °C. When required, antibiotics were included at the following concentrations: ampicillin, 100 μg/ml, chloramphenicol 7 μg/ml.

**Antibodies and Western Blot Techniques**—Murine polyclonal anti-MnSOD serum, produced as described (29), was used at 1:1000 in 4% Blotto. Rabbit polyclonal antiphosphoserine and antiphosphothreonine antibodies (Zymed Laboratories Inc.) were diluted at 1:1000 in blocking buffer (Zymed Laboratories Inc.). Rabbit polyclonal anti-Stp R96 (30), anti-ActA R32 (31) purified antibodies and anti-Listeria R11 (32), ant-InIC R117 sera were used at 1:1000 in 4% Blotto. After separation on 10% SDS-PAGE, proteins were detected by Western blotting as previously described (30).

**Expression and Purification of the *L. monocytogenes* MnSOD**—The coding region of sod (*lmo1439*), excluding the start codon, was amplified by PCR using genomic DNA from *L. monocytogenes* EGDe (BUG 1600) and the oligonucleotides AC38F and AC39F (supplemental Table S1). The PCR product was digested with NdeI and XhoI and cloned into the expression vector pET-28b (Novagen), creating pET-28b(*lmo1439*). The coding region of *sod* (*lmo1439*), excluding the start codon, was amplified by PCR using genomic DNA from *L. monocytogenes* EGDe (BUG 1600) and the oligonucleotides AC38F and AC39F (supplemental Table S1). The PCR product was digested with NdeI and XhoI and cloned into the expression vector pET-28b (Novagen), creating pET-28b(*lmo1439*). The product was digested by NdeI and XhoI and cloned into the expression vector pET-28b (Novagen), creating pET-28b(*lmo1439*).

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**Quantification of *L. monocytogenes* MnSOD Activity**—SOD activity was measured using the BSA assay (Pierce). Briefly, 4 μg of purified MnSOD were phosphorylated with PKA and dephosphorylated by Stp as described above. Samples and blanks were diluted in 40 μl of H2O and incubated in 900 μl of SOD-525 buffer at 37 °C. Thirty microliters of the R2 reagent were added and incubated at 37 °C for 1 min before addition of 30 μl of the R1 reagent. A525 nm was measured every 3 s for 2 min.

**Preparation of Total Bacterial Extracts and Supernatant Precipitation**—Cultures (10 ml) of the *L. monocytogenes* EGDe wild-type and Δstp mutant strains were harvested at different time points. Bacterial pellets were recovered after centrifugation at 4000 rpm for 20 min, washed twice in PBS and resuspended in 100 μl of B–PERII Bacterial Protein Extraction Reagent (Pierce) to extract total bacterial proteins. Bacterial supernatants were precipitated in 16% trichloroacetic acid on ice at 4 °C overnight. After centrifugation, the protein pellets were washed twice with 5 ml of cold acetone, dried, and resuspended in 350 μl of 1 M Tris (pH 8.8). Protein concentration of total bacterial extract and supernatant was determined by the conventional BCA assay (Pierce).

**MnSOD Immunoprecipitation**—Proteins (250 μg) from total bacterial extracts or from bacterial supernatants were immunoprecipitated with 2.5 μl of the anti-MnSOD serum using the protein G immunoprecipitation kit (Sigma) according to the manufacturer’s instructions. Briefly, after overnight incubation of protein samples with anti-MnSOD serum, protein G beads were transferred in the spin column and incubated for 4 h at 4 °C. Beads were washed six times with 1 × IP buffer, one time with 0.1 × IP buffer and incubated with 60 μl of Laemmli buffer. Immunoprecipitates were recovered after centrifugation. Equivalent volumes (30 μl) of immunoprecipitate were separated by SDS-PAGE and analyzed by Western blotting using anti-MnSOD serum or antiphosphothreonine antibodies.

**Immunofluorescence**—One milliliter of cultures of the *L. monocytogenes* strains growing in BHI at 6% O2 was harvested at A600 nm = 0.8. Pellets were washed and resuspended in unit from bovine heart (Sigma) in PKA buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35) overnight at 30 °C in the presence of 1 mM ATP. Two dialyses were performed in 2 liters of Stp buffer (50 mM Tris-HCl, pH 7.5, 1 mM of MnCl2, 0.1 mM Na2EDTA, 5 mM dithiothreitol, 0.01% Brij 35) for 4 h at 4 °C to eliminate residual ATP. Phosphorylated MnSOD (30 μg) was dephosphorylated by Stp (6 μg) for 1 h at 37 °C in Stp buffer. Ten micrograms of phosphorylated P-α-casein (Sigma) were dephosphorylated using 1 μg of alkaline phosphatase (AP, Roche Applied Science) in phosphatase buffer (Roche Applied Science) at 37 °C for 1 h. The phosphorylation level of the resulting α-casein was compared with that of the initial P-α-casein. BSA (Pierce) was used as a nonphosphorylated protein control for Pro-Q diamond staining. MnSOD, PKA, and Stp-purified proteins, MnSOD samples after PKA dephosphorylation and Stp dephosphorylation, P-α-casein-, α-casein-, and BSA-purified control proteins (2 μg) were separated by SDS-PAGE. Gels were stained with the Pro-Q and Sypro Ruby protein gel dyes (Molecular Probes) as previously described (35).

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5 E. Gouin, unpublished data.
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1 ml of PBS. Fifty microliters of suspension were loaded on a coverslip and placed in a 24-well microplate. Bacteria were fixed in 10% paraformaldehyde for 10 min. Cells were washed in PBS and incubated 5 min in 50 mM N\textsubscript{2}HCl. After blocking in PBS, 0.5% BSA for 1 h, anti-MnSOD, or anti-Listeria R11 sera, both diluted at 1:100 in 200 μl of PBS, 0.5% BSA, were added for 1 h. After washes in PBS, anti-mouse IgG-Alexa-conjugated or anti-rabbit IgG-FITC-conjugated secondary antibodies (Molecular Probes) diluted at 1:200 in 200 μl of PBS, 0.5% BSA, were used to detect L. monocytogenes MnSOD or total bacteria, respectively. Cover slips were mounted with Mowiol and analyzed with an AxioVert microscope (Zeiss) equipped with the Metamorph software (Universal Imaging Corporation).

Mutation and Complementation—Upstream and downstream 1-kb flanking sequences of the sod gene were amplified by PCR from genomic DNA from L. monocytogenes EGDe using the oligonucleotides SodKO1/SodKO2 and SodKO3/SodKO4 (supplemental Table S1). The upstream MluI-EcoRI and downstream EcoRI-NcoI fragments were cloned into EGDe genomic DNA with the oligonucleotides SodKO1/SodKO2 and SodKO3/SodKO4 (supplemental Table S1). The upstream sequence was amplified by PCR from genomic DNA from L. monocytogenes belonging to the agar and loaded with paraquat (570 μM, Sigma) and xanthine oxidase (0.2 μM, Sigma) and hydroxylamine. PEM were lysed with 0.2% Triton X-100 for 10 min, and the number of CFU was assessed as described in the previous section. For immunofluorescence labeling, PEM adhering onto glass coverslips were loaded over 20 min with 7.5 nM of Lyso-tracker Red DND-99 (Molecular Probes) at 37 °C. PEM were then infected as above for 4 h at 37 °C, washed once with PBS pH 7.5, and fixed with 4% paraformaldehyde for 20 min. After quenching with 50 mM N\textsubscript{2}HCl containing 0.05% saponin and 1% BSA for 10 min, nonspecific binding sites were blocked with 0.05% saponin and 5% horse serum during 45 min. Some coverslips were incubated for 30 min with anti-L. monocytogenes serum R11 followed by incubation during 30 min with secondary donkey anti-rabbit antibodies coupled to Alexa 647 and phalloidin coupled to Alexa 488 (Molecular Probes). All coverslips were mounted on Mowiol and analyzed with an AxioVert microscope (Zeiss) equipped with the Metamorph software (Universal Imaging Corporation). Student’s t and ANOVA tests were used for statistical analyses.

Immunoprecipitation of MnSOD from Infected Macrophages—PEM were activated with IFN-γ and infected with L. monocytogenes wild-type and Δsod mutant strains (MOI = 10) as described above. 15 min after adding gentamicin, PEM were lysed with 0.2% Triton X-100 for 10 min. Bacteria were recovered from cell lysates as previously described (39), and bacterial proteins were extracted with 100 μl of B-PER II Bacterial Protein Extraction Reagent. Proteins present in PEM were recovered in 500 μl of Tris, 1 M (pH 8.8) after precipitation of cell lysates with 16% trichloroacetic acid. Bacterial extracts (15 μg) and cellular extracts (250 μg) were immunoprecipitated with 2.5 μl of anti-MnSOD serum using the protein G immunoprecipitation kit (Sigma) as described above. Equivalent volumes (30 μl) of immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting.

Animal Studies—L. monocytogenes EGDe cultures were grown at 6% O\textsubscript{2} atmosphere. For quantification of bacterial multiplication, 8-week-old female BALB/c mice (Charles River) were injected intravenously with ~8 × 10\textsuperscript{3} CFU. Liver and spleen were recovered and disrupted in 3 ml PBS at 24 h, 48 h and 72 h after infection. Serial dilutions of organ homogenates were plated on BHI agar plates and CFU determined. Animal experiments were performed according to the Institut Pasteur guidelines for laboratory animal husbandry which comply with European regulations.

RESULTS

L. monocytogenes MnSOD Can Be Phosphorylated and Phosphorylation Down-regulates Its Activity—We previously demonstrated that Stp is a serine-threonine phosphatase involved in L. monocytogenes virulence and identified EF-Tu as its first target (30). Here, we identified L. monocytogenes MnSOD as the second target of Stp using a phosphoproteomic approach. Analysis of protein extracts of the L. monocytogenes Δstp mutant revealed the presence of a protein phosphorylated on...
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FIGURE 1. Regulation of *L. monocytogenes* MnSOD activity by phosphorylation. A, phosphoproteome analysis of protein extracts from wild-type EGDe strain (WT) and Δstp mutant (30). Phosphorylation of the *L. monocytogenes* MnSOD (dotted circle) on threonine residues (central panel) and on serine residues (right panel) was only detectable in the Δstp mutant by Western blotting. The spots, corresponding to phosphorylated MnSOD present in silver-stained two-dimensional gels (left panel), were analyzed by MALDI-TOF. Molecular masses are indicated on the left. B, phosphorylation and dephosphorylation of purified MnSOD in vitro. Phosphoproteins were detected on SDS-PAGE gel stained with Pro-Q Diamond fluorescent dye. The gel was further processed for total protein staining using Sypro Ruby fluorescent stain. Recombinant MnSOD was phosphorylated using PKA and dephosphorylated by Stp. Phosphorylated α-casein (P-α-casein), alkaline phosphatase (AP)-dephosphorylated α-casein and nonphosphorylated BSA were used as phosphoprotein controls. Molecular masses are indicated on the left. C, MnSOD activity in vitro. SOD activity was quantified by spectrophotometric measurement at 525 nm every 3 s for 2 min, using the Bioxytech SOD-525 kit. Recombinant MnSOD (4 μg) was phosphorylated by PKA and dephosphorylated by Stp. Mean values are expressed as the measure of A525 nm ± S.D. (n = 3).

threonine and serine residues, which was not phosphorylated in protein extracts of the wild-type strain (Fig. 1A). Mass spectrometry analysis of the corresponding spot identified the MnSOD.

To demonstrate a direct dephosphorylation of *L. monocytogenes* MnSOD by Stp, we produced and purified a recombinant MnSOD in *E. coli*. The recombinant MnSOD could be phosphorylated using PKA, a cAMP-dependent serine-threonine kinase (Fig. 1B). Phosphorylated MnSOD could be fully dephosphorylated in vitro by Stp (Fig. 1B).

We next examined the influence of MnSOD phosphorylation state on its activity. Dephosphorylation of the PKA-phosphorylated MnSOD more than doubled its activity, revealing that MnSOD activity can be down regulated by phosphorylation (Fig. 1C). Together, these results demonstrate that *L. monocytogenes* MnSOD which can be present in a phosphorylated form in bacteria is dephosphorylated by Stp, which thus increases its activity.

*L. monocytogenes* Cytoplasmic MnSOD Is Phosphorylated upon Entry in Stationary Phase and Is Secreted in a Nonphosphorylated State by the SecA2-dependent Machinery—We investigated MnSOD phosphorylation during *L. monocytogenes* growth (Fig. 2A) and performed immunoprecipitation experiments on bacterial extracts and culture supernatants. MnSOD could be immunoprecipitated from both bacterial extracts and culture supernatants, in both exponential and stationary phases (Fig. 2B, panel 1). Whereas the secreted MnSOD was constantly found in its nonphosphorylated state, MnSOD from bacterial extracts was nonphosphorylated in exponential phase and became phosphorylated upon entry in stationary phase (Fig. 2B, panel 1). Increased MnSOD phosphorylation was concomitant with decreased Stp production in bacteria (Fig. 2B, panel 2). We analyzed the global production of MnSOD during growth. The MnSOD level, which was high in bacterial extracts in exponential phase, decreased in stationary phase while the amount of MnSOD detected in culture supernatants increased (Fig. 2B, panel 2). Thus, upon entry in stationary phase, nonphosphorylated MnSOD is increasingly secreted while the remaining cytoplasmic MnSOD is progressively phosphorylated.

Using the Δstp mutant, we next addressed the role of Stp on MnSOD dephosphorylation and production (Fig. 2B, panel 3 and panel 4). As expected, in the bacterial extracts of the Δstp mutant, the phosphorylated form of MnSOD was
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A

![Graph showing OD_600nm over time](image)

B

1. OD_600nm 1.2 1.5 2.1 2.6 3.1 3.3
   - WT
   - IP (Total Extract): α-MnSOD
     - WB: α-MnSOD
   - IP (Total Extract): α-MnSOD
     - WB: α-phosphothreonine

2. OD_600nm 1.2 1.5 2.1 2.6 3.1 3.3 3.5 3.5
   - WT
   - WB (Total Extract): α-MnSOD
   - WB (Total Extract): α-Stp
   - WB (Total Extract): control α-ActA

3. OD_600nm 1.2 1.5 2.1 2.6 3.1 3.3
   - Δstp
   - IP (Total Extract): α-MnSOD
     - WB: α-MnSOD
   - IP (Total Extract): α-MnSOD
     - WB: α-phosphothreonine

4. OD_600nm 1.2 1.5 2.1 2.6 3.1 3.3 3.5 3.5
   - Δstp
   - WB (Total Extract): α-MnSOD
   - WB (Total Extract): control α-ActA

C

- WT
- ΔseqA2
- ΔseqA2 seqA2

- WT
- ΔseqA2
- ΔseqA2 seqA2

- MnSOD
- InIC
detected earlier, i.e. at mid-log phase, and at a higher level compared with the wild-type strain (Fig. 2B, panel 3). Strikingly, MnSOD immunoprecipitated from culture supernatants was still constantly detected in its nonphosphorylated form in the Δstp mutant. Because phosphorylated listeriolysin O and phosphorylated EF-Tu control proteins could be detected in supernatants from the wild-type and Δstp mutant respectively (supplemental Fig. S2), these results strongly suggest that secretion of the phosphorylated MnSOD cannot occur. We also observed that the level of MnSOD in both bacterial extracts and culture supernatants was higher in the Δstp mutant than in the wild-type strain while two controls proteins, ActA, the actin-based motility protein, and the secreted internalin InlC, were detected in similar amounts in both strains (Fig. 2B, panel 4). Thus, the presence of Stp controls not only MnSOD phosphorylation but also its production.

We then investigated the secretion mechanism of the MnSOD, whose amino acid sequence does not contain any signal peptide. Since the accessory secretion protein SecA2 had been shown to mediate secretion of proteins, which lack a signal peptide, in Gram-positive bacterial pathogens we analyzed supernatants from L. monocytogenes wild-type 10403S, from a ΔsecA2 mutant, and from a complemented strain (40) and found that the MnSOD was secreted by a SecA2-dependent machinery (Fig. 2C). Thus, MnSOD belongs, along with L. monocytogenes FbpA (41), to the increasing list of proteins lacking a signal peptide and secreted by the SecA2 pathway in pathogenic bacteria (40).

FIGURE 2. Analysis of MnSOD phosphorylation, secretion, and production during L. monocytogenes growth. A, growth curve of wild-type EGDe strain (WT) and Δstp mutant in BHI at 37 °C. Bacteria were harvested at various time points (arrows). Total bacterial proteins were extracted, and proteins from culture supernatants were precipitated. B, analysis of MnSOD phosphorylation and production in total extracts and culture supernatants from WT and Δstp mutant. At the chosen time points, MnSOD was immunoprecipitated from WT (panel 1) and Δstp mutant (panel 3) bacteria, using the anti-MnSOD serum. Immunoprecipitates from total bacterial extracts and culture supernatants were separated by SDS-PAGE. MnSOD was detected by Western blotting with the anti-MnSOD serum. The IgG small chain is indicated (*). Phosphorylated MnSOD was detected by Western blotting using the antiphosphoseryl-serine/threonine antibodies. Three additional phosphorylated proteins that could correspond to MnSOD partners, are indicated (**). Positions of the molecular mass markers are indicated on the right. At the time points indicated above, WT (panel 2) and Δstp mutant (panel 4) bacteria were harvested. Proteins from total extracts (20 μg) and culture supernatants (70 μg) were separated by SDS-PAGE. Anti-MnSOD serum and anti-Stp antibodies were used to detect MnSOD and Stp by Western blotting, respectively. Immunodetection of ActA and InlC control proteins was performed using the anti-ActA antibodies and the anti-InlC serum on the same samples. C, SecA2-dependent secretion of MnSOD. Proteins were precipitated from culture supernatants (80 μg) of the wild-type L. monocytogenes 10403S (WT), the ΔsecA2 mutant and complemented strains (ΔsecA2+secA2) (40). MnSOD was detected by immunoblotting using the anti-MnSOD serum. Immunodetection of the InlC control protein was performed using the anti-InlC antisera.

MnSOD Protects L. monocytogenes against Reactive Oxygen and Nitrogen Species-mediated Toxicity in Vitro—Because SOD is a major antioxidant enzyme, we investigated the sensitivity to ROS and reactive nitrogen species (RNS) of an isogenic Δsod mutant compared with the wild-type strain. We observed a large increase in the doubling time of the Δsod mutant grown...
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FIGURE 4. Role of L. monocytogenes MnSOD during infection. A, effect of the sod deletion on intracellular survival in macrophages. Peptone-elicited peritoneal macrophages (PEM, 10⁶ cells/well) from BALB/c mice were activated with IFN-γ and infected at a MOI of 10 with the wild-type strain (WT), the Δsod mutant and the complemented strain (Δsod + sod). The number of CFU was determined at 15 min, 1 h, and 4 h after addition of gentamicin. Mean values are expressed as CFU ± S.D., with p values of p < 0.05 (*) and p < 0.01 (**) corresponding to the comparison of WT and Δsod mutant CFUs. B, detection of MnSOD during PEM infection. PEM were activated with IFN-γ and infected for 15 min with the WT strain and the Δsod mutant. Infected PEM were lysed and centrifuged to separate BACTERIA and on PEM extracts of non infected macrophages (NI PEM) as described under “Experimental Procedures.” MnSOD immunoprecipitation was carried out on PEM extracts and bacterial extracts of infected PEM and on PEM extracts of non infected macrophages (NI PEM). Immunoprecipitates were analyzed by immunoblotting using the anti-MnSOD serum raised against L. monocytogenes MnSOD and with antiphosphothreonine antibodies. Molecular masses are indicated on the right. C, effect of the sod deletion on L. monocytogenes survival in BALB/c mice. Multiplication of the WT strain, the Δsod mutant and the Δsod + sod strain was determined in the spleen and liver of BALB/c mice infected intravenously with 8 × 10⁶ CFU. For each strain, CFU were counted in organs of 4 BALB/c mice at 24, 48, and 72 h after infection.

TABLE 1
Role of L. monocytogenes MnSOD on phagosomal escape in macrophages

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Δsod</th>
<th>Δsod + sod</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Infected PEM</td>
<td>99.2</td>
<td>99.2</td>
<td>100</td>
</tr>
<tr>
<td>% Infected PEM with intracytosolic bacteria labeled with phalloidin</td>
<td>22.3</td>
<td>14.3*</td>
<td>21.7</td>
</tr>
<tr>
<td>% Infected PEM with intracytosolic bacteria labeled with lysotracker</td>
<td>56.9</td>
<td>82.5b</td>
<td>63.8</td>
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*p < 0.05.
*b p < 0.0001.

in normal atmosphere (pO₂ = 21%) compared with the wild-type strain and the Δsod strain complemented with the sod gene that had a similar doubling time (Fig. 3A). In microaerophilic atmosphere (pO₂ = 6%), the doubling time of the Δsod mutant was similar to that of the wild-type and of the complemented strains (Fig. 3A).

We then tested the sensitivity of the Δsod mutant to ROS in these microaerophilic growth conditions using a disk diffusion assay. The Δsod mutant was hypersensitive to paraquat, a reagent that increases the intracellular level of superoxides. Indeed, the diameter of growth inhibitory area of the Δsod mutant (36.3 mm ± 0.8) was strongly increased compared with that of the wild-type and the complemented strains whose growth was not affected (0.0 mm and 0.1 mm ± 0.1, respectively). Alternatively, addition of hydrogen peroxide on filter disks resulted in a significant albeit less severe difference between growth inhibition of the Δsod mutant (5.3 mm ± 1.3) and that of the wild-type and the complemented strains (2.5 mm ± 0.5; 1.8 mm ± 1.0, respectively). We next analyzed growth of the Δsod mutant in liquid culture media in the presence of exogenous ROS and/or RNS. ROS were generated by xanthine oxidase (XO) from hypoxanthine (HX). We first verified that addition of HX did not inhibit L. monocytogenes growth (data not shown). Nitric oxide (NO) was produced from spermine/NO. The Δsod mutant had a significantly higher susceptibility to RNS (Fig. 3B, central panel) compared with that of the wild-type and complemented strains. No drastic effect of the production of exogenous ROS was observed in these conditions on the growth of the Δsod mutant (Fig. 3B, left panel). However, a strong synergistic effect of ROS and RNS on growth inhibition of the Δsod mutant was observed (Fig. 3B, right panel). Collectively, these results show that MnSOD protects L. monocytogenes from exogenous ROS and RNS.

MnSOD Is Found in a Phosphorylated Form in Macrophages—Because ROS and RNS are mediators of antibacterial activity in phagocytes, we next assessed the contribution of MnSOD to L. monocytogenes intracellular survival in macrophages. The wild-type, Δsod mutant and complemented strains had similar multiplication rates in the non listericidal macrophage-like RAW264.7 cell line and in peritoneal macrophages (data not shown). However, the Δsod mutant was killed more efficiently and significantly faster than the wild-type and complemented strains by listericidal macrophages, i.e. peritoneal macrophages activated with IFN-γ (Fig. 4A).

To further compare the intracellular behavior of the wild-type, Δsod mutant and complemented strains in peritoneal macrophages activated with IFN-γ, we indirectly analyzed bacterial escape from phagosomes, by counting after 4 h of
infection, the number of macrophages containing intracellular bacteria surrounded with polymerized actin in the cytosol. As shown in Table 1, all macrophages were infected with these bacterial strains. Significantly fewer Δsod mutant bacteria were able to polymerize cytosolic actin compared with wild-type and complemented strains, suggesting that a lower number of Δsod bacteria had reached the cytosol. To detect if actin-negative bacteria were present in a phagolysosomal compartment, we loaded macrophages with the lysosome marker LysoTracker Red DND-99. After 4 h of infection, the macrophages infected with the Δsod mutant contained a much higher number of LysoTracker Red-positive bacteria than the macrophages infected with the wild-type strain (Table 1). These results strongly suggested that MnSOD contributes to L. monocytogenes vacuole escape, possibly by preventing phagosomal bacterialicidal mechanisms.

We then determined the phosphorylation state of L. monocytogenes MnSOD in peritoneal macrophages activated with IFN-γ. We immunoprecipitated MnSOD from protein extracts of bacteria recovered from infected macrophages and from cellular extracts of infected macrophages (Fig. 4B). As shown in the control experiment with non infected macrophages, our anti-MnSOD antibodies cross-reacted with a cellular protein, probably a SOD. However, as expected, MnSOD was detected in the bacterial extracts from cells infected with the wild-type bacteria and it was not the case when Δsod bacteria had been used to infect cells. Strikingly, we detected a signal corresponding to a phosphorylated SOD in infected macrophages. Because this phosphorylated SOD was only present in macrophages infected with the wild-type strain and not with the Δsod mutant, it most likely corresponds to L. monocytogenes phosphorylated MnSOD (Fig. 4B), suggesting that L. monocytogenes MnSOD can be phosphorylated in macrophages. It is unlikely that phosphorylated MnSOD was secreted from the bacteria, as this has never been observed in vitro.

MnSOD Is Required for L. monocytogenes Pathogenesis—All our results were converging to indicate that MnSOD was critical for virulence of L. monocytogenes. We thus addressed the role of MnSOD in vivo. BALB/c mice were challenged intravenously with the wild-type, the Δsod mutant and the complemented strains. In spleen and liver, which are early sites of L. monocytogenes replication, growth of the Δsod mutant was strongly impaired compared with that of the wild-type strain (Fig. 4C). This difference could be detected as soon as 1 day after infection in the spleen and 2 days post-infection in the liver (Fig. 4C). At 3 days post-infection, there were 10 times fewer mutant bacteria in the spleen compared with the wild-type strain and growth of the mutant was controlled by the animals in the liver. The wild-type and complemented strains behaved similarly in animals. Thus, MnSOD is a novel virulence factor of L. monocytogenes significantly contributing to listeriosis in mice.

DISCUSSION

In this article, we have characterized a new virulence factor in Listeria and the first example of a SOD down-regulated by phosphorylation. In addition, we provide evidence that MnSOD, which is secreted as an active, nonphosphorylated form by the SecA2-dependent machinery, plays a critical role in intracellular survival in macrophages and is required for full virulence of L. monocytogenes. The first unexpected result and the starting point of this study was that Listeria MnSOD can be phosphorylated on serine and threonine residues. The second important result was that Listeria MnSOD can be secreted and that only the nonphosphorylated and most active form is secreted. Strikingly, MnSOD can become phosphorylated inside infected cells, highlighting a possible cross-talk between the host cell and the incoming microbe.

That L. monocytogenes MnSOD was present in culture supernatants as well as in the bacterial cytoplasm was at first unexpected since its amino acid sequence does not reveal any standard signal peptide. However, several pathogenic bacteria, including L. monocytogenes and Mycobacterium tuberculosis, have now been shown to secrete proteins lacking a signal peptide, through the SecA2 auxiliary secretion system (40–43). Thus MnSOD secretion occurs by the well established mechanism, the SecA2-dependent mechanism.

ROS are produced during growth in all living cells and thus the cytoplasmic form of MnSOD protects Listeria from endogenously produced ROS. During the oxidative burst that occurs after phagocytosis, ROS are among the main mediators of the antibacterial activity of phagocytes. Superoxides produced in the acidic phagolysosome can directly damage targets on the bacterial surface (44). They can also penetrate inside bacteria and react with cytosolic targets such as [4Fe-4S] cluster proteins or DNA (45). In addition, superoxides can indirectly be deleterious by reacting with nitric oxide to form peroxynitrite, a toxic oxidant that can freely enter bacteria. Thus targets of ROS and RNS are present both inside bacteria and on its surface. Interestingly, our preliminary results show that L. monocytogenes MnSOD can be detected on the bacterial surface (supplemental Fig. S3), as reported for SODs from Mycobacterium leprae (46), Mycobacterium avium (47), and Mycobacterium bovis BCG (48). Therefore Listeria and mycobacterial MnSODs may protect bacterial surface components from superoxide damage as documented for well-characterized periplasmic CuZnSOD from pathogenic Gram-negative bacteria (13). As shown by the increase in the bacterial doubling time in vitro, ROS and RNS are indeed toxic for Listeria and the Δsod mutant was much more sensitive than the wild type to ROS and RNS, definitively establishing the role of the MnSOD in protection against these radicals.

The generation of superoxides into the phagocytic vacuole has been reported to induce a potassium influx which increases the pH to the optimal value for activation of bactericidal proteases (49), which also participate to the phagocytic process. We observed that the Δsod mutant was associated with acidic compartments of infected macrophages, failing to escape from phagosomes, strongly suggesting that MnSOD allows L. monocytogenes to resist phagolysosomal degradation in perfect agreement with the report that localized reactive oxygen and nitrogen intermediates inhibit escape from vacuoles in activated macrophages (22). As expected from its location in the infected cell, survival of the Δsod mutant was impaired in listericidal macrophages. Together these results demonstrate that
MnSOD counteracts a host defense mechanism by destroying superoxide radicals generated by the activated macrophages, as reported for SODs produced by other pathogenic bacteria (50–53). In line with the role of MnSOD in intracellular survival in macrophages, this enzyme was required for full virulence in mice.

We also provide here evidence that *Listeria* MnSOD can be phosphorylated and that phosphorylation controls its activity. Post-translational modifications of SODs affecting their enzymatic activity have been reported in eukaryotes. For instance, tyrosine nitration of human MnSOD decreases its activity (54) and glycation of human CuZnSOD, which is associated to diabetes and aging, inactivates the enzyme (55–57). More recently, Csar et al. (58) reported for the first time, through a proteomic analysis, transient phosphorylation of a cytoplasmic CuZnSOD after treatment of myeloid cells by the granulocyte colony stimulating factor. However, whether phosphorylation could regulate SOD activity remained elusive. We demonstrate here that complete dephosphorylation of *L. monocytogenes* MnSOD by the serine-threonine phosphatase Stp dramatically increases its activity, possibly through conformational changes affecting the active site. *Listeria* growing exponentially produced the most active, nonphosphorylated MnSOD, thereby preventing damage from ROS produced during aerobic respiratory metabolism (Fig. 5A) (59). In contrast, when bacterial division and metabolism slow down upon entry in stationary phase, MnSOD is found in its less active phosphorylated state. The increased phosphorylation of MnSOD observed in stationary phase is concomitant with a decreased production of the serine-threonine phosphatase Stp, confirming the regulatory link between the two proteins (Fig. 5A). Along these lines, MnSOD phosphorylation, which occurs earlier and at a higher level during growth of a Δstp mutant, correlated with an increased, possibly compensatory, production of MnSOD. Interestingly, in mammalian cells, a link between phosphorylation of antioxidant proteins and growth seems to exist as inactivation of the antioxidant human peroxiredoxin I by Cdc2-dependent phosphorylation, has been hypothesized to be important for cell cycle progression (60). Strikingly, a phosphorylated form of *L. monocytogenes* MnSOD was detected in the cytoplasm of infected macrophages. Since secreted MnSOD is constantly found in its nonphosphorylated form in *L. monocytogenes*, we propose that a cellular kinase down-regulates MnSOD activity by phosphorylation (Fig. 5B). Secretion of proteins interfering with phagosomal maturation, e.g. *M. bovis* BCG serine-threonine kinase G (61) and *Salmonella* pathogenicity island 2 proteins (62), is a strategy used by pathogenic bacteria to promote intracellular survival. Inactivation of secreted proteins important for bacterial survival could be a strategy used by the host phagocytes to control intracellular infection. Up to now, and to our knowledge, this is the first reported case of bacterial protein down-regulation through phosphorylation in the host. A previous report has nevertheless documented the differential ubiquitination/degradation of *Salmonella* type III effectors (63).

Are other bacterial SODs phosphorylated and would phosphorylation also occur during infection with other bacteria? Our preliminary results show unambiguously that the mycobacterial FeSOD from *M. bovis* BCG is phosphorylated on both serine and threonine residues and can be dephosphorylated *in vitro* (supplemental Fig. S4). Interestingly, it has been reported that *M. tuberculosis* FeSOD, which is essentially identical to

![FIGURE 5. Regulation of MnSOD activity by Stp in *L. monocytogenes*. A, during exponential growth phase, phosphorylation of MnSOD by an unidentified bacterial kinase is counteracted by Stp, generating a pool of highly active MnSOD. In stationary phase, decrease in the level of Stp leads to an increase in phosphorylated MnSOD, while nonphosphorylated MnSOD is actively secreted by the SecA2 system. B, within host macrophages, phagocytosed *L. monocytogenes* neutralizes the oxidative burst-generated ROS by secretion of MnSOD, which in turn is possibly down-regulated by phosphorylation mediated by an unknown cellular kinase.]
M. bovis BCG FeSOD, can be secreted by the SecA2 pathway and is critical for survival in the host (43, 64). Whether this FeSOD activity is tightly regulated by phosphorylation in mycobacteria has not been determined. Given the present high interest in mycobacterial kinases and phosphatases as potential drug targets, this issue clearly deserves investigation.

In conclusion, we have shown that post-translational regulation of Listeria MnSOD represents a key level of control of this important enzyme. It would be interesting to investigate if a similar post-translational level of regulation also exists in humans. Activation and inactivation of SODs or of proteins regulating SODs may provide tools to fight against diseases associated to superoxide-mediated cell injury such as cancer, neurodegenerative, inflammatory, and cardiovascular diseases.

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REFERENCES

SOD Phosphorylation and Listeria Virulence


