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The Zinc Metalloprotease of *Listeria monocytogenes* Is Required for Maturation of Phosphatidylcholine Phospholipase C: Direct Evidence Obtained by Gene Complementation

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The maturation of the 33-kDa proenzyme to the 29-kDa phosphatidylcholine phospholipase C (PC-PLC) of *Listeria monocytogenes* requires the production of the zinc metalloprotease encoded by *mpl*, the proximal gene of the lecithinase operon. We recently described a low-virulence lecithinase-deficient mutant of *L. monocytogenes* EGD-SmR, designated JL762, generated by a single insertion of transposon Tn*1545* in *mpl*. This mutant failed to produce the 29-kDa PC-PLC, an exoenzyme probably involved in cell-to-cell spreading. The role of the product of the *mpl* gene in production of PC-PLC was investigated in *trans*-complementation experiments. The entire *mpl* gene was cloned in a plasmid able to replicate in *L. monocytogenes*. This recombinant plasmid was introduced into JL762 and restored the lecithinase phenotype on egg yolk agar and the production of the active 29-kDa PC-PLC in culture supernatants and partially restored the level of virulence. These results demonstrate that zinc-dependent metalloprotease of *L. monocytogenes* is involved in the virulence of this bacteria at least through its action on PC-PLC.

There is strong evidence that most virulence genes of the intracellular pathogen *Listeria monocytogenes* are very closely linked on the chromosome (reviewed in reference 24). These genes act synergistically, enabling the bacteria to survive and replicate inside the host cells (17). The main steps of this intracellular parasitism include entry into epithelial cells (7, 8), evasion of the phagolysosomal compartment (27, 28), actin polymerization, cell-to-cell spreading (5, 13, 21, 32, 33), and lysis of the two-membrane vacuole of infected adjacent cells (36). Transcriptional analysis indicates that there are two adjacent operons surrounding the *hly* gene (24), which encodes listeriolysin O, a key virulence factor (2, 4, 9, 10, 12, 25). The first operon includes *prfA*, a positive activator of virulence genes (16, 19), and *plcA*, encoding a phosphatidylinositol phospholipase C (15, 18). The second, the lecithinase operon, is composed of several genes, including *mpl*, encoding a zinc metalloprotease (6, 20), *actA*, encoding a surface protein required for actin polymerization (13), and *plcB*, encoding a precursor of the phosphatidylcholine phospholipase C (PC-PLC), which contributes to lysis of the two-membrane vacuoles (36).

Although data on the functions of most of the virulence genes are now available, the role of *mpl*, the proximal gene of the lecithinase operon, remains unknown. It is only found in the pathogenic species *L. monocytogenes* (6, 20), suggesting that it may be involved in intracellular parasitism. Sequence data indicate that the *mpl* product is a 57-kDa protein with an N-terminal signal sequence (6, 20). The amino acid sequence of the protein shows strong similarities to that of the thermolysin family, a group of metalloproteases produced by *Bacillus* species (14, 30, 31, 35) and some gram-negative bacteria, including *Serratia marcescens* (22), *Legionella pneumophila* (3), and *Pseudomonas aeruginosa* (1). These proteases are all proenzymes, and their proteolytic activities are easily detected (14, 30, 31, 35). In a recent work, we isolated a mutant from *L. monocytogenes* EGD-SmR, JL762, which was obtained by a single insertion of the transposon Tn*1545* into the *mpl* gene (29). This mutant expressed a lecithinase-deficient phenotype and was less virulent in mice than the wild type. JL762 does not produce the 29-kDa mature form of PC-PLC, although the 33-kDa precursor was detected in culture supernatants. The product of the *mpl* gene may thus be responsible for the maturation of the 33-kDa precursor into the active PC-PLC product (29). In this article, we demonstrate by *trans*-complementation experiments that the zinc metalloprotease, encoded by the *mpl* gene, is required for the maturation of PC-PLC.

The *mpl* gene was amplified by the polymerase chain reaction from total crude DNA of *L. monocytogenes* EGD by using the following primers: 5′-GAATTCGAATATTCTG ACTGTTTATC-3′ and 5′-GAATTCAATTGACTAATTGT TTACTT-3′ (6). The annealing temperature was 55°C. This reaction generated a 1.74-kb *Eco*RI fragment. Restriction analysis and partial sequencing of this polymerase chain reaction product confirmed that the fragment amplified carried the *mpl* gene and its promoter (data not shown). Several attempts to clone this fragment into low- or high-copy-number gram-negative vectors and then transform it into *Escherichia coli* led to structurally unstable recombinant plasmids. Therefore, the 1.74-kb *Eco*RI purified insert was ligated to *Eco*RI-linearized and dephosphorylated pAT28 DNA, a shuttle vector able to replicate in gram-negative and gram-positive bacteria (34), and introduced by electroporation into *L. monocytogenes*. The protocol used was that of Park and Stewart (23), modified as follows. Overnight cultures of *L. monocytogenes* in brain heart infusion (BHI) broth were diluted (2.5:100) in fresh medium and grown at 37°C with shaking to 0.5 optical density unit. Penicillin was then added to a final concentration of 12 μg/ml, and incuba-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference
L. monocytogenes		
EGD-SmR	Sm^r	9
JL762	Sm^r Er^r Tc^r Km^r; (EGD-SmR::Tn*1545*)	29
Plasmids		
pAT28	Sp^r Tra^- Mob^+ *lacZ*α^+; *oriR* pUC, *oriR* pAMβ1, multiple cloning site of pUC18	34
pNec1	pAT28Ω 1.7-kb *Eco*RI fragment containing *mpl* from EGD-SmR	This study

tion was continued to an optical density of 1.2. Cells were then harvested by centrifugation, washed with ice-cold electroporation buffer (1 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 500 mM sucrose), resuspended in 0.5 ml of the same buffer supplemented with 15% glycerol, mixed with 1 μg of plasmid DNA in a 0.2-cm cuvette, and electroporated at 2,500 V and 25 μF (with a time constant of approximately 9 ms), and a 200-Ω pulse with the Gene Pulser Apparatus (Bio-Rad Laboratories, Richmond, Calif.). The samples were immediately added to 1 ml of BHI broth, incubated for 4 h at 37°C, and then plated on BHI agar containing spectinomycin (60 μg/ml). The plasmid content of randomly selected transformants analyzed by agarose gel electrophoresis after digestion by *Eco*RI revealed that all transformants contained a single plasmid, designated pNec1, consisting of pAT28 plus a 1.74-kb insert (data not shown). For *trans*-complementation experiments, plasmid pNec1, or pAT28 as the control, was introduced by electroporation into the parental strain EGD-SmR and its lecithinase-negative mutant JL762 (Table 1).

The following features of the six strains constructed (EGD-SmR, EGD-SmR/pAT28, EGD-SmR/pNec1, JL762, JL762/pAT28, and JL762/pNec1) were investigated: morphology of the bacilli, colony aspect, motility at 22°C, serovar (1/2a), catalase and oxidase activity, utilization of L-rhamnose and D-xylose, growth curves in BHI broth, and patterns of fermentation of 50 different carbohydrates by using the API-50 CH system (BioMérieux). There were no differences between the strains. The appearances of the colonies on 5% horse blood agar were similar, as were the hemolytic titers in supernatants obtained after culture in charcoal-treated tryptic-glucose-yeast broth (~2,000 U/ml) as described previously (10). As expected, transformed strains EGD-SmR/pAT28, EGD-SmR/pNec1, JL762/pAT28, and JL762/pNec1 were resistant to streptomycin and spectinomycin, and the JL762 derivatives were resistant to tetracycline, erythromycin, and kanamycin because of the presence of Tn*1545*. All of the strains were plated on 2.5% egg yolk-BHI agar containing spectinomycin for strains harboring pAT28 or pNec1 to test for lecithinase activity as previously described (11). After incubation for 24 to 48 h at 37°C, JL762/pNec1 colonies produced opalescent halos that were absent around the parental lecithinase-deficient mutant JL762 (Fig. 1). Colonies of EGD-SmR, EGD-SmR/pAT28, EGD-SmR/pNec1, and JL762/pNec1 expressing the lecithinase activity produced similar halos (Fig. 1). Phospholipase activity was assayed in culture supernatants as previously described (11): EGD-SmR, EGD-SmR/pAT28, EGD-SmR/pNec1, and JL762/pNec1 each produced about 50 U/ml. No phospholipase activity was detected with JL762 and JL762/pAT28. Thus, *mpl* is able to restore the expression of the lecithinase activity to culture supernatants of *L. monocytogenes* JL762.

We then studied the secretion of the 29-kDa PC-PLC to culture supernatants by Western blot (immunoblot) analysis (29). Trichloroacetic acid-precipitated proteins from culture supernatants of bacteria grown for 9 h at 37°C in charcoal-treated tryptic-glucose-yeast broth supplemented with 0.1 mM $ZnSO_4$ were probed with an immunoabsorbed rabbit antiserum raised against highly purified 29-kDa PC-PLC (29). The antiserum revealed a 29-kDa band corresponding to the active PC-PLC in the supernatants of cultures of EGD-SmR, EGD-SmR/pAT28, and EGD-SmR/pNec1 (Fig. 2). The 33-kDa doublet corresponding to pro-PC-PLC was detected in all six strains. As expected, the 29-kDa PC-PLC was not detectable in supernatants of JL762 and JL762/pAT28. This protein was present in JL762/pNec1, indicating that *mpl* induced the production of an active 29-kDa PC-PLC in the supernatant. However, no bands were detected (29 or 33 kDa) in the supernatant of the PC-PLC-negative mutant (36) resulting from the insertion of Tn*917-lac* into *plcB* (data not shown).

We also studied the virulence of the strains by determining the 50% lethal dose for specific-pathogen-free ICR Swiss mice (Charles River, St-Aubin-lès-Elboeuf, France). Groups of five mice, 6 to 8 weeks old, were infected intravenously (i.v.) with increasing doses of bacteria. Because plasmids pAT28 and pNec1 were segregationally unstable in vivo, infected mice were treated simultaneously for 4 days with

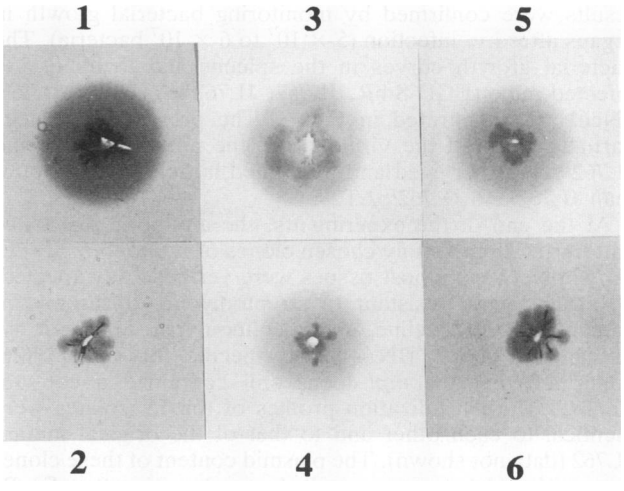

FIG. 1. Detection of the lecithinase activity on egg yolk agar. *L. monocytogenes* EGD-SmR (panel 1), JL762 (panel 2), EGD-SmR/pNec1 (panel 3), JL762/pNec1 (panel 4), EGD-SmR/pAT28 (panel 5), and JL762/pAT28 (panel 6) were grown on 2.5% egg yolk agar for 48 h at 37°C. Opalescent halos are visible around the parental strain EGD-SmR and the *trans*-complemented mutant JL762/pNec1.

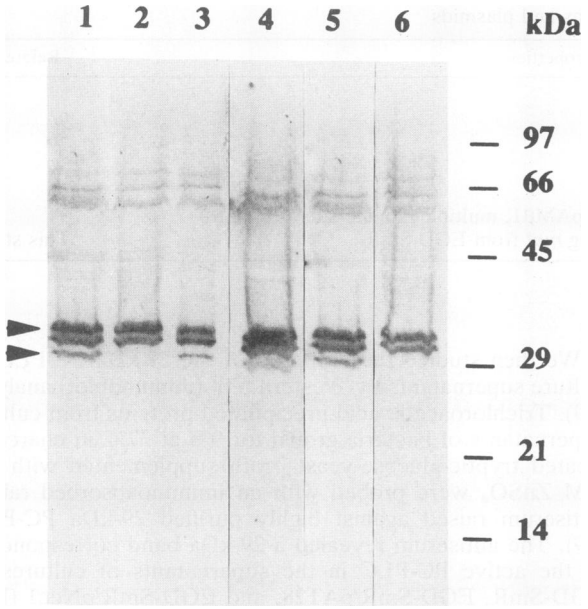

FIG. 2. Immunoblot analysis of culture supernatants from *L. monocytogenes* EGD-SmR and *trans*-complemented strains. Trichloroacetic acid-precipitated supernatant proteins (5 μg per well) were separated on a sodium dodecyl sulfate-13% polyacrylamide gel, electrotransferred to nitrocellulose sheets, and probed with a rabbit immunoadsorbed anti-29-kDa PC-PLC serum diluted 1:5. Bound antibodies were detected with peroxidase-labelled goat anti-rabbit immunoglobulin. Supernatants were prepared from EGD-SmR (lane 1), JL762 (lane 2), JL762/pNec1 (lane 3), EGD-Sm/pNec1 (lane 4), EGD-SmR/pAT28 (lane 5), and JL762/pAT28 (lane 6). The positions of the 29-kDa PC-PLC and the doublet 33-kDa band are indicated with arrowheads.

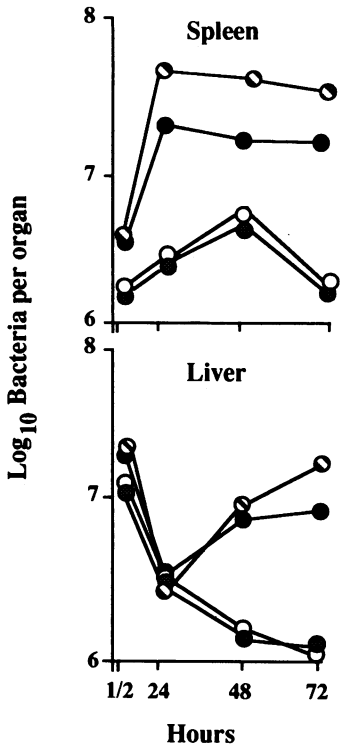

FIG. 3. Bacterial growth curves of EGD-SmR (⊘) and the lecithinase-deficient mutant JL762 (○) and its transformants JL762/pAT28 (●) and JL762/pNec1 (●) in the spleens and livers of infected mice inoculated i.v. with 5×10^7 to 6×10^7 bacteria. Each time point corresponds to the mean value for a group of five mice. Mice were sacrified at 0.5, 24, and 72 h after inoculation.

spectinomycin injected subcutaneously twice a day (25 mg, two times), thus selecting in vivo the plasmid-bearing bacteria. The 50% lethal doses of JL762 and EGD-SmR have been previously reported to be $10^{7.6}$ and $10^{6.2}$, respectively (29); the 50% lethal dose of JL762/pNec1 was $10^{7.25}$. These results were confirmed by monitoring bacterial growth in organs after i.v. infection (5×10^7 to 6×10^7 bacteria). The bacterial growth curves in the spleens and livers of i.v. infected mice (EGD-SmR, JL762, JL762/pAT28, or JL762/pNec1) are illustrated in Fig. 3. The presence of pNec1 partially restored the virulence of the mutant JL762, and JL762/pNec1 survived and multiplied in host tissues better than JL762 and JL762/pAT28.

At the end of the experiments, the antibiotic resistance patterns of 15 randomly chosen clones of *L. monocytogenes* JL762/pNec1 from host tissues were verified. As expected, all strains were resistant to streptomycin, erythromycin, kanamycin, tetracycline, and spectinomycin. Southern hybridization with a DNA probe specific for Tn*1545* (26) confirmed that the *mpl* locus still contained a copy of Tn*1545*. The hybridization profiles of the 15 isolates were identical to each other and to that of the original mutant JL762 (data not shown). The plasmid content of these clones was analyzed by agarose gel electrophoresis after *Eco*RI digestion to study the structural stability of pNec1. The restriction patterns obtained were indistinguishable from that of pNec1, thus demonstrating the structural stability of this plasmid in vivo (data not shown). Thus, the restoration of lecithinase activity and virulence of the JL762/pNec1 isolates was due to *trans*-complementation with *mpl* and not to precise excision of Tn*1545* from the *mpl* locus nor to the allelic replacement of chromosomal *mpl*::Tn*1545* by the plasmid-borne *mpl*.

This study shows that the zinc metalloprotease encoded by *mpl*, the proximal gene of the lecithinase operon, is produced in vivo and is required for the maturation of the 29-kDa PC-PLC of *L. monocytogenes*. The introduction of a multicopy plasmid harboring *mpl* restored the production of an active extracellular 29-kDa PC-PLC to the lecithinase-deficient mutant JL762. The *plcB* product appears, therefore, to be a subtrate for the zinc metalloprotease of *L. monocytogenes*. It has been reported that *plcB* encodes a 298-amino-acid protein homologous to the PC-PLC of *Bacillus cereus* and to the alpha-toxin of *Clostridium perfringens* (36). Sequence comparisons suggest that *L. monocytogenes* PC-PLC is subject to two cleavages: (i) at Ala-25 after the peptide signal, and (ii) between Ser-51 and Try-52 residues to produce the 238-amino-acid mature protein corresponding to the 29-kDa PC-PLC. This active enzyme might contribute to virulence by lysing the two-membrane vacuoles that enclose the bacteria of newly infected cells (36). The partial restoration of virulence in *mpl*-complemented JL762 is in agreement with this hypothesis. Whether the only biological function of the zinc metalloprotease is the maturation of PC-PLC remains unknown: other bacterial proteins or even eucaryotic proteins might be substrates for this enzyme, since some metalloproteases of the thermolysin family possess broad substrate specificity (1, 3). This protease might therefore be

directly involved in the infectious process, for example, through a scavenging function allowing degradation of eucaryotic membrane proteins. Finally, the products of two genes of the lecithinase operon, *mpl* and *plcB*, are both zinc-dependent metalloenzymes and are secreted as proenzymes which require proteolytic cleavage for their maturation. In conclusion, the results presented in this work reveal an example of refined regulatory mechanisms in *L. monocytogenes*, namely, that the expression of virulence factors is coordinated not only at the transcriptional level through *prfA* but also at the posttranslational level through maturation processes of proenzymes such as the 29-kDa PC-PLC.

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