Mutagenesis of Active-Site Histidines of *Listeria monocytogenes*
Phosphatidylinositol-Specific Phospholipase C: Effects on Enzyme Activity and Biological Function

TRUDI BANNAM† AND HOWARD GOLDFINE*
Department of Microbiology, University of Pennsylvania School of Medicine,
Philadelphia, Pennsylvania 19104-6076

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*L. monocytogenes*, a gram-positive facultative intracellular pathogen, produces two distinct phospholipases C. PC-PLC, encoded by *plcB*, is a broad-range phospholipase, whereas PI-PLC, encoded by *plcA*, is specific for phosphatidylinositol. It was previously shown that PI-PLC plays a role in efficient escape of *L. monocytogenes* from the primary phagosome. To further understand the function of PI-PLC in intracellular growth, site-directed mutagenesis of *plcA* was performed. Two potential active-site histidine residues were mutated independently to alanine, serine, and phenylalanine. With the exception of the activity of the enzyme containing H38F, which was unstable, the PI-PLC enzyme activities of culture supernatants containing each mutant enzyme were <1% of wild-type activity. In addition, the levels of expression of the mutant PI-PLC proteins were equivalent to wild-type expression. Derivatives of *L. monocytogenes* containing these specific *plcA* mutations were found to have phenotypes similar to that of the *plcA* deletion strain in an assay for escape from the primary vacuole, in intracellular growth in a murine macrophage cell line, and in a plaquing assay for cell-to-cell spread. Thus, catalytic activity of PI-PLC is required for all its intracellular functions.

Phosphatidylinositol-specific phospholipases C (PI-PLC) are secreted by several gram-positive bacteria, including *Bacillus cereus*, *Bacillus thuringiensis*, *Listeria monocytogenes*, *Clostridium novyi*, and *Staphylococcus aureus*. Except for the two proteins from the *Bacillus* spp., which are almost identical in amino acid sequence, amino acid identities among the other proteins for which sequences are known range from 21 to 40% (25). Bacterial PI-PLC are highly specific for PI and show no activity on PI-4-P or PI-4,5-P$_2$. The latter two are cleaved by eukaryotic phospholipases, giving rise to the important intracellular messengers diacylglycerol (DAG) and inositol-1,4,5-P$_3$ from PI-4,5-P$_2$ (26, 27). Bacterial PI-PLC can also cleave the glycerol-P bond in glycosyl-PI anchors (GPI anchors) (8, 22).

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**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Bacterial strains used in this study are listed in Table 1. The wild-type *L. monocytogenes* strain 10403S (2). All *L. monocytogenes* derivatives were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) and were maintained on BHI agar. The *Escherichia coli* strain DH5α (Life Technologies) was used for routine maintenance and construction of plasmids and was grown in liquid Luria-Bertani or on Luria-Bertani agar. *E. coli* strains containing pKSV7 derivatives were grown in the presence of 30 µg of ampicillin per ml.

**Construction of *L. monocytogenes* PI-PLC mutant derivatives.** The *L. monocytogenes* 10403S *plcA* gene used in this study originated from the chromosomal clone pDP1228 obtained from Daniel A. Portnoy (University of California, Berkeley). The plasmid pDP1228 was originally derived from a Tn917-LTV3 insertion into the 10403S chromosome as previously described (4). Restriction mapping of pDP1228 determined that this clone contained complete copies of the *plcA* and *hlyA* genes and an incomplete *mpl* gene. The *plcA* gene was subcloned from pDP1228 into pBlueScript II KS (+) on a 2.1-kb fragment utilizing a Sall site located within Tn917-LTV3 and an EcoRI site located in hlyA. This clone, pDP2910, contains a complete copy of the *plcA* gene. The nucleotide sequence of the entire *plcA* gene was determined by using the Sequenase version 917.

† Present address: Department of Microbiology, Monash University, Clayton 3168, Australia.

* Corresponding author. Mailing address: Department of Microbiology, University of Pennsylvania School of Medicine, 301C Johnson Pavilion, Philadelphia, PA 19104-6076. Phone: (215) 898-6384. Fax: (215) 573-4856. E-mail: goldfinh@mail.med.upenn.edu.

† Present address: Department of Microbiology, Monash University, Clayton 3168, Australia.
PI-PLC activity assays. L. monocytogenes supernatants were analyzed for PI-PLC enzymatic activity by using the previously described procedure (5, 13). Briefly, [3H-inositol]PI is suspended in deoxycholate-Tris buffer, pH 7.2, and incubated with bacterial supernatants from cells grown for 5 to 6 h in BHI broth with shaking. The unhydrolyzed lipid is extracted with chloroform–methanol–HCl, and the water-soluble [3H]inositol phosphate is measured in a liquid scintillation spectrometer. Each strain was assayed three times, and the results are presented as percentages of the PI-PLC activity present in supernatants of the wild-type strain which were measured in parallel.

Plaquing of PI-PLC mutants in L2 cells. Plaque formation in mouse fibroblast L2 cells was evaluated as previously described (20, 35). Plaques were visualized in infected monolayers by overlaying with medium containing 1% agarose and 0.2% neutral red. The resultant plaques were measured after projection of the autoradiograph. Plaque size is indicated.

RESULTS

L. monocytogenes PI-PLC mutant derivatives. (i) Effects on catalytic activity. For these studies, a cloned copy of the wild-type plcA gene from L. monocytogenes 10403S was obtained and completely sequenced. This revealed, as expected, a high degree of similarity to the previously sequenced plcA gene from L. monocytogenes LO28, except that the mature protein contained I47M, N83K, and F90Y (24). In keeping with previous nomenclature, residue 1 is the first amino acid of the mature form of PI-PLC (13).

Alignment of the primary sequences of PI-PLC proteins from B. cereus, S. aureus (6), and L. monocytogenes revealed two histidine residues that were completely conserved, and mutation of related histidines in a mammalian PLC had been shown to result in loss of activity (34). Thus, these residues were targeted for site-directed mutagenesis. For examination of the requirement of the conserved histidine residues for PI-PLC activity, H38 and H86 were independently mutated to alanine, phenylalanine, and serine. In each case, the wild-type plcA gene was replaced by the mutant derivative by allelic exchange. This was done in both the 10403S wild-type strain and in the strain with ΔplcB, DP-L1935, previously derived from 10403S. The strains constructed are listed in Table 1.

To determine the effects of mutating H38 and H86 on PI-PLC catalytic activity, assays were performed on culture supernatants. It was found that each of the mutant strains produced a less-than-detectable level of PI-PLC activity in the wild-type background with the exception of H38F, which was not studied further (see below). The expression of PI-PLC protein by each of these derivatives was examined by immunoblotting of culture supernatants. Strains harboring the PI-PLC derivatives bearing H38A, H38S, H86A, and H86S were found to produce a PI-PLC protein in amounts equivalent to that of the wild-type strain (1). However, mutation of H38 and H86 to phenylalanine led to the production of unstable forms of PI-PLC. Therefore, these strains were excluded from further experiments.

(ii) Effects on escape from a primary vacuole. Before comparison of the host cell interactions of site-specific mutants with those of the previously studied in-frame deletion mutants, the normal expression of the mutant derivatives of PI-PLC was confirmed by immunoprecipitation following L. monocytogenes infection of J774 cells (Fig. 1). Stable proteins having the predicted size of PI-PLC were detected from strains harboring the PI-PLC derivatives H38A, H86A, H38S, and H86S.

Intracellular growth of L. monocytogenes in macrophages consists of four distinct stages: initial entry into the cell by phagocytosis, escape from the primary vacuole, replication in the cytoplasm, and subsequent intercellular spread. The abilities of different L. monocytogenes derivatives to escape from the primary vacuole can be conveniently studied by using an immunofluorescence actin nucleation assay (20). This assay was shown to produce data similar to those found in earlier studies.
electron microscopic studies implicating PI-PLC in escape of *L. monocytogenes* from the primary vacuole (5, 33). The fraction of the histidine-mutant bacteria that was stained with fluorescent phalloidin was approximately 50% of that for the wild-type strain and indistinguishable from that for the derivative harboring an in-frame deletion of *plcA* (Fig. 2).

(iii) Effects on intracellular growth of *L. monocytogenes*. It is well documented that wild-type *L. monocytogenes* is able to replicate in bone marrow-derived macrophages, and previous studies showed that an in-frame deletion of *plcA* led to decreased growth (5). Most of the apparent difference reflects the deficiency in escape from the primary vacuole. We investigated the abilities of the PI-PLC mutant derivatives to grow in these cells and found that they also showed decreased growth. The observed growth rates of the strains containing PI-PLC H38A, H86A, H38S, and H86S mutations were all indistinguishable from each other and were identical to the growth rate of the wild-type strain.

(iv) Effects on cell-to-cell spread. Mutation of the broad-range phospholipase C of *L. monocytogenes* (PC-PLC) is known to decrease the ability of the bacterium to spread from cell to cell by 34%, as measured by plaque size on monolayers of L2 fibroblast cells, whereas mutation of PI-PLC results in only a 10% reduction. A double mutant lacking both phospholipases shows a 68% reduction in plaque size (5, 33). We tested the stable histidine mutants for their ability to spread from cell to cell in both the wild-type background and in a strain containing an additional in-frame *plcB* deletion. The plaque sizes observed for the histidine mutants in both backgrounds were identical to those obtained with strains containing in-frame deletions in *plcA* or in both *plcA* and *plcB* (Fig. 3).

The original objectives of this research were (i) to construct stably expressed active-site mutations in *L. monocytogenes* PI-PLC, an important virulence factor; (ii) to verify the contributions of two conserved histidines to the active site of *L. monocytogenes* PI-PLC; and (iii) to learn whether a single amino acid substitution provided the same defects in biological activities of *L. monocytogenes* PI-PLC that had previously been observed with an in-frame deletion in this gene (5, 33). Both sequence alignments (6) and mutagenesis of conserved histidines in mammalian PLC-1 (34) had implicated these histidines in enzymatic activity. More-recent studies have revealed that these histidines are present in the active sites of rat PLC-δ1 (9), *B. cereus* PI-PLC (12, 17), and *L. monocytogenes* PI-PLC (25). The work presented in this paper demonstrates the need for the two mutated histidines for both the enzymatic and biological activities of *L. monocytogenes* PI-PLC.

Previous work done in this laboratory had indicated that *L. monocytogenes* PI-PLC had activities that could contribute to its biological activities other than catalysis of PI hydrolysis (14). Our finding that all the characteristics of the deletion mutant were displayed by stable histidine mutants led us to reconsider our earlier finding that *L. monocytogenes* PI-PLC possesses a membrane-permeabilizing activity that is separate from its catalytic activity. We produced a C-terminal his-tagged version of PI-PLC, expressed it in *E. coli*, and purified it by standard methods. This protein, while possessing good catalytic activity, was inactive in the membrane permeabilization assay. We then obtained recombinant wild-type *L. monocytogenes* PI-PLC (a generous gift of D. Heinz, University of Freiburg) and found that it too lacked membrane permeabilization activity. Lastly, we carried out additional purification of *L. monocytogenes* PI-PLC and were able to separate catalytic and membrane-permeabilizing activities by hydrophobic-interaction chromatography (38).

These findings and the data contained in the present work show conclusively that the catalytic activity of *L. monocytogenes* PI-PLC is essential for its biological activity and that two hist-
tides conserved in all prokaryotic and eukaryotic glycerophosphoinositol-specific PLCs (16) are essential for the catalytic activity of these enzymes. This work does not rule out other features, such as specific membrane interactions, that may be unique to L. monocytogenes PI-PLC and important for its biological roles, but it provides no support for this concept.

LLO is absolutely required for escape from the primary vacuole of cells of most types (28), and mutants lacking both phospholipases manage to escape, albeit in reduced numbers (33). Thus, we are led to consider that the pore-forming ability of LLO may lead to autolysis of the phagocyte membrane as the pores become larger and more numerous or as a result of equilibration of vacuolar contents with the cytosol. It is also possible that lysis requires activation and recruitment of host proteins. The role of L. monocytogenes PI-PLC in escape from a vacuole and in cell-to-cell spread remains enigmatic. Earlier membranes, its hydrolysis in the phagosome is unlikely to progress to lysis of the invading bacterium. The availability of site-specific mutations will be of value in these future studies.

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REFERENCES


