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The Broad-Range Phospholipase C and a Metalloprotease Mediate Listeriolysin O-Independent Escape of *Listeria monocytogenes* from a Primary Vacuole in Human Epithelial Cells

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Intracellular growth of *Listeria monocytogenes* begins after lysis of the primary vacuole formed upon bacterial entry into a host cell. Listeriolysin O (LLO), a pore-forming hemolysin encoded by *hly*, is essential for vacuolar lysis in most cell types. However, in human epithelial cells, LLO$^-$ mutants are capable of growth, suggesting that gene products other than LLO are capable of mediating escape from a vacuole. In this study, we investigated the role of other bacterial gene products in lysis of the primary vacuole in the human epithelial cell line Henle 407. Double internal in-frame deletion mutants were constructed by introducing a mutated *hly* allele into strains harboring deletions in either of the phospholipase C (PLC)-encoding genes or a metalloprotease-encoding gene. Bacterial escape from the primary vacuole, intracellular growth, and cell-to-cell spread were evaluated in Henle 407 cells. The results indicated that, in the absence of LLO, the broad-range PLC and the metalloprotease were both required for lysis of the primary vacuole in Henle 407 cells. Although phosphatidylinositol-specific PLC was not required, the efficiency of escape was reduced in an LLO phosphatidylinositol-specific PLC double mutant. These observations suggest that the relative importance of LLO, the phospholipases, and the metalloprotease may vary in different cell types or in cells from different species. In addition, these studies provide insight into the mechanisms of action of virulence determinants involved in the lysis of vacuolar membranes.

Listeria monocytogenes is a rapidly growing, facultative intracellular bacterial pathogen. Escape from a primary vacuole and growth in the host cell cytosol are essential determinants of *L. monocytogenes* pathogenesis (9, 32). It is well documented that the secreted bacterial hemolysin, listeriolysin O (LLO), encoded by *hly* is largely responsible for mediating escape from the primary vacuole (1, 9, 26, 32). Consequently, mutants lacking LLO are unable to grow intracellularly in most cell types (4, 9, 19, 27) and are 5 logs less virulent in a murine model of infection (6, 10, 17, 25, 27).

L. monocytogenes secretes two phospholipases C (PLC), phosphatidylinositol-specific PLC (PI-PLC) encoded by *plcA* (3, 20, 23) and a broad-range PLC (PC-PLC, which is most active on phosphatidylcholine), encoded by *plcB* (8, 18, 33). PC-PLC is synthesized as an inactive precursor which is activated in broth culture by a secreted metalloprotease (Mpl) encoded by *mpl* (7, 24, 28, 29). Whereas PI-PLC has a very restrictive specificity (11, 14, 20), PC-PLC is capable of hydrolyzing all of the major mammalian cell phospholipids (12, 13). The phospholipases are believed to be involved in membrane lysis during infection and to share some functions (5, 30, 33). A recent study has shown that a mutant lacking both PLCs is 500-fold less virulent in a murine model of infection, forms very small plaques in murine L2 cells, and is significantly defective in escape from a primary vacuole in primary murine macrophages (30).

In 1988, we made the perplexing observation that nonhemolytic mutants of *L. monocytogenes* are still capable of growth in human epithelial cells (27), although it was not determined whether these mutants actually escaped from a vacuole or rather grew in a vacuole. Also, the precise nature of the mutations carried by these nonhemolytic strains was not known. In Fig. 1, an LLO$^-$ deletion mutant (16) is shown growing in the cytosol of Henle 407 cells, a human epithelial cell line. It is clear that the mutant is capable of escape into the cytosol as bacteria are surrounded by host actin filaments (Fig. 1c) and can be seen occasionally in pseudopod-like structures (Fig. 1a).

In this study, we investigated the role(s) of PI-PLC, PC-PLC, and Mpl in mediating the escape of *L. monocytogenes* from a vacuole in human epithelial cells in the absence of LLO. Mutant strains used in this study were constructed by introducing a previously described deletion *hly* allele (16) into strains already harboring deletions in either *plcA* or *plcB* as previously described (5, 30). The strain containing a deletion in *mpl* was constructed as part of this study and resulted in a mutant lacking approximately one-half of the structural gene including the putative active site of the Mpl. All of the mutants generated had internal in-frame gene deletions minimizing polar effects on downstream genes. In addition, to rule out the possible role of unlinked mutations, the wild-type allele was reinserted by allelic exchange in all deletion mutants which failed to grow in Henle 407 cells.

The strains listed in Table 1 were evaluated for growth in Henle 407 cells as previously described (21, 27). The single LLO$^-$ and double LLO$^-$ PI-PLC$^-$ mutants had intracellular growth rates similar to that of wild-type *L. monocytogenes* (Fig. 2a). In contrast, mutants lacking LLO and either PC-PLC or Mpl were defective for growth (Fig. 2a), and introduction of the wild-type *plcB* or *mpl* allele into the chromosome corrected the growth defect (22). In the presence of LLO, both PC-PLC and Mpl were dispensable for intracellular growth in Henle 407 cells (Fig. 2b) (30). The results were entirely consistent during three separate experiments.

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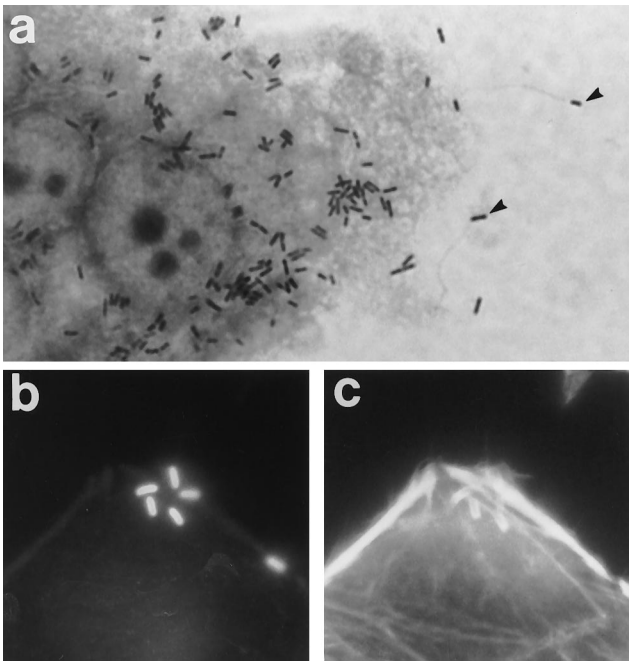

FIG. 1. Henle 407 cells infected with LLO$^-$ *L. monocytogenes* (DP-L2161). (a) Light micrograph showing bacteria in pseudopod-like structures (arrowheads) at 8.5 h postinfection. (b and c) Fluorescence micrographs of intracellular bacteria at 2 h postinfection. Bacteria were stained with an antiserum to *L. monocytogenes*, followed by a rhodamine-labeled secondary antibody (b), and double stained with fluorescein-phalloidin to identify intracytosolic bacteria coated with F-actin (c).

The formation of plaques in a confluent monolayer of cells reflects the ability of *L. monocytogenes* to spread from cell to cell. Accordingly, the mutants were evaluated for the ability to form plaques in Henle 407 cells. The assay was performed as described for L2 cells (21, 31). Evaluation of plaque size at 4 days postinfection further showed that LLO is not necessary for cell-to-cell spread in Henle 407 cells (Table 1). However, the total number of plaques was reduced by approximately 50% (22). It should be noted that plaques formed in Henle 407 cells were qualitatively inferior to those formed in L2 cells. The edges of the plaques were not as smooth, and the color contrast between the red-stained cells and the clear, unstained plaques was not as sharp as it is with L2 cells. For this reason, it was not possible to accurately measure the diameter of the small plaques formed by double phospholipase mutant DP-L1936.

The results presented in Fig. 2 and Table 1 indicate that both PC-PLC and Mpl were required for growth in Henle 407 cells in an LLO$^-$ background, presumably because the mutants were unable to escape from the primary vacuole. To precisely evaluate the relative contribution of each gene product to escape from the primary vacuole, the percentage of bacteria present in the cytosol 2 h after infection was estimated by counting bacteria surrounded by actin filaments, as nucleation of host actin around bacteria occurs shortly after entry into the cytosol. This was accomplished by performing triple immunofluorescence staining to distinguish adherent-extracellular bacteria from intracellular bacteria and intravacuolar bacteria from intracytosolic bacteria. Infected cells, fixed in formalin, were reacted with a guinea pig antiserum to *L. monocytogenes* before permeabilization of the cells and then reacted with a rabbit antiserum to *L. monocytogenes* after the cells were permeabilized with Triton X-100. Coumarin-labeled anti-guinea pig immunoglobulin G and rhodamine-labeled anti-rabbit immunoglobulin G were used as secondary antibodies. Intracytosolic bacteria were stained with fluorescein-phalloidin, which binds to F-actin, and the resulting differential staining was evaluated by immunofluorescence microscopy. Bacteria labeled with rhodamine but not with coumarin were considered intracellular, and those labeled with fluorescein and rhodamine were considered intracytosolic. The results showed that although the LLO$^-$ mutant was capable of growth in Henle 407 cells, the efficiency of escape from the primary vacuole was decreased twofold (Table 1). Furthermore, in the absence of LLO, PI-PLC was also required for efficient lysis of the vacuole. Incidentally, a role for PI-PLC in bacterial escape from a vacuole in murine macrophages has previously been reported (5, 30). Mutants which failed to grow in Henle 407 cells were also negative for F-actin staining, indicating that they are probably trapped within host cell vacuoles. Lastly, the double phospholipase mutant showed a much lower efficiency of escape than either of the single phospholipase mutants, emphasizing the overlapping functions of these phospholipases as recently reported (30).

The results of this study confirm that LLO$^-$ mutants can

TABLE 1. *L. monocytogenes* strains and relevant characteristics

Strain	Genotype	Avg plaque size in Henle 407 cells (%) ± SD[a]	Avg % escape from vacuole in Henle 407 cells ± SD (no. of assays)[b]	Reference or source of strain
10403S	Wild type	100	62 ± 8 (9)	2
DP-L1552	Δ*plcA*[c]	105 ± 6	60 ± 13 (5)	5
DP-L1935	Δ*plcB*	59 ± 6	48 ± 12 (6)	30
DP-L1936	Δ*plcA* Δ*plcB*	ND[d]	36 ± 10 (6)	30
DP-L2161	Δ*hly*	119 ± 12	30 ± 5 (4)	16
DP-L2296	Δ*mpl*	70 ± 1	53 ± 4 (6)	This study
DP-L2317	Δ*hly* Δ*plcA*	90 ± 12	16 ± 5 (4)	This study
DP-L2318	Δ*hly* Δ*plcB*	U[e]	0 ± 0 (4)	This study
DP-L2404	Δ*hly* Δ*mpl*	U	<1 ± 1 (4)	This study
DP-L2486	Δ*hly*[f]	117 ± 11	ND	This study
DP-L2585	Δ*hly*[g]	114 ± 9	ND	This study

[a] Relative percent diameter of plaques formed by mutant strains in comparison with those of wild-type strain. Plaques were measured at 4 days postinfection. Average results of four separate experiments are shown.

[b] Percentage of intracellular bacteria staining for F actin at 2 h postinfection. One hundred intracellular bacteria were counted per strain per assay.

[c] Δ, in-frame deletion in the structural gene.

[d] ND, not determined.

[e] U, undetectable.

[f] DP-L2404 in which a wild-type *mpl* gene copy was introduced by allelic exchange.

[g] DP-L2318 in which a wild-type *plcB* gene copy was introduced by allelic exchange.

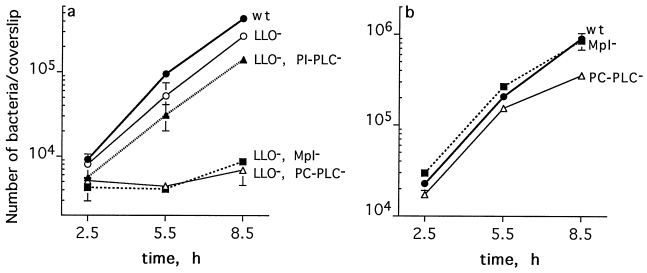

FIG. 2. Growth of *L. monocytogenes* strains in Henle 407 cells. Each datum point and error bar represents the mean number of viable bacteria recovered from three coverslips and the standard deviation. wt, wild type.

escape from the primary vacuole in human epithelial cells and further show that PC-PLC and Mpl are required. PC-PLC presumably acts by mediating hydrolysis of host vacuolar phospholipids, whereas the role of Mpl is less clear. The most obvious explanation is that Mpl is necessary to activate PC-PLC, as previously shown in broth culture (28). Alternatively, PC-PLC is activated by another protease and Mpl has a substrate distinct from PC-PLC. Thirdly, Mpl may activate PC-PLC and have another substrate(s) as well. We are unable to resolve which of these explanations is correct, although we favor the first. PI-PLC, although not required, does play a role and may act by a nonenzymatic mechanism, as recently proposed (15).

The observation that LLO is dispensable for escape from a primary vacuole in Henle 407 cells suggests that the nature of the primary vacuole of human epithelial cell lines is different from that of all of the murine cells evaluated. Therefore, it is possible that bacterial gene expression may be influenced by different environmental cues. Normally, PC-PLC cannot be detected in *L. monocytogenes* 10403S after growth in broth or in tissue culture medium (22). Perhaps in Henle 407 cells early synthesis of PC-PLC in the primary vacuole results in lysis. However, some strains of *L. monocytogenes* express PC-PLC in large amounts in broth culture (5, 17), yet in the absence of LLO, these overexpressing strains still fail to escape from a vacuole in murine macrophage cell lines (22). It is also possible that the primary vacuole of Henle 407 cells has an elevated pH compared with most other cells. PC-PLC has a broad pH range of activity (13), but Mpl may have a more restrictive pH range. However, the addition of weak bases such as NH_4Cl, which elevate the pH of vacuolar compartments, does not permit escape of LLO^- mutants from the primary vacuole in murine cells (22). Alternatively, vacuolar membranes may differ in susceptibility to PC-PLC, possibly because of the composition or accessibility of membrane phospholipids. Lastly, perhaps the requirement for LLO is bypassed in Henle 407 cells because of either a defect in the maturation of the vacuole into a phagolysosome or a difference in the permeability of the vacuolar membrane.

In a murine model of infection, it is well established that LLO is an essential determinant of *L. monocytogenes* pathogenicity required for escape from the primary vacuole. However, the formation of a pore in the vacuolar membrane may not be itself sufficient for disruption of the vacuole. The results of this study indicate that, in some cells, LLO is dispensable as long as PC-PLC is present. It is not unreasonable to conclude from these and other data (30) that the combination of LLO and PLCs is probably important, although their relative importance may vary in different cell types, in vivo as well as in vitro. We speculate that in a wild-type background, LLO acts first by introducing a pore into the vacuolar membrane. The presence of one or more pores affects the further maturation and content of the vacuole. This may have two consequences: (i) it affects further bacterial gene expression, and (ii) it affects the composition of the vacuole. Phospholipases, of either bacterial or host origin, may then be responsible for dissolution of the vacuolar membrane, acting by a nonenzymatic mechanism, as recently proposed (15), and/or by phospholipid hydrolysis.

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