**Brief Definitive Report**

**pH-dependent Perforation of Macrophage Phagosomes by Listeriolysin O from Listeria monocytogenes**

By Kathryn E. Beauregard,*† Kyung-Dall Lee,* R. John Collier,‡ and Joel A. Swanson*†

From the *D department of C cell Biology and 1D department of M microbiology and M ol ecular G enetics,
 Harvard Medical School, Boston, M assachusetts 02115

**Summary**

The pore-forming toxin listeriolysin O (LLO) is a major virulence factor implicated in escape of Listeria monocytogenes from phagocytic vacuoles. Here we describe the pH-dependence of vacuolar perforation by LLO, using the membrane-impermeant fluorophore 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) to monitor the pH and integrity of vacuoles in mouse bone marrow–derived macrophages. Perforation was observed when acidic vacuoles containing wild-type L. monocytogenes displayed sudden increases in pH and release of HPTS into the cytosol. These changes were not seen with LLO-deficient mutants. Perforation occurred at acid vacuolar pH (4.9–6.7) and was reduced in frequency or prevented completely when macrophages were treated with the lysosomotropic agents ammonium chloride or bafilomycin A1. We conclude that acidic pH facilitates LLO activity in vivo.

After entry into cells by phagocytosis, Listeria monocytogenes lysys its vacuole and escapes into the cytosol (1–3), a process that is important for its growth and cell-to-cell spread within a host (1, 4–6). A critical factor implicated in the escape of L. monocytogenes from vacuoles is listeriolysin O (LLO), a sulfhydryl-activated pore-forming toxin secreted by the bacterium (1, 7, 8). Mutants lacking LLO show decreased virulence in mice (9–14), and, in certain types of cultured cells, LLO-deficient mutants remain in vacuoles and do not proliferate (7, 9, 10). Furthermore, Bacillus subtilis strains expressing LLO are able to enter the cytosol of J774 cells, indicating that LLO is sufficient for this process (8).

The action of LLO may be triggered by vacuolar acidification (15, 16). Phagosomes of macrophages containing L. monocytogenes acidify (17), and LLO is more active at an acidic pH in vitro (15). Here we describe the relationship between pH and LLO activity inside L. monocytogenes-containing vacuoles. Including the pH-sensitive, membrane-impermeant dye 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS; reference 18) during macrophage infections permitted labeling of the lumen of endocytic vesicles and measurement of the pH and integrity of their membranes. This method allowed us to visualize perforation of L. monocytogenes-containing vacuoles and indicated a role for acidic vacuolar pH in the activity of LLO in vivo.

**Materials and Methods**

Reagents and Cells. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Molecular Probes (Eugene, OR). Mouse bone marrow–derived macrophages were obtained from the femurs of female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) and cultured in vitro as described (19). Strains of L. monocytogenes were kindly provided by Dr. Daniel Portnoy (University of Pennsylvania, Philadelphia, PA) and were grown to stationary phase in Luria-Bertani broth. Just before use, bacteria were washed three times in Ringer’s buffer (RB; 155 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 2 mM NaH2PO4, 10 mM Hepes, 10 mM glucose, pH 7.2). Fresh bacteria were prepared every 2 h.

Macrophage Infections. Macrophages were kept on a temperature-controlled stage set to 37°C, and coverslips containing 1.5–2.0 × 105 cells were incubated with 1 ml 5 mM HPTS in RB and 5–10 μl of a washed L. monocytogenes culture (~10 CFU/cell). The macrophages were incubated for up to 10 min, during which time an L. monocytogenes–infected cell was located. The coverslip was then washed six to seven times with RB, and pH recordings were begun immediately (time 0). Washes with RB were repeated as necessary to reduce background fluorescence.

To inhibit vacuole acidification, 10 mM NH4Cl was included in the first RB wash and all subsequent solutions. Alternatively, 0.5 μM bafilomycin A1 was added to the cells 30 min before infection and throughout the experiment.

Microscopy. Fluorescent images were collected on an IM-35 inverted microscope (Carl Zeiss Inc., Thornwood, NY) equipped for epifluorescence using an ×100 lens, numerical aperture 1.32. The fluorescence excitation system included a 50 W mercury arc lamp and filter changer (Stephen Baer, Photome, Cambridge, MA), which provided alternating 405 and 440 nm excitation wavelengths (exc.); emission was measured at 520 nm. Neutral density filters were sometimes added to reduce photodamage. Images were collected through a video camera, intensified through a multichannel plate intensifier (Video Scope Int. Ltd., Cambridge, MA).
Vacuolar Perforation by Wild-type L. monocytogenes. L. mono-
cytogenes were internalized into spacious phagosomes by a
process resembling macropinocytosis. This was different
from the reported mechanism of L. monocytogenes entry into
epithelial cells (21), but similar to the way Salmonella typh-
imurium enter macrophages (22). Immediately after phago-
cytosis, individual bacteria could be seen by phase-contrast
microscopy as phase-dense rods moving freely inside phase-
bright phagosomes; these phagosomes also contained HPTS
which was included during the infection (see Fig. 1, a, ves-
cicle 2; Fig. 2 a, vesicle 1; and Fig. 3 a, vesicle 3). No tight-fit-
ing phagosomes were observed, although older phagosomes
became smaller and restricted the movement of bacteria.

The pH of L. monocytogenes-containing vacuoles and of
phase-bright, fluorescent vacuoles that did not contain bac-
teria was monitored by collecting phase contrast and flu-
orescence images at regular intervals (Fig. 1). Fluorescent
images of HPTS in vacuoles were taken at exc. 405 and
440 nm. The ratio of fluorescence from these two record-
ings indicated the pH of the probe environment (18): bright
fluorescence at exc. 440 nm and dim fluorescence at exc. 405 nm indicated higher pH (>7.2), whereas bright
fluorescence at exc. 405 nm and dim fluorescence at 440
nm indicated low pH (<7.0).

Phagosomes began to acidify soon after phagocytosis,
with larger ones acidifying more slowly. A typical infection
with wild-type L. monocytogenes (Fig. 1), showed one or
more macropinosomes (Fig. 1, a–c, vesicle 1) and phago-
somes (Fig. 1, a–c, vesicle 2) labeled with HPTS. Whereas
the macropinosome remained acidic throughout the period
of observation, acidified phagosomes often showed a sud-
den increase of fluorescence at exc. 440 nm, reflecting an
increase in pH (Fig. 1, b and c, frame 3, vesicle 2). Some-
times a slow decrease in pH was followed by an abrupt alkali-

Figure 1. HPTS fluorescence indicates phagosomal pH and
membrane integrity. The mac-
roframe shown by phase contrast
microscopy (a) contained two
prominent phase-bright vesicles.
Vesicle 1 was a macropinosome
and vesicle 2 was a spacious pha-
gosome containing three bacteria.
Visible as phase-dense rods, the
fluorescence of HPTS contained
in these vesicles is shown for exc. 440
nm (b) and 405 nm (c). The four
frames in each series show images
taken at 30-s intervals, with the left-most image corresponding to the phase contrast image. At low pH, the fluorescence of HPTS is greater at exc. 440 nm than
at 440 nm; conversely, at neutral pH, fluorescence is greater at exc. 440 nm than at 405 nm. Hence, both vesicles appeared acidic in the early time points. Ves-
icle 1 remained acidic through the series, but vesicle 2 showed an abrupt increase in pH (b and c, third frame), followed by loss of dye (b and c, fourth frame).
A Requirement for LLO.

To determine the optimal pH for LLO activity in vivo, we measured the lowest pH achieved by each vacuole before perforation. Perforation occurred over a range of acidic pH values from 4.9 to 6.7, with a mean near 6.0 (mean = pH 5.94, SD = 0.45; Fig. 5), consistent with earlier suggestions that vacuole acidification creates optimal conditions for LLO activity in vivo (15–17).

There are conflicting reports as to whether inhibitors of vacuole acidification prevent L. monocytogenes from moving to the cytosol (5, 29, 30). To determine whether low pH was required for LLO to induce vacuolar perforation, macrophages were infected with wild-type L. monocytogenes in the presence of NH₄Cl, a weak base that raises the pH of acidic vesicles (31), or bafilomycin A₁, an inhibitor of vacuolar proton ATPases (32). In the presence of bafilomycin A₁, phagosomes maintained a steady, alkaline pH (Fig. 4 C). Vacular perforation, as monitored by leakage of the HPTS to the cytoplasm, was completely prevented by bafilomycin A₁ (0 out of 16; Table 1) and was less frequent in NH₄Cl (3 out of 16). Consistent with the results of Okhuma and Poole (33), 10 mM NH₄Cl elevated pH but did not completely prevent acidification of macrophage vacuoles. 65% of L. monocytogenes-containing vacuoles in NH₄Cl-treated cells were pH 7 or less, as opposed to 33% of vacuoles in bafilomycin A₁-treated cells. The vacuoles that showed perforation in the presence of NH₄Cl were within the range of pH at which perforation occurred without this compound. These results show that the level of acidification corresponded with the amount of LLO activity observed (46% for buffer, 19% for NH₄Cl, and 0% for bafilomycin A₁; Table 1). This is consistent with the fact that weak bases cause a slight decrease in intracellular growth of its pH (b and c, frame 4) before losing the dye (b and c, frame 5). Vesicle 2 contained L. monocytogenes; it was initially at a relatively high pH (b and c, frame 1). It then acidified (b and c, frame 2), became abruptly alkaline again (b and c, frame 3), and lost its dye (b and c, frame 4). Vesicle 3, another phagosome, was initially at high pH and remained so until it lost most of its dye.
L. monocytogenes (5, 29), as the number of bacteria that could escape to the cytosol was reduced in the presence of NH₄Cl. Although it is established that L. monocytogenes escapes its vacuole, the mechanism of this process remains ill-defined. Evidence presented here shows that one of the earliest detectable steps in vacuole lysis is perforation of the membrane, indicated by an abrupt increase in vacuolar pH and by leakage of its contents. These changes required LLO and acidic pH. We conclude that acidic pH facilitates LLO activity in vivo through an undefined mechanism.

Table 1. Vacuolar Perforation Requires LLO and Is Prevented by Raising pH

|          | WT * | DP-L2161 | DP-L2864 | WT + NH₄Cl | WT + bafilomycin A₁ |
|----------|------|----------|----------|------------|-------------------|
|          | 24/52| 0/19     | 0/10     | 3/16       | 0/16              |

The fractions shown here represent the number of vacuoles that scored positive for vacuole perforation over the total number of vacuoles observed for that particular condition.

*Wild-type L. monocytogenes

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Address correspondence to Dr. Joel A. Swanson, Department of Anatomy and Cell Biology, University of Michigan Medical School, 1335 Catherine Ave., Ann Arbor, MI 48109-0616. Phone: 313-647-6399; FAX: 313-763-1166; E-mail: jswan@umich.edu. K.-D. Lee’s current address is the College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.

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