JCB Comment

Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite

Shaynoor Dramsi and Pascale Cossart

Unité des Interactions Bactéries Cellules, Institut Pasteur, 75724 Paris Cedex 15, France

Cholesterol-dependent cytolysins (CDCs)* are produced by a large number of pathogenic gram–positive bacteria. A member of this family, listeriolysin O (LLO), is produced by the intracellular pathogen *Listeria monocytogenes*. A unique feature of LLO is its low optimal pH activity (~6) which permits escape of the bacterium from the phagosome into the host cell cytosol without damaging the plasma membrane of the infected cell. In a recent study (Glomski et al., 2002, this issue), Portnoy's group has addressed the molecular mechanism underlying the pH sensitivity of LLO. Unexpectedly, a single amino acid substitution in LLO L461T results in a molecule more active at neutral pH and promoting premature permeabilization of the infected cells, leading to attenuated virulence. This finding highlights how subtle changes in proteins can be exploited by bacterial pathogens to establish and maintain the integrity of their specific niches.

L. monocytogenes has emerged as a model system for the molecular study of intracellular parasitism. It can enter into a wide variety of cells by phagocytosis. Subsequent to entry into a host cell, *L. monocytogenes* lyses its vacuole and escapes into the cytosol, where it can multiply and spread from cell to cell (Cossart and Lecuit, 1998). There is overwhelming evidence that the primary *L. monocytogenes* determinant responsible for escape from a vacuole and thus entrance in the cytosol, two key events for virulence, is LLO, encoded by the *hly* gene. First, mutants lacking LLO fail to escape from a vacuole and are absolutely avirulent. Complementation with *hly* restores virulence (Cossart et al., 1989). Second, expression of LLO by *Bacillus subtilis* confers to these extracellular nonpathogenic bacteria the capacity to escape from a vacuole and grow in the cytosol (Bielecki et al., 1990). Third, purified LLO encapsulated into pH-sensitive liposomes can mediate dissolution of a vacuole (Lee et al., 1996).

Several members of the pore-forming CDCs family, formerly called thiol-activated toxins, have been characterized in detail. They include streptolysin O (SLO) of *Streptococcus pyogenes* and perfringolysin O (PFO) of *Clostridium perfringens*. The crystal structure of PFO has been solved recently (Fig. 1 and Rossjohn et al. [1997]) and a mechanism for membrane insertion has been proposed (Shatursky et al., 1999). Based on the high overall degree of similarity in the primary structure, all members of the family are thought to share a common mechanism of action that involves binding to cholesterol-containing membranes, followed by insertion, oligomerization of 20–80 monomers, and formation of a pore of 20–30-nm diameter. CDCs are composed of four domains of which the first three are involved in toxin oligomerization and membrane disruption and the fourth primarily in binding to the membranes. A highly conserved undecapeptide, ECTGLAWEWWR, in the fourth domain is essential for cytolytic activity. Substitution of the tryptophan residues for alanine in the undecapeptide of LLO strongly impairs its hemolytic activity (Michel et al., 1990). Interestingly, LSO, the hemolysin of the nonpathogenic *L. seeligeri*, which has a lower hemolytic activity, displays a phenylalanine in place of the alanine in this conserved motif (Ito et al., 2001).

The major known difference between LLO and other members of the family is that LLO has an optimum activity at acidic pH (Geoffroy et al., 1987). This raises the question of whether this unique trait has any physiological relevance in the infectious process. To start to address this question, PFO has been cloned and expressed in *L. monocytogenes* in place of LLO under the control of the endogenous *hly* promoter (Jones and Portnoy, 1994). PFO was able to mediate vacuolar escape at ~50% of the efficiency of LLO. However, after a small number of bacterial divisions in the cytosol, the host cell became permeabilized and died. Determination of virulence in mice showed that the strains expressing PFO were avirulent. These results indicate that LLO has evolved some specific properties to prevent cytotoxicity that are not shared by PFO, a protein from an extracellular pathogen. By selecting PFO-expressing *L. monocytogenes* "mutants" able to grow normally in the host cytosol, the same group identified three classes of PFO variants. The first class had a severe defect in hemolytic activity. The second class had a change in the optimal pH activity of PFO due to a single amino acid substitution L462F. The third class showed a

Address correspondence to Pascale Cossart, Unité des Interactions Bactéries Cellules, Institut Pasteur, 28 rue du Dr Roux, 75724, Paris cedex 15, France. Tel.: 33-1-4568-8841. Fax: 33-1-4568-8706. E-mail: pcossart@pasteur.fr

*Abbreviations used in this paper: CDC, cholesterol-dependent cytolysin; LLO, listeriolysin O; PFO, perfringolysin O; SLO, streptolysin O.

Key words: listeria; virulence; toxin; pH; phagosome

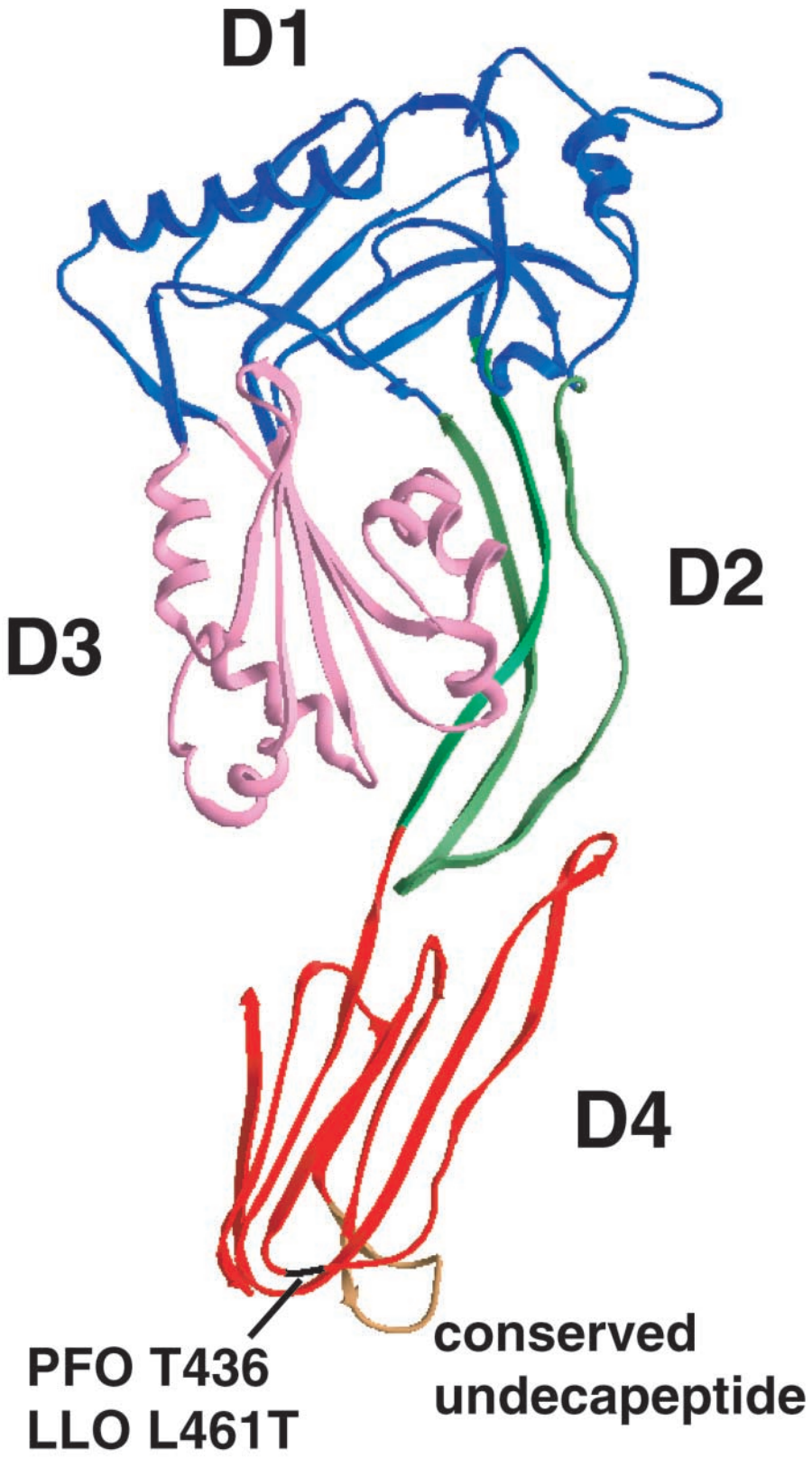

Figure 1. **Structure of perfringolysin with each domain in a different color (in brown the undecapeptide conserved in all CDCs).** The position of the amino acid critical for pH sensitivity is highlighted in black (adapted from Rossjohn et al., 1997).

decrease in PFO half-life in the host cytosol (Jones et al., 1996). These experiments strongly supported the idea that LLO has acquired a number of fail-safe mechanisms, including protein instability in the cytosol and a low optimal pH activity.

Protein instability

A recent study demonstrated that a PEST-like sequence present at the NH_2 terminus of LLO and absent in PFO is responsible for the rapid degradation of LLO in the host cell cytosol (Decatur and Portnoy, 2000). PEST-like sequences

are thought to target eukaryotic proteins for phosphorylation and degradation, and deletion or specific amino acid substitution of this sequence in LLO led to increased cytotoxicity and lower virulence in a mouse model. When the sequence was introduced in PFO and the chimeric toxin expressed in *L. monocytogenes*, bacteria were less toxic than those expressing wild-type PFO and were able to multiply intracellularly in J774 macrophages. Thus, introduction of a PEST motif in LLO is a strategy used by *L. monocytogenes* to restrict the activity of this powerful toxin to the host cell vacuole, thereby preserving the intracellular niche for bacterial multiplication. Intriguingly, another report (Lety et al., 2001) has also shown that *L. monocytogenes* mutants expressing a PEST-deleted *hly* allele, although fully hemolytic, are strongly impaired in virulence in a mouse model. However, in contrast to the previous report, in which J774 nonbactericidal macrophages were used, the authors of this second report used bone marrow–derived macrophages and showed that PEST-deleted or -substituted mutants were unable to escape from the phagocytic vacuole raising the interesting possibility that the PEST motif may play different roles in different cells.

Low optimal pH activity

Direct evidence for a link between the optimum activity at low pH and compartment-specific pore-forming activity of LLO has been provided by a recent study using a pH-sensitive and membrane-impermeant fluorophore 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS). It was shown that *L. monocytogenes* containing phagosomes (average pH ~5.9) rapidly acidify after bacterial uptake, followed by an increase in pH and dye release from the vacuole. Perforation of the vacuole was inhibited by lysosomotropic agents, such as ammonium chloride and bafilomycin A1 (Beauregard et al., 1997). Bafilomycin A1 was also shown to inhibit *L. monocytogenes* escape from the primary vacuole in epithelial cells (Conte et al., 1996). These experiments indicate that LLO activity is maximal in the phagosome lumen, requires this low pH for activity and leads to membrane disruption, increasing pH, and inactivation of LLO activity, an autoswitch process.

In their recent study, Glomski et al. (2002), by swapping dissimilar residues from the pH-insensitive ortholog perfringolysin O into LLO, identified leucine 461 of LLO as a key residue responsible for the low optimum pH activity of LLO. Changing this residue to threonine results in a molecule highly active at pH 7. If one assumes that LLO shares with PFO, a general structure similarity, the L461T mutation is located in the outer loop of the fourth domain (Fig. 1). That a single amino acid residue is sufficient to increase the activity at pH 7.0 is quite astonishing, although it is important to note that activity at pH 5.0 is also increased in the mutant. How this single amino acid change LLO L461T affects pH sensitivity thus awaits further biochemical analysis. Interestingly, the mutation that altered the optimum pH activity of PFO L462F described above was located in the conserved undecapeptide, which is very close to the region of the corresponding L461T mutation (Jones et al., 1996). It is particularly worth noting that while the LLO L461T mutant can efficiently permeabilize the host cell membrane from the cytosolic compartment, it only promotes efficient escape from the vacuolar compartment if this latter has been acidified, suggesting that additional bacterial or host factors activated by low pH are needed to act in concert with LLO to mediate escape from the phagosomal compartment. These findings could also reflect the difference in lipid composition of the internal versus external leaflets of the plasma membrane lipid bilayer, resulting in a difference in the behavior of LLO depending on the nature of the first leaflet with which it is in contact.

While it is clear that LLO is largely responsible for mediating escape from the vacuole in most cell lines, two *L. monocytogenes* PLCs also play a role. Mutants lacking both the broad-range PLC (PLCB) and a phosphatidylinositol-specific PLC (PLCA) escape from a macrophage vacuole at 50% of the efficiency of the wild-type. PLCb can even replace LLO in some cell lines, such as the human epithelial cell lines HeLa and Henle 407 (Vazquez-Boland et al., 2001).

Together, available data are consistent with the following model for vacuolar escape. Subsequent to internalization, LLO is secreted and binds to cholesterol-containing membranes. Optimal pore formation occurs between pH 5.5 and 6.0, the pH of an early endosome. One consequence of the pore formation is an elevation of the vacuolar pH, which may prevent vacuolar maturation, thus allowing the *L. monocytogenes* PLCs, perhaps in concert with additional host factors, to mediate vacuolar dissolution. LLO activity is then progressively switched off and degraded and bacterial multiplication can take place in the host cytosol. Meanwhile, LLO may have induced a series of signaling events since it is now well established that in addition to its role in escape from the phagosomal vacuole, LLO is one of the most potent *L. monocytogenes* signal-inducing molecules (Vazquez-Boland et al., 2001).

We sincerely acknowledge Rod Tweten for preparation of Fig. 1.

Submitted: 26 February 2002
Accepted: 27 February 2002

References

Beauregard, K.E., K.D. Lee, R.J. Collier, and J.A. Swanson. 1997. pH-dependent perforation of macrophage phagosomes by listeriolysin O from *Listeria monocytogenes*. *J. Exp. Med.* 186:1159–1163.

Bielecki, J., P. Youngman, P. Connelly, and D.A. Portnoy. 1990. *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. *Nature.* 345:175–176.

Conte, M.P., G. Petrone, C. Longhi, P. Valenti, R. Morelli, F. Superti, and L. Seganti. 1996. The effects of inhibitors of vacuolar acidification on the release of *Listeria monocytogenes* from phagosomes of Caco-2 cells. *J. Med. Microbiol.* 44:418–424.

Cossart, P., and M. Lecuit. 1998. Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. *EMBO J.* 17:3797–3806.

Cossart, P., M.F. Vicente, J. Mengaud, F. Baquero, D.J. Perez, and P. Berche. 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infect. Immun.* 57:3629–3636.

Decatur, A.L., and D.A. Portnoy. 2000. A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. *Science.* 290:992–995.

Geoffroy, C., J.L. Gaillard, J.E. Alouf, and P. Berche. 1987. Purification, charac-

terization and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes. Infect. Immun.* 55:1641–1646.

Glomski, I.J., M.M. Gedde, A.W. Tsang, J.A. Swanson, and D.A. Portnoy. 2002. The *L. monocytogenes* hemolysin has an acidic pH optimum to compartimentalize activity and prevent damage to infected host cells. *J. Cell Biol.* In press.

Ito, Y., I. Kawamura, C. Kohda, H. Baba, T. Kimoto, I. Watanabe, T. Nomura, and M. Mitsuyama. 2001. Difference in cholesterol-binding and cytolytic activities between listeriolysin O and seeligeriolysin O: a possible role of alanine residue in tryptophan-rich undecapeptide. *FEMS Microbiol. Lett.* 203: 185–189.

Jones, S., and D.A. Portnoy. 1994. Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. *Infect. Immun.* 62:5608–5613.

Jones, S., K. Preiter, and D.A. Portnoy. 1996. Conversion of an extracellular cytolysin into a phagosome-specific lysin which supports the growth of an intracellular pathogen. *Mol. Microbiol.* 21:1219–1225.

Lee, K.D., Y.K. Oh, D.A. Portnoy, and J.A. Swanson. 1996. Delivery of macromolecules into cytosol using liposomes containing hemolysin from *Listeria monocytogenes. J. Biol. Chem.* 271:7249–7252.

Lety, M.A., C. Frehel, I. Dubail, J.L. Beretti, S. Kayal, P. Berche, and A. Charbit. 2001. Identification of a PEST-like motif in listeriolysin O required for phagosomal escape and for virulence in *Listeria monocytogenes. Mol. Microbiol.* 39:1124–1139.

Michel, E., K.A. Reich, R. Favier, P. Berche, and P. Cossart. 1990. Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitutions in listeriolysin O. *Mol. Microbiol.* 4:2167–2178.

Rossjohn, J., S.C. Feil, W.J. McKinstry, R.K. Tweten, and M.W. Parker. 1997. Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell.* 89:685–692.

Shatursky, O., A.P. Heuck, L.A. Shepard, J. Rossjohn, M.W. Parker, A.E. Johnson, and R.K. Tweten. 1999. The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell.* 99:293–299.

Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14: 584–640.