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Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis

Andrew Camilli[†], Lewis G. Tilney[‡], and Daniel A. Portnoy^{*}

[†]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

[‡]Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018, USA

Summary

The *plcA* gene of *Listeria monocytogenes* encodes a secreted phosphatidylinositol-specific phospholipase C (PI-PLC). Recent studies have established that transposon mutations within *plcA* result in avirulence for mice and pleiotropic effects when examined in tissue-culture models of infection. Genetic analysis reveals that many of the effects of the transposon insertions are due to loss of readthrough transcription from *plcA* into the downstream gene *prfA*, which encodes an essential transcription factor of numerous *L. monocytogenes* virulence genes. Construction of an in-frame deletion within *plcA* had no effect on expression of *prfA* thus allowing direct assignment of a role of the PI-PLC in pathogenesis. PI-PLC was shown to play a significant role in mediating escape of *L. monocytogenes* from phagosomes of primary murine macrophages. Interestingly, this defect manifested itself *in vivo* in the liver but not in the spleen of infected mice.

Introduction

Listeria monocytogenes is a Gram-positive, food-borne human and animal pathogen responsible for serious infections in immunocompromised individuals and pregnant women (Seeliger, 1988). Murine listeriosis has been extensively studied as a model for infections in which antibody plays little or no role (Mackaness, 1962; Hahn and Kaufman, 1981; North, 1970). Immunity to *L. monocytogenes* requires prior administration of sublethal doses of live bacteria or, alternatively, adoptive transfer of immune CDS⁺ T-cells, although innate immune mechanisms are also crucial (Portnoy, 1992). Thus, murine listeriosis is an excellent system for the study of host–parasite interactions for infections which require the induction of cell-mediated immunity. During the past few years there has been a surge of interest in the pathogenesis of *L. monocytogenes* as a model intracellular parasite. Much of this interest reflects the observations that this bacterium enters the host cytoplasm and uses a host system of actin-based motility to mediate movement both within a cell and from cell to cell without leaving the cytoplasm (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Mounier *et al.*, 1990; Theriot *et al.*, 1992).

The cell biology of *L. monocytogenes* intracellular parasitism can be broadly divided into five stages, including (i) internalization, (ii) escape from a vacuole, (iii) intracellular growth, (iv) intracellular movement and pseudopod formation using host actin filaments, and (v)

^{*}For correspondence. Tel. (215) 898 8913; Fax (215) 898 9557.

cell-to-cell spread which proceeds through an intermediate double-membraned vacuole (Tilney and Portnoy. 1989). Several bacterial genes whose products are required for these processes have recently been identified using transposon mutagenesis and characterization of the resulting defects in tissue-culture models of infection (Portnoy *et al.*, 1992a). One region of the *L. monocytogenes* genome encodes a number of these virulence factors, including a pore-forming haemolysin, Listeriolysin O (LLO), which is largely responsible for lysis of the initial vacuole; two distinct phospholipases C including one, PI-PLC, specific for phosphatidylinositol and the other, PC-PLC, having a broad substrate specificity that includes phosphatidyl-choline (PC); a metalloprotease, Mpl, which apparently can activate PC-PLC; ActA, a surface protein required for actin assembly; and lastly, PrfA, which appears to be a specific transcription factor required for the regulated expression of genes for the above-mentioned proteins (Portnoy *et al.*, 1992a). The precise functions, sites of action, and regulation of these determinants of pathogenicity are current topics of investigation.

During a screen for *L. monocytogenes* transposon mutants which formed small plaques in tissue-culture monolayers, we identified a mutant which had suffered an insertion within the structural gene encoding PI-PLC (Sun *et al.*, 1990). The mutant was avirulent, was delayed in escape from host vacuoles and, within the cytoplasm, had a severe defect in cell-to-cell spread (Sun *et al.*, 1990; Camilli *et al.*, 1991). The defects of this mutant were due to either loss of PI-PLC activity and/or a polar effect on expression of the downstream *prfA* gene. Using a genetic approach, we now show that readthrough transcription of *prfA* from the *plcA* gene promoter is necessary for expression of genes required for cell-to-cell spread. We also show that PI-PLC is required for efficient escape of *L. monocytogenes* from host-cell vacuoles and for wild-type levels of growth in the livers of infected mice.

Results

Genetic complementation of *plcA*::Tn917-LTV3 mutation

L. monocytogenes DP-L1054 contains a Tn917-LTV3 insertion in *plcA* (see Fig. 1A, vertical arrow), fails to secrete PI-PLC activity, is defective for cell-to-cell spread and consequently produces small plaques in tissue-culture monolayers, and is avirulent (Camilli *et al.*, 1991; Sun *et al.*, 1990). Transformation of DP-L1054 with a plasmid containing *plcA* under the expression of its own promoter (Fig. 1B, strain DP-L1341) resulted in a ninefold higher level of secreted PI-PLC activity than that of the wild-type strain (Table 1). The increase in PI-PLC activity over wild-type levels was probably due to an increased gene dosage of *plcA* on the multicopy plasmid (Wirth *et al.*, 1986), whose replication functions have been reported to maintain a copy number of approximately 10 in *Streptococcus sanguis* (Behnke and Gilmore, 1981). However, possession of this plasmid (by DP-L1054) failed to complement the defect in cell-to-cell spread as determined by measuring plaque size in L2 fibroblast monolayers (Fig. 2). In contrast, transformation of DP-L1054 with a plasmid containing *plcA* and the immediate downstream gene *prfA* (Fig. 1B, strain DP-L1480) resulted in complementation of PI-PLC activity to a level 73 times that of the wild type (Table 1) and partially restored the wild-type plaque phenotype (Fig. 2). Surprisingly, transformation of DP-L1054 with a plasmid containing only *prfA* (Fig. 1B, strain DP-L1530) complemented plaque formation to nearly wild-type levels (Fig. 2), although it was

unable to complement for loss of PI-PLC expression (Table 1). Thus, only plasmids containing *prfA* could complement the defect in cell-to-cell spread, suggesting that the defective cell-to-cell spread by DP-L1054 was due to loss of *prfA* expression and not loss of PI-PLC secretion. The most likely explanation for this was that the transposon insertion in DP-L1054 was exerting a polar effect on expression of the downstream *prfA* gene.

The observation that the complemented strain containing both *plcA* and *prfA* (DP-L1480) made smaller plaques than that containing *prfA* alone (DP-L1530) (see Fig. 2) may have been due to the presence of the *plcA* promoter in multicopy in the former strain. To test this hypothesis, we transformed the wild-type strain 10403S with a plasmid containing a 616 bp fragment that included the *plcA* promoter, to generate DP-L1503. DP-L1503 formed smaller plaques than 10403S or 10403S transformed with vector alone (strain DP-L1483) (Fig. 2), suggesting that the presence of the *plcA* promoter in multicopy was slightly deleterious for cell-to-cell spread of *L. monocytogenes*. The most likely explanation for this effect was titration of PrfA by the palindromic DNA site recognized by PrfA (Freitag *et al.* 1992) which was present in the *plcA* promoter on the multicopy plasmid.

Loss of readthrough transcription in the transposon-containing strain DP-L1054 was difficult to confirm using Northern blot analysis because of low levels of transcription of *plcA* in the 10403S genetic background. However, we were able to show loss of readthrough transcription from *plcA* into *prfA* in another *L. monocytogenes* genetic background, the hyper-haemolytic isolate SLCC 5764, which exhibited significantly higher levels of *plcA* and *prfA* transcription during *in vitro* growth (compare lanes 1 and 5 in Fig. 3). In wild-type *L. monocytogenes* *plcA* and *prfA* are both transcribed monocistronically as RNA transcripts of approximately 1.1, 0.8 and 0.9 kb, respectively, as well as together in a bicistronic RNA transcript of approximately 2.0 kb (Fig. 3, lane 1) (Leimeister-Wachter *et al.*, 1992; Mengaud *et al.*, 1991). However, the bicistronic RNA transcript was absent in DP-L1387 (Fig. 3, lane 2), which has a Tn917-LTV3 insertion in the extreme 3' end of *plcA* such that the monocistronic RNA transcript was not noticeably reduced in mobility (a predicted loss of approximately 93 bases from the 3' end). Note that monocistronic transcription of *prfA* was unaffected in DP-L1387 (Fig. 3, lane 2). In contrast, monocistronic transcription of *plcA* was greatly reduced (Fig. 3, lane 2), suggesting that readthrough transcription of *prfA* contributes significantly to PrfA levels (PrfA activates transcription from the *plcA* promoter). The loss of secreted PI-PLC activity by DP-L1387 (Table 1) was not due to the greatly reduced transcription of *plcA* (Fig. 3, lane 2) but instead was due to truncation of the C-terminal 7 amino acids of the PI-PLC polypeptide caused by the transposon insertion (data not shown). The presence of the bicistronic RNA transcript in 10403S and its corresponding absence in DP-L1054 (*plcA*: Tn-917-LTV3) were confirmed in separate Northern blot experiments after longer autoradiogram exposure times (data not shown).

Loss of readthrough transcription of *prfA* by intergenic disruption results in small plaque formation

To directly test whether loss of readthrough transcription of *prfA* affects cell-to-cell spread, a plasmid having temperature-sensitive replication properties was integrated into the *plcA*–*prfA* intergenic region on the chromosome of wild-type *L. monocytogenes* to act, in effect,

as a transcriptional terminator (Fig. 4). Northern blot analysis confirmed the loss of readthrough transcription in the resulting mutant strain, DP-L1510 (data not shown), and also demonstrated the unaffected monocistronic transcription of both *plcA* and *prfA* via their respective promoters (Fig. 3, lane 7). The same plasmid integration mutation made in the SLCC 5764 background more clearly demonstrates loss of the bicistronic RNA transcript (Fig. 3, lane 3). In addition, DP-L1510 secreted wild-type levels of PI-PLC and PrfA-induced haemolytic activity into culture supernatants (Table 1), further suggesting that the plasmid integration did not noticeably affect expression of the adjacent genes in the 10403S genetic background *in vitro*. However, *in vivo*, DP-L1510 was defective for cell-to-cell spread forming plaques in L2 cell monolayers that measured less than 25% of wild-type diameter (Fig. 5). As controls, two random integration strains, DP L1532 and DP-L1534, formed medium-sized plaques measuring 62% and 69% of wild-type plaque diameter, respectively (Fig. 5). Thus, readthrough transcription from *plcA* into *prfA* was required for large plaque formation in tissue-culture cells. The medium-sized plaques formed after random integration of the plasmids were consistent with our observations that *L. monocytogenes* strains containing integrated temperature-sensitive plasmids are somewhat defective for cell-to-cell spread (A. Camilli and D. A. Portnoy, unpublished).

Disruption of *plcA* by internal deletion does not affect plaque size

To independently confirm that PI-PLC was not required for cell-to-cell spread, we developed a method using allelic exchange to generate a strain containing an internal, in-frame, deletion in *plcA* (Fig. 6) to abolish PI-PLC expression but maintain readthrough transcription of *prfA*. The resulting strain, DP-L1552, had no detectable PI-PLC activity in culture supernatants (Table 1), secreted wild-type levels of haemolytic activity (Table 1) and, transcribed wild-type levels of the now truncated monocistronic and bicistronic RNA transcripts (Fig. 3, lanes 4 and 8), i.e., due to the internal *plcA*. As shown in Fig. 2, DP-L1552 made large plaques indistinguishable from those of the wild-type strain thus confirming that (i) PI-PLC plays no apparent role in cell-to-cell spread, and (ii) that readthrough transcription of *prfA* from the upstream *plcA* gene promoter plays a major quantitative role in plaque formation. Hence, loss of readthrough transcription of *prfA* in the original transposon mutant strain, DP-L1054, appears to have been entirely responsible for its defective cell-to-cell spread.

Loss of readthrough transcription of *prfA* in *L. monocytogenes* SLCC 5764 results in reduced secretion of several polypeptides *in vitro*

In addition to *plcA*, at least four other linked *L. monocytogenes* genes encode secreted proteins including the structural genes for LLO, PC-PLC, Mpl, and ActA (Mengaud *et al.*, 1989; Vazquez-Boland *et al.*, 1992; Domann *et al.*, 1992). The *prfA* gene product has recently been shown to be necessary for transcriptional activation of all five of these linked genes, and perhaps others (Freitag *et al.*, 1992; Chakraborty *et al.*, 1992; Mengaud *et al.*, 1991). The experiments described in the sections above demonstrated that readthrough transcription of *prfA* was required for cell-to-cell spread. Accordingly, the defect in cell-to-cell spread may have resulted from reduced expression of *prfA*-activated genes. However, strains DP-L1054 and DP-L1510, in which readthrough transcription was blocked, still expressed wild-type levels of the *prfA*-activated gene product LLO *in vitro* (Table 1 and Sun

et al., 1990) and had secreted protein profiles indistinguishable from that of the wild-type strain (data not shown). Thus, the polar effect was manifested while inside host cells but had no measurable effect on secreted levels of *prfA*-induced polypeptides *in vitro*.

The majority of *L. monocytogenes* clinical isolates resemble 10403S in the levels of secreted proteins *in vitro*. However, a minority of isolates such as SLCC 5764 exhibit a hypersecreting phenotype that includes high levels of secretion of the PrfA-induced gene products LLO, PC-PLC, PI-PLC, and ActA (A. Camilli and D. A. Portnoy, unpublished). Although not proven, the hypersecretory phenotype of SLCC 5764 *in vitro* may approximate the *in vivo* behaviour of 10403S at an intracellular stage of infection with respect to secreted levels of PrfA-induced proteins. Given the technical difficulties of assessing the levels of protein secretion of 10403S and derivative strains when intracellular, we therefore examined whether blocking readthrough transcription of *prfA* in SLCC 5764 would result in reduced secretion of *prfA*-regulated proteins *in vitro*. A SLCC 5764 derivative strain, DP-L1387, containing a Tn917-LTV3 insertion in *plcA*, had readthrough transcription blocked (Fig. 3, lane 2). This mutant strain secreted into culture supernatants only 6% and 62% of the wild-type levels of PC-PLC and haemolysin activities, respectively (Table 1), and accordingly showed reduced amounts of PC-PLC and LLO polypeptides on an SDS-PAGE gel of secreted proteins (compare lanes B and D in Fig. 7 and see legend). In addition to LLO and PC-PLC, at least six other polypeptide species were present in reduced amounts compared to the parental strain or to DP-L1553 which had an inframe internal deletion in *plcA* (Fig. 7, lane C). Furthermore, DP-L1535, which had readthrough transcription blocked by integration of a temperature-sensitive plasmid into the *plcA-prfA* intergenic region, had a secreted protein profile similar to that of DP-L1387 and, in addition, showed reduced secretion of PI-PLC (Table 1; Fig. 7, lane E and legend), while a random integration control strain, DP-L1536, had essentially a wild-type profile (Fig. 7, lane F). Thus, readthrough transcription of *prfA* does occur *in vitro* in the hypersecreting strain SLCC 5764 and was necessary for full expression of several secreted polypeptides, some of which represent known virulence factors.

PI-PLC is necessary for full virulence in mice

The small plaque mutant DP-L1054 (*plcA*: Tn917-LTV3) was previously reported to have a 50% lethal dose (LD₅₀) in mice three logs higher than that of the wild-type strain (Camilli *et al.*, 1991. and Table 1). Given the large plaque phenotype of the *plcA* strain, DP-L1552 (Fig. 2), we predicted that its LD₅₀ would be close to that of the wild-type strain. Indeed, the LD₅₀ for DP-L1552 was only two- to fourfold higher than that of 10403S (Table 1). Recall that DP-L1552 had no detectable secretion of PI-PLC but was competent for readthrough transcription from *plcA* into *prfA*.

To determine whether or not the reduced virulence of DP-L1552 in mice was due to a decreased ability to multiply within the murine host, we determined the ratios of 10403S to DP-L1552 in the livers and spleens of mice that had been simultaneously co-infected with equal numbers of the two strains 48 h previously (see *Experimental procedures*). It is known that ~90% of intravenously administered *L. monocytogenes* are immediately sequestered in the livers and ~10% in the spleens of mice and that these organs are the primary sites of

subsequent bacterial multiplication during murine listeriosis (Mandel and Cheers, 1980). The ratios of 10403S to DP-L1552 in the spleens were not significantly different from a value of 1 ($P>0.5$) (see Fig. 8, closed circles). However, in the livers the ratios of 10403S to DP-L1552 were greater than 1 ($P<0.001$) with a mean ratio of 33 (Fig. 8, open circles). Thus, although PI-PLC was not required for growth *in vitro* (DP-L1552 exhibited growth kinetics identical to wild-type in rich medium; data not shown) or in the spleens of mice, this secreted factor was required for wild-type levels of net bacterial growth in the murine liver following intravenous infection.

PI-PLC is necessary for wild-type growth in primary cultures of murine macrophages

DP-L1552 (*plcA*) formed plaques whose sizes were almost indistinguishable from those of the wild-type strain in L2 cell monolayers (Fig. 2), suggesting that PI-PLC was not required for intracellular growth or cell-to-cell spread in this fibroblast cell line. However, it remained possible that PI-PLC would play a role in survival and/or growth within macrophages, which are the first host cells to encounter *L. monocytogenes* after i.v. infection. Therefore, we compared the growth of the wild-type strain 10403S with that of its isogenic derivative DP-L1552 in both established and primary cultures of murine macrophages. As previously reported (Portnoy *et al.*, 1988; Sun *et al.*, 1990) 10403S and DP-L1054 (*plcA*: Tn917-LTV3) grew logarithmically in the J774 macrophage-like cell line while a non-haemolytic derivative, DP-L1044, incapable of lysing the phagosomal membrane (Sun *et al.*, 1990), did not grow (Fig. 9A). The growth of DP-L1552 was similar to that of 10403S in this cell line, confirming that PI-PLC was not necessary for growth in J774 cells.

We next tested bacterial growth in the more stringent cell types: peritoneal- and bone marrow-derived macrophages. In contrast to the J774 cell-line, these primary cell populations are capable of killing *L. monocytogenes* in tissue culture (Fig. 9B and C; Portnoy *et al.*, 1988; 1989) and thus are thought to have more physiological relevance to *in vivo* macrophage populations. DP-L1054, DP-L1044, and DP-L1552 were continually killed in peritoneal macrophages while 10403S showed limited growth after 3 h (Fig. 9B). Light-microscopic examination of the 10403S-infected peritoneal macrophage monolayers 8h after infection (data not shown) revealed occasional foci of infection resulting from bacterial multiplication and cell-to-cell spread. In contrast, no foci of infection were found for the negative control strain DP-L1044 which is non-haemolytic and thus incapable of escaping from host vacuoles (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989). Strains DP-L1054 and DP-L1552, despite net killing over the course of the experiment, made rare foci of infection. Although in DP-L1054 and DP-L1552 foci occurred less frequently than in 10403S-infected monolayers, they were, nonetheless, identical in appearance to wild-type foci with respect to the number of bacteria and extent of their cell-to-cell spread which, for DP-L1552, suggested that PI-PLC was not important for events subsequent to escape from the initial host cell phagosome. In bone marrow-derived macrophage cultures all strains tested except for the non-haemolytic strain DP-L1044 showed net growth (Fig. 9C). Again, microscopic examination of infected monolayers revealed less frequent foci of infection for both DP-L1054 and DP-L1552 compared to 10403S infected monolayers, i.e. 9.5% and 38% of the foci per unit area for 10403S-infected monolayers, respectively (data not shown). The reduced net growth and less frequent foci of infection formed by DP-L1552 compared to

10403S in peritoneal and bone marrow-derived macrophages was consistently seen in multiple experiments (data not shown).

The possibility existed that an unlinked spontaneous mutation had occurred during the construction of DP-L1552 which was responsible for defective intracellular growth in macrophages. To test for this possibility a revertant of DP-L1552 was constructed using allelic exchange to replace the *plcA* allele with the wild-type *plcA* allele (essentially the reverse of the method shown schematically in Fig. 6). The revertant strain secreted wild-type levels of PI-PLC into culture supernatants and exhibited wild-type intracellular growth in peritoneal-derived macrophages (data not shown), thus confirming that the *plcA* was responsible for loss of PI-PLC expression and defective intracellular growth of DP-L1552.

PI-PLC is required for efficient lysis of host vacuoles in bone marrow-derived macrophages

The reduced frequency of infectious foci for DP-L1552 compared to 10403S-infected peritoneal- and bone marrow-derived macrophages was not due to reduced invasiveness, as the number of intracellular bacteria at early time points was essentially identical for all strains tested (Fig. 9, A, B and C). It was possible that inefficient lysis of phagosome membranes by DP-L1552 led to increased residence times within the potentially harsh phagosomal environment thus resulting in increased killing of the bacteria. Accordingly, inefficient escape from phagosomes may have been responsible for the reduced number of infectious foci seen for DP-L1552 versus wild-type- infected monolayers. We tested this hypothesis by direct quantification of membrane-bound versus cytoplasmic bacteria after infecting bone marrow-derived macrophages with 10403S or DP-L1552. Peritoneal-derived macrophages were not used for this experiment because the number of cytoplasmic bacteria was too low (because of the extensive killing of *L. monocytogenes* by these cells; Fig. 9B) to allow electron-microscopic quantification of significant numbers. Both stationary and log-phase cultures of *L. monocytogenes*, representing two different bacterial physiological states which may occur *in vivo* during infection, were used to infect bone marrow-derived macrophages in separate experiments. Electron-microscopic examination of infected monolayers fixed *in situ* clearly revealed the presence or absence of a vacuolar membrane surrounding the intracellular bacteria (Fig. 10). At 1.5 h after identical infections with stationary-phase bacteria, 7.5% of wild-type bacteria had lysed the phagosomal membrane and were found free in the cytoplasm while only one DP-L1552 bacterium (0.4%) was found free in the cytoplasm (Table 2). At 1.5h after identical infections with log-phase bacteria, 44% of wild-type bacteria had lysed the phagosomal membrane and were found free in the cytoplasm while only 15% of DP-L1552 bacteria were found free in the cytoplasm (Table 2). Thus, PI-PLC was necessary for efficient lysis of the phagosomal membrane in primary cultures of bone marrow-derived macrophages. In addition, log-phase *L. monocytogenes* appears to escape the phagosomal compartment of bone marrow-derived macrophages more efficiently than stationary-phase bacteria.

Electron micrographs of cytoplasmic 10403S or DP-L1552, 4 h after infection, clearly revealed the presence of electron-dense actin tails trailing the bacteria (Fig. 10, E and F) (Tilney and Portnoy, 1989), suggesting that loss of PI-PLC did not affect actin assembly. In

addition, video microscopy of infected PTK2 cells revealed no measurable difference in intracellular movement of DP-L1552 versus wild type (J. Theriot and D. A. Portnoy, unpublished).

Discussion

The data presented here show that the *L. monocytogenes plcA* gene plays a dual role in pathogenesis. Perhaps its most critical role is in regulation, as transcripts which initiate at the *plcA* promoter and read through a putative terminator (Mengaud *et al.*, 1989) *in vivo* are required for full expression of the positive regulatory factor PrfA which, in turn, regulates genes necessary for efficient cell-to-cell spread. A second role of *plcA* is to encode PI-PLC which is required for bacterial growth in the murine liver, which may reflect its role in lysis of macrophage phagosomes. Thus, *L. monocytogenes* has adopted a strategy for coupling the expression of one virulence factor to that of a positive regulatory factor which is necessary for expression of other genes.

plcA provides readthrough transcription of *prfA* which is required for cell-to-cell spread

In a previous study, we found that a *L. monocytogenes plcA*:Tn917-LTV3 mutant (DP-L1054) was defective in the nucleation of host actin filaments and showed impaired cell-to-cell spread (Sun *et al.*, 1990). Since the results of the present study show that PI-PLC has no apparent role in cell-to-cell spread, we suggest that readthrough transcription of *prfA* occurs in the host cell cytoplasm and is required for actin-based motility and cell-to-cell spread. In this respect, an intact *actA* gene has recently been shown to be required for bacterial-mediated nucleation of host actin (Kocks *et al.*, 1992; Domann *et al.*, 1992) and requires *prfA* for its transcription activation (Mengaud *et al.*, 1991; Chakraborty *et al.*, 1992). Thus, it is likely that *actA* is one of the downstream genes not properly expressed in loss of *prfA* readthrough transcription mutant strains. Indeed, DP-L1054 makes greatly reduced levels of ActA in infected J774 cells (R. A. Brundage and D. A. Portnoy, unpublished). Our data showing reduced secretion of at least eight polypeptides after blocking of readthrough transcription in a hypersecreting *L. monocytogenes* strain suggests that PrfA also regulates expression of other bacterial factors which may interact with host molecules at other stages of intracellular parasitism. Indeed, four of the eight polypeptides of reduced expression are known virulence factors. These include LLO, which is required for lysis of the host phagosome, two forms of PC-PLC, which are required for efficient escape from the double-membraned vacuole formed after spreading cell-to-cell, and PI-PLC (Vazquez-Boland *et al.*, 1992), which we now show plays a role in lysis of the host phagosome formed after the initial invasion event. Our data showing reduced expression of the above-mentioned virulence factors are consistent with previous reports indicating that PrfA activates transcription of *plcA*, *hly*, *actA*, and *plcB* (Chakraborty *et al.*, 1992; Mengaud *et al.*, 1991; Freitag *et al.*, 1992). Interestingly, the differing levels of reduction of PC-PLC versus LLO expression after blocking of readthrough transcription of *prfA* (94% and 38% reduction compared with control strain activities, respectively) suggest a model of differential regulation for these two determinants *in vitro* and perhaps *in vivo*.

Readthrough transcription of a gene that encodes a positive transcription factor of the upstream gene as well as other genes involved in virulence was, to our knowledge, unprecedented in bacteria. In addition to the promoter provided by the *plcA* gene, *prfA* has its own two promoters immediately upstream of the PrfA coding sequence, providing other possible levels of PrfA expression *in vivo* (Mengaud *et al.*, 1991; N. E. Freitag and D. A. Portnoy, unpublished). The relative contributions of these three promoters in pathogenesis are currently under investigation. It is likely that the expression of *prfA* is highly regulated via all three promoters to allow precise control over the levels of PrfA in the bacterial cell during various stages of intracellular parasitism. Readthrough transcription may be one mechanism that *L. monocytogenes* uses to temporally regulate PrfA levels during infection.

Role of PI-PLC in pathogenesis

Our studies show that PI-PLC is required for full virulence of *L. monocytogenes* in a murine model of infection. Interestingly, PI-PLC was necessary for growth in the liver but not the spleen. In the liver, resident macrophages (Kupffer cells) ingest the majority of intravenously injected *L. monocytogenes* (Lepay *et al.*, 1985). The surviving bacteria spread into hepatocytes and grow logarithmically until host defence mechanisms are sufficiently mobilized to limit bacterial growth (Rosen *et al.*, 1989). The precise role of PI-PLC *in vivo* is not known, but our results using bone marrow-derived macrophages suggest that PI-PLC plays a role in mediating efficient lysis of host phagosomes. Why PI-PLC has no apparent role in the spleen remains a mystery. In contrast to the liver, innate immunity to *L. monocytogenes* in the spleen is rather ineffective, as shown by a lack of bacterial killing in the spleen during the first hours of infection (Havell, 1989). Thus, the lack of a demonstrable role for PI-PLC in growth within the spleen may simply reflect a lack of efficient bactericidal properties of spleen cells harbouring *L. monocytogenes*. Specifically, delayed escape from phagosomes by PI-PLC-deficient *L. monocytogenes* may be tolerated in spleen cells perhaps because of inefficient listericidal activity of the phagosome compartments. This may reflect a different population of cells infected by *L. monocytogenes* in the spleen from that in the liver, and/or a different cellular environment.

What are the host target molecules of *L. monocytogenes* PI-PLC? Our results showing that PI-PLC was required for efficient bacterial escape from phagosomes in primary cultures of bone marrow-derived macrophages strongly suggests that PI-PLC may hydrolyse host PI and/or glycosyl-phosphatidylinositol (GPI)-anchored proteins within the extracytoplasmic leaflet of the phagosomal membrane to facilitate escape into the cytoplasm. Goldfine and Knob (1992) have purified the *L. monocytogenes* PI-PLC to homogeneity and found that it specifically hydrolyses PI with high activity levels extending into the acidic pH range. In addition, LLO haemolytic activity has been found to have a low pH optimum (Geoffroy *et al.*, 1987; Portnoy *et al.*, 1992b). Thus, it is likely that PI-PLC may act in concert with LLO, possibly within acidified vacuoles of the host cell to mediate lysis of the vacuolar membrane.

The precise molecular events mediated by LLO and PI-PLC to cause lysis of host vacuoles are not known. LLO is a pore-forming protein which requires cholesterol as a membrane receptor (Smyth and Duncan, 1978; Geoffroy *et al.*, 1987). Cholesterol is usually equally

distributed between the cytoplasmic and extracytoplasmic leaflets of eukaryotic plasma cell membranes (Fisher, 1976). In contrast, PI is predominantly found on the cytoplasmic leaflet but can be found in the extracytoplasmic leaflet in large quantities in some cell types such as hepatocytes (Higgins *et al.*, 1989). In addition, PI can be found in the extra-cytoplasmic leaflet in small quantities as lipid anchors for GPI-linked proteins (Cross, 1990). Interestingly, Ikezawa and Taguchi (Ikezawa and Taguchi, 1981) have reported that erythrocytes treated with purified *Bacillus cereus* PI-PLC are more sensitive to osmotic lysis. It is conceivable that *L. monocytogenes* PI-PLC may function similarly on phagosome membranes within other cell types of haematopoietic origin in order to increase their sensitivity to lysis by LLO. In addition, PI-PLC may at some stage of lysis gain access to and hydrolyse PI within the cytoplasmic leaflet to facilitate dissolution of the phagosomal membrane. Whatever its role(s) may be, PI-PLC is not absolutely required for lysis of the phagosomal membrane, as PI-PLC-deficient *L. monocytogenes* can escape to the cytoplasm in all tissue-culture cells examined thus far (D. A. Portnoy, unpublished). In addition to PI-PLC, *L. monocytogenes* secretes a broad-substrate PLC (PC-PLC) which may function redundantly with PI-PLC to mediate lysis of phagosomal membranes in concert with LLO (see below).

The importance of PI-PLC, as well as LLO, in escape from the double-membraned vacuoles formed during cell-to-cell spread is not known. The presence of two host membranes (inverted with respect to each other) surrounding the bacteria may necessitate two distinct mechanisms for sequential membrane lysis. A *L. monocytogenes* PC-PLC mutant strain has been shown to be partially defective in escape from these double-membraned vacuoles, suggesting that PC-PLC plays a role in lysis of the inner membrane (Vazquez-Boland *et al.*, 1992). PI-PLC-deficient *L. monocytogenes* appears to have little or no defect in cell-to-cell spread in tissue-culture. However, recent studies using *plcA plcB* single and double mutant strains have shown a major role for these two PLCs in production of wild-type size plaques, i.e. a synergistic reduction of plaque size was noted for the double mutant strain versus either single mutant strain alone (G. A. Smith and D. A. Portnoy, unpublished). Thus, PI-PLC and PC-PLC appear to have redundant functions.

Given that *L. monocytogenes* is an oral pathogen that must cross through the intestinal epithelial barrier, it is possible that PI-PLC may act at other stages of infection that were bypassed in the present studies using intravenous infections. In this respect, it is known that GPI-anchored proteins are found exclusively on the apical surface of intestinal epithelial cells (Lisanti *et al.*, 1990). Therefore, *L. monocytogenes* PI-PLC may act on intestinal epithelia to somehow promote survival and/or invasion. However, it has recently been found that the purified *L. monocytogenes* PI-PLC has relatively little activity on eukaryotic GPI-anchored proteins relative to other bacterial PI-PLCs (H. Goldfine, personal communication). In addition, in preliminary experiments we were unable to detect a defect in the ability of a PI-PLC-deficient strain (DP-L1552) to gain access to and multiply within the livers and spleens of mice after oral challenge (data not shown).

The use of PI-PLC by *L. monocytogenes* to directly facilitate lysis of host phagosomes appears to be unique among intracytoplasmic pathogens. Although other bacterial species, including *B. cereus*, *Bacillus thuringiensis*, *Clostridium novyi*, and *Staphylococcus aureus*,

secrete related PI-PLC enzymes, none of these is known to be an intracellular pathogen. However, this does not preclude action of these enzymes on host membranes extracellularly, particularly in the case of *S. aureus* for which there is evidence that PI-PLC contributes to the pathogenesis of infections (Marques *et al.*, 1989). Interestingly, several protozoan intracellular parasites express developmentally regulated PI-PLCs hypothesized to mediate invasion of host cells indirectly. The most studied systems are the merozoite stage of malarial parasites (Breton *et al.*, 1990) and *Trypanosoma cruzi* (Fenton Hall *et al.*, 1992), which apparently use PI-PLCs to release endogenous GPI-anchored substrates which, in turn, act on host cell membrane to mediate invasion of erythrocytes (malarial parasites) and on host glycoproteins to mediate invasion of the cytoplasm of macrophages (*T. cruzi*).

Although PI-PLC is not an essential determinant of pathogenicity in the mouse model (in contrast to LLO) it nevertheless clearly plays a significant role in the growth of *L. monocytogenes* in some host tissues. It is reasonable that many virulence determinants will play subtle roles in pathogenesis when assayed in the laboratory, but in fact may contribute significantly to the overall fitness of a pathogenic species in nature. Molecular dissection of virulence determinants of this class will be necessary for a detailed understanding of host–parasite interactions.

Experimental procedures

Plasmid constructions

The *Escherichia coli* host for all plasmid constructions was DH5 α MCR (F[−] *mcrA*, *mcrB*, *mrr*, ϕ 80*lacZ* M15, (*lacZYA*–*argF*)*U169*, *recA1*, *endA1*, *hsdR*, *hsdM*, *supE44*, λ –*thi-1*, *gyrA*, *relA1*) (Bethesda Research Laboratories Life Technologies, Inc.). Derivatives of pAM401 (Wirth *et al.*, 1986) were maintained in *E. coli* by growth in the presence of 10 μ g of chloramphenicol per ml of media. Plasmid pKSV7 is a shuttle vector capable of replication in *E. coli* and temperature-sensitive replication in *B. subtilis* and other Gram-positive bacteria such as *L. monocytogenes* (Smith and Youngman, 1992) and was maintained in *E. coli* in the presence of 50 μ g of ampicillin per ml of media.

The *L. monocytogenes plcA* gene, bases 279 to 1349 (Mengaud *et al.*, 1989), was amplified from 10403S chromosomal DNA using the polymerase chain reaction (PCR), and ligated into pAM401 using *Sa*I- and *Xba*I-generated DNA ends to generate pDP1339. The *Sa*I and *Xba*I restriction sites of the amplified product were created as non-complementary ends of the amplification primers. Primers used were 5'-

GGGTCGACTAGGAATAATATATGTTGTTG-3' and 5'-

GGTCTAGATTCTAGTCCTGCTGTCC-3'. The *plcA* and *prfA* genes, bases –810 to 1349 (Mengaud *et al.*, 1989), were amplified together from 10403S chromosomal DNA using the second primer above and 5'-GGGTCGACCAGCTCTTCTTGGTGAAG-3'. The amplified product was then ligated into pAM401 using *Sa*I- and *Xba*I-generated DNA ends to generate pDP1462. Plasmid PDP1500, containing *prfA* alone, was constructed by deleting the *plcA* gene, bases 429 to 1349 (Mengaud *et al.*, 1989), from pDP1462 after restriction with *Xba*I and *Pst*I, treatment of the DNA ends with T4 DNA polymerase to make them blunt, and intramolecular ligation. Plasmid pDP1499, containing the *plcA* promoter and a portion of the 3' end of *plcA*, was constructed by deleting a *plcA* internal fragment, bases

428 to 882 (Mengaud *et al.*, 1989). from pDP1339 after restriction with *Pst*I and *Nsi*I and intramolecular ligation. pDP1526 (pKSV7: : *plcA*) was constructed by a single three-part ligation of pKSV7 restricted with *Bam*HI and *Xba*I, the 468 bp *Xba*I and *Nsi*I-generated fragment from pAM401: :*plcA* containing the 5' end of *plcA* (bases 882 to 1351; Mengaud *et al.*, 1989) and the 501 bp *Pst*I- and *Bam*HI -generated fragment from pAM401: :*plcA prfA* containing the 3' end of *plcA* (bases -77 to 429; Mengaud *et al.*, 1989). The *prfA* promoter, bases 1–429 (Mengaud *et al.*, 1989), was isolated by *Eco*RI and *Pst*I double digestion of pDP1462 and the fragment was subsequently ligated into *Eco*RI- and *Pst*I-restricted pKSV7 to generate pDP1498. Two random *Hind*III-generated 10403S chromosomal DNA fragments, approximately 3kb in length, were ligated into *Hind*III-restricted pKSV7, to generate the random integration control plasmids pDP1519 and pDP1521.

Construction of *L. monocytogenes* mutant strains

L. monocytogenes strain DP-L1387 was isolated as a mutant with reduced lecithinase (PC-PLC) from a Tn917-LTV3 bank of SLCC 5764, constructed as previously described (Camilli *et al.*, 1990). The site of Tn917-LTV3 insertion was determined by sequencing one transposon-chromosomal DNA junction as previously described (Sun *et al.*, 1990). *L. monocytogenes* was transformed with plasmid DNA as previously described (Camilli *et al.*, 1990). Selective pressure for maintenance of pAM401, pKSV7, and their derivatives in *L. monocytogenes* was exerted in the presence of 10 µg of chloramphenicol per ml of media. In addition, maintenance of pKSV7 derivatives required growth at 30°C, a permissive temperature for plasmid replication in Gram-positive bacteria.

Integration of pKSV7 derivatives into the *L. monocytogenes* chromosome occurred by homologous recombination between *L. monocytogenes* DNA sequences on the plasmids and their corresponding chromosomal alleles (see Figs 4 and 6). Integration mutants were enriched by growth for approximately 30 generations at 40°C, a non-permissive temperature for pKSV7 replication, in Brain Heart Infusion (BHI) broth containing 10 µg chloramphenicol per ml of media. Each integration strain was subsequently colony purified on BHI agar containing 10 µg chloramphenicol per ml of media and incubated at 40°C. Southern blot analyses of chromosomal DNA isolated from each integration strain confirmed the presence of the integrated plasmid.

Construction of DP-L1552 containing a *plcA* allele on the chromosome is shown schematically in Fig. 6. Integration of the pKSV7 derivative, pDP1526, to generate a merodiploid intermediate was done as described above. Spontaneous excision of the integrated plasmid, through intramolecular homologous recombination, occurred at a low frequency. Bacteria in which the plasmid had excised from the chromosome were enriched by growth at 30°C in BHI broth for approximately 50 generations. The nature of the selective pressure during this step was not known but may be due to a slight growth defect of strains containing integrated temperature-sensitive plasmids. Approximately 50% of excision events, i.e. those resulting from homologous recombination between sequences 3' of the deletion, resulted in allelic exchange of *plcA* for the wild-type allele on the chromosome. The excised plasmids were cured by growing the bacteria at 40°C in BHI for approximately 30 generations. Cured bacteria retaining the *plcA* allele on the chromosome

were identified by their failure to produce a zone of turbidity surrounding colonies after growth on BHI agar plates containing a 5 ml overlay of BHI agar/2.5% egg yolk/2.5% phosphate-buffered saline (PBS) (BHI/egg yolk agar). The turbid zones resulted from PI-PLC hydrolysis of PI in the egg yolk, giving an insoluble diacylglycerol precipitate. The correct *plcA* deletion on the *L. monocytogenes* chromosome was confirmed by amplifying the deleted allele using PCR and sequencing across the deletion.

SDS-PAGE and activity assays

Each *L. monocytogenes* strain was grown overnight, from a single fresh colony, in a 2 ml BHI stationary culture containing 10 µg of chloramphenicol per ml of media either at 30°C for plasmid containing strains or at 40°C for integration strains; 1.5 ml of each overnight culture was subsequently used to inoculate 13.5 ml of BHI and the inoculated cultures were incubated with aeration either at (i) 37°C for 6h for 10403S integration derivative strains, SLCC 5764, and SLCC 5764 plasmid-containing derivatives, (ii) 37°C for 7h for SLCC 5764 integration derivative strains or, (iii) 37°C for 5h for all other strains. These variable times of incubation were empirically determined, by optical density measurements of growing cultures of all strains used, prior to producing final cultures of approximately the same stage of growth (early stationary phase). The resulting cultures were immediately chilled on ice for 5 min, and the bacteria sedimented at $4300 \times g$ for 10 min at 4°C; 1 ml of each clear supernatant was transferred to a sterile polypropylene tube and kept on ice to be used immediately for haemolytic, PI-PLC, and PC-PLC activity assays. Haemolytic assays were done as previously described (Portnoy *et al.*, 1989), PI hydrolysis assays were done essentially as described (Low, 1981) except that [^3H -inositol]-PI, 0.045 µCi, 17.4 Ci mmol⁻¹ (Amersham Corp.), and 13.5µg of PI (Sigma Chemical Co.) were sonicated in 100µl of 40 mM Tris-HCl, pH 7.2, and 0.2% deoxycholate (per assay), and the PI-sonicate was then incubated with culture supernatant in a final volume of 200 µl at 37°C. PC hydrolysis assays were done exactly as above for PI hydrolysis except that [^3H]-PC, 0.045µCi, 17.4 Ci mmol⁻¹ (Amersham Corp.), and 13.5µg of PC (Sigma Chemical Co.) were used. When necessary, BHI broth was used as a diluent for high-activity supernatants; 9 ml of each clear supernatant was used for trichloroacetic acid precipitation and SDS-PAGE analysis of secreted polypeptides as previously described (Portnoy *et al.*, 1988). Multiple activity and SDS-PAGE experiments were performed using separately grown cultures of the strains listed in Table 1 to confirm the relative activity levels and secreted protein profiles.

Northern blot analysis

L. monocytogenes wild-type and mutant strains were grown to early stationary phase as described in the section above (*SDS-PAGE and activity assays*). RNA was prepared from each strain according to the following method (E. Domann and T. Chakraborty, personal communication). Bacteria were sedimented from 10 ml of culture fluid at $4300 \times g$ for 5 min at 20°C and the cell pellet resuspended in 1 ml of 25% sucrose, 25 mM Tris-HCl (pH 8), and 10 mg ml⁻¹ lysozyme. The bacteria were incubated at 37°C with vigorous shaking (220 r.p.m.) for 1h, at which time 1 ml of 2% SDS and 100µl of 5 mg ml⁻¹ proteinase K was added, and the bacteria were incubated at 37°C with vigorous shaking for an additional 30 min; 800µl of the viscous suspension (due to lysis of bacteria) was aliquoted to a micro-centrifuge tube and bacterial lysis was then brought to completion by freeze-thawing three

times by incubation for 4 min each time in a dry ice-ethanol bath and then a 37°C water bath. The lysate was incubated at 65°C for 4min, at which time 400µl of 65°C phenol (previously equilibrated with 50 mM sodium acetate, pH 5.5) was added. The tube was mixed briefly by inversion and incubated for 5 min at 65°C. The aqueous and phenol phases were separated by centrifugation at $12000 \times g$ for 5 min at room temperature. The aqueous phase was subsequently transferred to a clean microcentrifuge tube and extracted two more times with 400 µl of room-temperature phenol equilibrated as above. A final extraction of the aqueous phase was performed with an equal volume of water-saturated ether. RNA was precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 2vols 100% ethanol at -70°C.

Twenty-five micrograms of RNA from each *L. monocytogenes* strain was separated on 1.1% agarose formaldehyde gels, blotted to nylon, and hybridized with random-primed *plcA* and *prfA*-specific DNA probes according to standard methods (Sambrook *et al.*, 1989). The *plcA*-specific DNA probe extended from base number 882 to 1350 (Mengaud *et al.*, 1989), which includes the *plcA* promoter and the 5' end but does not include the site of Tn917-LTV3 insertion in DP-L1054. The *prfA*-specific DNA probe extended from base number -396 to 1 (Mengaud *et al.*, 1989), which includes the first two-thirds of the *prfA* coding sequence.

Virulence and plaquing assays

LD₅₀ experiments were performed to assess the precise virulence of *L. monocytogenes*. LD₅₀ values were determined after intravenous injection of BALB/c mice (Charles River) with bacteria grown overnight in Trypticase soy broth (TSB) as described (Portnoy *et al.*, 1988), with the following modification; DP-L1341 was grown overnight in TSB containing 10 µg of chloramphenicol per ml of media.

Plaquing of *L. monocytogenes* strains was done as described previously (Sun *et al.*, 1990), with the following modifications; all plasmid-containing strains were grown overnight at 30°C in BHI containing 10 µg chloramphenicol per ml of media, and all integration strains were grown overnight at 40°C in BHI containing 10 µg of chloramphenicol per ml of media. Plaques of plasmid-containing and integration strains were grown in the presence of 10 µg of chloramphenicol per ml of media at 37°C in a 5% CO₂ atmosphere. All plaques were grown for 3d before staining with neutral red (Sigma).

Tissue-culture growth assays

Intracellular growth assays in J774 cells were done as previously described (Sun *et al.*, 1990). Growth in resident peritoneal macrophages and bone marrow-derived macrophages was performed as described previously (Portnoy *et al.*, 1988; 1989).

Growth in liver and spleen

10403S and DP-L1552 were grown overnight, from a single fresh colony, in 2 ml of BHI stationary cultures at 30°C; 1 ml of each overnight culture was used to inoculate 19 ml of BHI and the cultures were incubated at 37°C with aeration until an optical density of 0.8 was attained, representing $\approx 10^9$ cfu per ml of log-phase bacteria. The cultures were immediately

chilled on ice for 5 min, and the bacteria kept at 4°C in all subsequent steps. An equal volume of each of the 10403S and DP-L1552 cultures were combined and the bacteria washed twice in lipopolysaccharide-free PBS in a microcentrifuge. The bacteria were diluted to a final concentration of 1×10^9 cfu ml⁻¹ in lipopolysaccharide-free PBS and the bacterial titre measured by plating serial dilutions on BHI agar. A 1:1 ratio of 10403S to DP-L1552 in the final suspension was confirmed by testing 200 colonies from the BHI titre plates for their PI-PLC reaction on BHI/egg yolk agar. Approximately 1×10^3 cfu were given intravenously by tail-vein injection into BALB/c female mice 7–10 weeks old. The mice were given food and water *ad libitum*, and were sacrificed after 48 h by CO₂-suffocation. The liver and spleen from each mouse were immediately removed and each homogenized in 12 ml of 4°C sterile water. Serial dilutions of each homogenate were plated onto Luria-Bertani agar containing 150 µg of streptomycin per ml of media, 10403S (and derivatives) is a spontaneous streptomycin-resistant derivative of 10403. The plates were incubated at 37°C for approximately 30 h, then 200 single colonies from each organ homogenate were patched using sterile toothpicks onto BHI/egg yolk agar to score for their PI-PLC-reaction. The ratio of PI-PLC-positive to PI-PLC negative bacteria represented the ratio of 10403S to DP-L1552 in the organs.

Electron microscopy

Bone marrow-derived macrophages were infected with stationary-phase bacteria as previously described (Portnoy *et al.*, 1988) such that every cell contained approximately 10–20 bacteria after 30 min of incubation. Alternatively, log-phase bacteria, OD (600 nm) = 0.8 in BHI broth at 37°C with aeration, were used for infection such that every cell contained approximately 10–20 bacteria after 30 min of incubation. After 1.5 h of incubation, the infected monolayers were fixed *in situ*, and subsequently examined by electron microscopy as previously described (Tilney *et al.*, 1990).

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References

- Behnke D, Gilmore MS. Location of antibiotic resistance determinants, copy control, and replication functions on the double-selective streptococcal cloning vector pGB301. *Mot Gen Genet.* 1981; 184:115–120.
- Breton, CB.; Langsley, G.; Barale, J.; Pereira da Silva, LH. A malaria phosphatidylinositol-specific phospholipase C: a possible role in merozoite maturation and erythrocyte invasion. In: Cohen, CM.; Palek, J., editors. *Cellular and Molecular Biology of Normal and Abnormal Erythroid Membranes.* New York: Wiley-Liss; 1990. p. 315-332.
- Camilli A, Portnoy DA, Youngman P. Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn917 derivative that allows direct cloning of DNA flanking transposon insertions. *J Bacteriol.* 1990; 172:3738–3744. [PubMed: 2163385]
- Camilli A, Goldfine H, Portnoy DA. *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. *J Exp Med.* 1991; 173:751–754. [PubMed: 1847723]

- Chakraborty T, Leimeister-Wachter M, Domann E, Hartl M, Goebel W, Nichterlein T, Notermans S. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *J Bacteriol.* 1992; 174:568–574. [PubMed: 1729245]
- Cross GAM. Glycolipid anchoring of plasma membrane proteins. *Annu Rev Cell Biol.* 1990; 6:1–39. [PubMed: 2148872]
- Dabiri GA, Sanger JM, Portnoy DA, Southwick FS. *Listeria monocytogenes* moves rapidly through the host cytoplasm by inducing directional actin assembly. *Proc Natl Acad Sci USA.* 1990; 87:6068–6072. [PubMed: 2117270]
- Domann E, Wehland J, Rohde M, Pistor S, Hartl M, Goebel W, Leimeister-Wachter M, Wuenscher M, Chakraborty T. A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO J.* 1992; 11:1981–1990. [PubMed: 1582425]
- Fisher KA. Analysis of membrane halves: cholesterol. *Proc Natl Acad Sci USA.* 1976; 73:173–177.
- Freitag NE, Youngman P, Portnoy DA. Transcriptional activation of the *Listeria monocytogenes* hemolysin gene in *Bacillus subtilis*. *J Bacteriol.* 1992; 174:1293–1298. [PubMed: 1735720]
- Gaillard JL, Berche P, Mounier J, Richard S, Sansonetti P. *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect Immun.* 1987; 55:2822–2829. [PubMed: 3117693]
- Geoffroy C, Gaillard JL, Alouf JE, Berche P. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. *Infect Immun.* 1987; 55:1641–1646. [PubMed: 3110067]
- Goldfine H, Knob C. Purification and characterization of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C. *Infect Immun.* 1992; 60:4059–4067. [PubMed: 1398918]
- Hahn H, Kaufmann SHE. The role of cell-mediated immunity in bacterial infections. *Rev Inf Dis.* 1981; 3:1221–1250.
- Fenton Hall BF, Webster P, Ma AK, Joiner KA, Andrews NW. Desialylation of lysosomal membrane glycoproteins by *Trypanosoma cruzi*: a proposed role for the surface neuraminidase in facilitating parasite entry into the host cell cytoplasm. *J Exp Med.* 1992; 176:313–325. [PubMed: 1500849]
- Havell EA. Evidence that tumor necrosis factor has an important role in antibacterial resistance. *J Immunol.* 1989; 143:2894–2899. [PubMed: 2509559]
- Higgins JA, Hitchin BW, Low MG. Phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis* as a probe for the distribution of phosphatidylinositol in hepatocyte membranes. *Biochem J.* 1989; 259:913–916. [PubMed: 2543374]
- Ikezawa H, Taguchi R. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* and *Bacillus thuringiensis*. *Meth Enzymol.* 1981; 71:731–741.
- Kathariou S, Metz P, Hof H, Goebel W. Tn916-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. *J Bacteriol.* 1987; 169:1291–1297. [PubMed: 3029033]
- Kathariou S, Pine L, George V, Carlone GM, Holloway BP. Nonhaemolytic *Listeria monocytogenes* mutants that are also noninvasive for mammalian cells in culture: evidence for coordinate regulation of virulence. *Infect Immun.* 1990; 58:3988–3995. [PubMed: 2123830]
- Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, Cossart P. *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. *Cell.* 1992; 68:521–531. [PubMed: 1739966]
- Leimeister-Wachter M, Goebel W, Chakraborty T. Mutations offering hemolysin production in *Listeria monocytogenes* located outside the listeriolysin gene. *FEMS Microbiol Lett.* 1989; 65:23–30. [PubMed: 2558949]
- Leimeister-Wachter M, Domann E, Chakraborty T. The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. *J Bacteriol.* 1992; 174:947–952. [PubMed: 1732227]
- Lepay DA, Steinman RM, Nathan CF, Murray HW, Cohn ZA. Liver macrophages in murine listeriosis. *J Exp Med.* 1985; 161:1503–1512. [PubMed: 4009118]
- Lisanti MP, Rodriguez-Boulon E. Glycophospholipid membrane anchoring provides clues to the mechanism of protein sorting in polarized epithelial cells. *Trends Biochem Sci.* 1990; 15:113–118. [PubMed: 2139258]

- Low MG. Phosphatidylinositol-specific Phospholipase C from *Staphylococcus aureus*. Meth Enzymol. 1981; 71:741–746. [PubMed: 7278681]
- Mackaness GB. Cellular resistance to infection. J Exp Med. 1962; 116:381–406. [PubMed: 14467923]
- Mandel TE, Cheers C. Resistance and susceptibility of mice to bacterial infection: histopathology of Listeriosis in resistant and susceptible strains. Infect Immun. 1980; 30:851–861. [PubMed: 6785235]
- Marques MB, Weller PF, Parsonnet J, Ransil BJ, Nicholson-Weller A. Phosphatidylinositol-specific phospholipase C, a possible virulence actor of *Staphylococcus aureus*. J Clin Microbiol. 1989; 27:2451–2454. [PubMed: 2808668]
- Mengaud J, Vicente MF, Cossart P. Transcriptional mapping and nucleotide sequence of the *Listeria monocytogenes* *hlyA* region reveal structural features that may be involved in regulation. Infect Immun. 1989; 57:3695–3701. [PubMed: 2509367]
- Mengaud J, Dramsi S, Gouin E, Vazquez-Boland JA, Milon G, Cossart P. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. Mol Microbiol. 1991; 5:2273–2283. [PubMed: 1662763]
- Mounier J, Ryter A, Coquis-Rondon M, Sansonetti PJ. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect Immun. 1990; 58:1048–1058. [PubMed: 2108086]
- North RJ. The relative importance of blood monocytes and fixed macrophages to the expression of cell-mediated immunity to infection. J Exp Med. 1970; 132:521–534. [PubMed: 5002519]
- Portnoy DA. Innate immunity to a facultative intracellular bacterial pathogen. Curr Opin Immunol. 1992; 4:20–24. [PubMed: 1596365]
- Portnoy DA, Jacks PA, Hinrichs DJ. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J Exp Med. 1988; 167:1459–1471. [PubMed: 2833557]
- Portnoy DA, Schreiber RD, Connelly P, Tilney LG. Gamma interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. J Exp Med. 1989; 170:2141–2146. [PubMed: 2511268]
- Portnoy DA, Chakraborty T, Goebel W, Cossart P. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect Immun. 1992a; 60:1263–1267.
- Portnoy DA, Tweten RK, Kehoe M, Bielecki J. Capacity of Listeriolysin O, Streptolysin O, and Perfringolysin O to mediate growth of *Bacillus subtilis* within mammalian cells. Infect Immun. 1992b; 60:2710–2717. [PubMed: 1612739]
- Raveneau J, Geoffroy C, Beretti J, Gaillard J, Alouf JE, Berche P. Reduced virulence of a *Listeria monocytogenes* phospholipase-deficient mutant obtained by transposon insertion into the zinc metalloprotease gene. Infect Immun. 1992; 60:916–921. [PubMed: 1311708]
- Rosen H, Gordon S, North RJ. Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. Absence of monocytes at infective foci allows *Listeria* to multiply in nonphagocytic cells. J Exp Med. 1989; 170:27–37. [PubMed: 2501445]
- Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.
- Seeliger HPR. Listeriosis-history and actual developments. Infection. 1986; 16:81–85.
- Smith K, Youngman P. Use of a new integrational vector to investigate compartment-specific expression of the *Bacillus subtilis* *spoII*M gene. Biochimie. 1992; 74:705–711. [PubMed: 1391050]
- Smyth, CJ.; Duncan, JL. Thiol-activated (oxygenlabile) cytolysins. In: Jeljaszewicz, J.; Wadström, T., editors. Bacterial Toxins and Cell Membranes. New York: Academic Press Inc; 1978. p. 129-183.
- Sun A, Camilli A, Portnoy DA. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. Infect Immun. 1990; 58:3770–3778. [PubMed: 2172168]
- Theriot JA, Mitchison TJ, Tilney LG, Portnoy DA. The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. Nature. 1992; 357:257–260. [PubMed: 1589024]

- Tilney LG, Portnoy DA. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J Cell Biol. 1989; 109:1597–1608. [PubMed: 2507553]
- Tilney LG, Connelly PS, Portnoy DA. The nucleation of actin filaments by the bacterial intracellular pathogen, *Listeria monocytogenes*. J Cell Biol. 1990; 111:2979–2988. [PubMed: 2125302]
- Vazquez-Botand J, Kocks C, Dramsi C, Ohayon H, Geoffrey C, Mengaud J, Cossart P. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect Immun. 1992; 60:219–230. [PubMed: 1309513]
- Wirth R, An FY, Clewell DB. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli*-*S. faecalis* shuttle vector. J Bacteriol. 1986; 165:831–836. [PubMed: 3005240]
- Zar, JH. Biostatistical Analysis. 2. Vol. Chapter 10. Englewood, NJ: Prentice-Hall, Inc; 1974. p. 124

Fig. 1.

A Physical map of the *L. monocytogenes* chromosomal region containing *plcA* and *prfA*. The site of Tn917-LTV3 insertion in DP-L1054 is indicated by the arrow above the *plcA* gene (Sun *et al.*, 1990). Stem and loop structures represent the locations of rho-independent terminator-like sequences (Mengaud *et al.*, 1989; 1991). Promoters are indicated below the genes by arrows (Mengaud *et al.*, 1989; 1991. N, Freitag and D. A. Portnoy, unpublished). B. Complementation derivative strains of DP-L1054 and the respective *L. monocytogenes* DNA sequences (solid lines) contained by their complementing plasmids

Fig. 2.

Plaque formation by *L. monocytogenes* strains in L2 cell monolayers. Plaques were stained with neutral red after 3 d. Strains are indicated above each well. The mean plaque diameter formed by each strain is shown at the bottom of each well as a percentage of the 10403S (wild-type) mean plaque diameter. The standard deviations of all means was 9% of the wild-type mean plaque diameter.

Fig. 3.

Upper panel. Physical map of the chromosomal region of *L. monocytogenes* containing the *plcA* and *prfA* genes. The directions of transcription and RNA transcripts are indicated schematically below each gene. DNA probes used in the detection of RNA transcripts are lettered A and B. Lower panel. Northern blot analysis autoradiograms of RNA transcripts from strains SLCC 5764 (lane 1), DP-L1387 (lane 2), DP-L1535 (lane 3), DP-L1553 (lane 4), 10403S (lane 5), DP-L1054 (lane 6), DP-L1510 (lane 7), and DP-L1552 (lane 8). After a 24 h exposure to generate the first autoradiogram on the left, the Northern filter membrane was washed to remove probe A and then rehybridized with probe B and exposed for 48 h to generate the second autoradiogram on the right (see the *Experimental procedures*). Equivalent amounts of RNA (25 µg) were analysed for each strain. The positions and approximate sizes of the 23S, 16S, and 5S ribosomal RNAs served as molecular weight markers and are indicated between the autoradiograms. The slowly migrating bands present for DP- L1054 (lane 6, probe A) represent chimaeric RNA transcripts resulting from a transcriptional fusion with Tn917-LTV3 which was inserted into the *plcA* gene in this strain.

Similarly, the faint band of approximately 2.4 kb present for DP-L1510 (lane 7, probe A) resulted from a transcriptional fusion of *plcA* with one end of the integrated plasmid in this strain. A chimaeric RNA transcript of identical mobility was seen for DP-L1535 (with probe A) upon longer exposures (data not shown).

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Fig. 4.

Schematic diagram for construction of the isogenic strains DP-L1510 and DP-L1535, in which readthrough transcription of *prfA* was blocked.

A. Chromosomal integration of pDP1498 by homologous recombination between the cloned *Pst*I (P)- and *Eco*RI (E)-generated fragment present on the plasmid and the homologous chromosomal sequence. The designated cross-over points are arbitrary. P₁ and P₂ indicate the two promoter elements immediately upstream of *prfA* while P_{plcA} indicates the single *plcA* promoter (Mengaud *et al.*, 1989; 1991; N. Freitag unpublished).

B. The resulting integration structure on the chromosome of both mutant strains. The integration strains were selected for, and maintained by, growth at a non-permissive temperature for plasmid replication in the presence of chloramphenicol. The plasmid was shown by Southern blot analysis to have integrated multiple times in a head-to-head configuration (data not shown).

Fig. 5. Plaque formation by wild-type *L. manocyctogenes* (10403S), a mutant strain having readthrough transcription of *prfA* blocked by an integrated plasmid (DP-L1510), and two control random integration strains (DP-L1532 and DP-L1534). Plaques were stained with neutral red after 3 d. Strains are indicated above each well. The mean plaque diameter formed by each strain is shown at the bottom of each well as a percentage of the 10403S (wild-type) mean plaque diameter. The standard deviations of all means was 9% of the wild-type mean plaque diameter.

Fig. 6.

Schematic diagram for construction of the isogenic strains DP-L1552 and DP-L1553 each containing an in-frame Internal deletion in *plcA*.

A. Chromosomal integration of pDP1526 by homologous recombination between the *plcA* allele on the plasmid, which had the internal *N* to *P* fragment deleted, and the wild-type *plcA* chromosomal allele. The designated cross-over points are arbitrary; however, recombination occurred on either side of the internal deletion.

B. The resulting integration structure on the chromosome which was selected for by growth at a non-permissive temperature for plasmid replication in the presence of chloramphenicol. Upon passage of the merodiploid intermediate strains for several generations without

selective pressure, spontaneous excision of the integrated plasmid from the chromosome occurred (C). After curing of the excised plasmids at a non-permissive temperature for plasmid replication (D), *L. monocytogenes* Cm^s revertants were recovered at a frequency of = 1 %. Approximately 50% of the Cm^s revertants retained the *plcA* allele on the chromosome. These resulted from excision of the integrated plasmid via homologous recombination on the opposite side of the deletion allele as shown in (C).

Fig. 7.

Coomassie-brilliant-blue stained SDS-PAGE of secreted polypeptides of mutant and wild-type *L. monocytogenes* SLCC 5764. Proteins were precipitated from culture supernatant fluids with 10% trichloroacetic acid and separated on a 7% SDS-polyacrylamide gel. A. Molecular mass markers of the sizes indicated (in kDa) on the left; B, parental strain SLCC 5764; C, DP-L1553; D, DP-L1387; E, DP-L1535; F, DP-L1536. Arrows indicate polypeptide species of reduced expression in DP-L1387 and DP-L1535. LLO is the major band migrating at ≈ 58 kDa indicated by the third arrow from the top (Kathariou *et al.*, 1987). The two secreted forms of PC-PLC are indicated by the bottom and fourth from bottom arrows (Raveneau *et al.*, 1992; Kathariou *et al.*, 1990; D. A. Portnoy, unpublished). The second arrow from the bottom indicates a doublet consisting of PI-PLC (top band), which

accordingly is absent from DP-L1553 (compare lanes B and C), and an unknown polypeptide (bottom band).

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Fig. 8.

Ratio of wild-type *L. monocytogenes* to DP-L1552 in livers (open circles) and spleens (closed circles) of mice 48 h after infection. Note that the ratio ordinate is a log scale. Fifteen mice were injected intravenously with a sublethal dose of log-phase bacteria, sacrificed after 48 h, and the number of colony-forming units per organ was determined by plating serial dilutions of organ homogenates on agar media. The ratios of wild-type (PI-PLC⁺) to DP-L1552 (PI-PLC⁻) were determined by patching 200 colonies (derived from each organ homogenate) onto BHI/egg yolk agar and scoring for an opaque zone surrounding each PI-PLC⁺ isolate, after 24 h of bacteria growth (see the *Experimental procedures*). The mean ratios in liver and spleen are indicated on the right side of the figure. The ratio values for liver and spleen were statistically analysed for deviation from a value of 1 by the Wilcoxon paired sample test (Zar, 1974).

Fig. 9.

Intracellular growth of *L. monocytogenes* strains in primary and established murine macrophages cells. A, J774 cell-line; B, peritoneal-derived macrophages; C, bone marrow-derived macrophages, ○, wild-type strain 10403S; ●, DP-L1552; □, DP-L1054; ■, DPL1044. Data points and error bars represent the mean and standard deviations of the number of viable bacteria recovered from three coverslips.

Fig. 10.

Representative electron micrographs of thin sections of portions of bone marrow-derived macrophages infected with stationary-phase *L. monocytogenes* and fixed *in situ*. A, C, E, wild-type strain 10403S after 1, 1.5, and 4 h of infection, respectively; B, D, F, DP-L1552 after 1, 1.5, and 4 h of infection, respectively. The presence of host membrane surrounding bacteria is shown in (A) and (B), while bacteria free in the cytoplasm are shown in the remaining panels. Actin tails were seen trailing both the wild-type and mutant *L. monocytogenes* bacteria after 4 h of infection (panels E and F). It is worth noting the presence in panel E, and to a lesser extent in panel F, of many small vesicles ‘coating’ the periphery of the actin tails. This was observed infrequently in electron micrographs from this experiment as well as from other experiments using different host cell types (data not shown), and thus may be highly dependent upon variable fixation conditions. The function and/or significance of these small vesicles is not known. Bars = 0.1 μm .

Table 1

Bacterial strains and properties.

Strain	Genotype	Haemolytic activity ^a	PI-PLC S. A.	LD ₅₀	PC-PLC S. A.	Source/Reference
10403S	Wild-type Isolate	220	7100	9.7×10^3 ^b	ND	Portnoy <i>et al.</i> , (1988)
DP-L1054	10403S <i>plcA</i> :Tn917LTV3	200	0	1.4×10^7	ND	Sun <i>et al.</i> (1990)
DP-L1341	DP-L1054 (pAM401: <i>plcA</i>)	130	62000	ND	ND	This work
DP-L1480	DP-L1054 (pAM401: <i>plcA prfA</i>)	3300	520000	ND	ND	This work
DP-L1530	DP-L1054 (pAM401: <i>prfA</i>)	2500	0	ND	ND	This work
DP-L1503	10403S (pAM401: <i>plcA</i> promoter)	170	6400	ND	ND	This work
DP-L1483	10403S (pAM401)	200	7000	ND	ND	This work
DP-L1510	10403S <i>plcA-prfA</i> intergenic integration with pKSV7: <i>prfA</i> promoter	170	7800	ND	ND	This work
DP-L1532	10403S random Integration with pKSV7: random chromosomal DNA fragment	170	9600	ND	ND	This work
DP-L1534	10403S random integration with pKSV7: random chromosomal DNA fragment	140	9600	ND	ND	This work
DP-L1552	10403S <i>plcA</i>	230	0	2.2×10^4 ^b	ND	This work
DP-L1044	10403S <i>hly</i> :Tn917LTV3	0	8700	6.3×10^8	ND	Sun <i>et al.</i> (1990)
SLCC 5764	Hypersécréting wild-type isolate	1250	88 000	1.3×10^5	3600	Leimeister-Wachter <i>et al.</i> (1989)
DP-L1387	SLCC 5764 <i>plcA</i> :Tn917LTV3 ^d	770	0	ND	200	This work
DP-L1553	SLCC 5764 <i>plcA</i>	1700	0	ND	3600	This work
DP-L1535	SLCC 5764 <i>plcA-prfA</i> intergenic integration with pKSV7: <i>prfA</i> promoter	770	5100	ND	54	This work
DP-L1536	SLCC 5764 random integration with pKSV7: random chromosomal DNA fragment	1100	19000	ND	1900	This work

^aAll biochemical assays were done using early stationary-phase culture supernatant fluids after sedimenting the bacteria by centrifugation (see *Experimental procedures*). Haemolytic activities are expressed as the reciprocal of the dilution of each culture supernatant at which 50% lysis of erythrocytes was observed. The specific activities (S. A.) of PI-PLC and PC-PLC hydrolysis are in units of nanomoles of product formed per hour per ml of supernatant. LD₅₀ values represent the number of cotony-forming units of each *L. monocytogenes* strain resulting in death of half of the mice challenged intravenously.

^bExperiment 1.

^cExperiment 2.

^dDP-L1387 has a single Tn917LTV3 insertion between base numbers 318 and 319 (Mengaud *et al.*, 1989) which maps to the 3' end of *plcA*.

ND, not determined.

Table 2

Electron-microscopic quantification of vacuolar versus cytoplasmic bacteria in bone marrow-derived macrophages after infection with stationary- or log-phase *L. monocytogenes*.

Strain	Bacterial phase of growth ^a	Total number of bacteria examined	Number of bacteria in vacuoles	Number (% of total) of bacteria in cytoplasm
10403S	Stationary	322	298	24 (7)
DP-L1552	Stationary	262	261	1 (0.4)
10403S	Logarithmic	186	105	81 (44)
DP-L1552	Logarithmic	170	144	26 (15)

^aTwo separate experiments were done, one using stationary-phase, and the second, log-phase *L. monocytogenes* cultures for infection of bone marrow-derived macrophages. 1.5 h after Infection, the infected monolayers were fixed *in situ*, thin-sectioned, and examined by electron microscopy to quantify vacuolar versus cytoplasmic bacteria.