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Phosphatidylcholine synthesis is required for optimal function of *Legionella pneumophila* virulence determinants

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Abstract

The function of phosphatidylcholine (PC) in the bacterial cell envelope remains cryptic. We show here that productive interaction of the respiratory pathogen *Legionella pneumophila* with host cells requires bacterial PC. Synthesis of the lipid in *L. pneumophila* was shown to occur via either phospholipid *N*-methyltransferase (PmtA) or phosphatidylcholine synthase (PcsA), but the latter pathway was demonstrated to be of predominant importance. Loss of PC from the cell envelope caused lowered yields of *L. pneumophila* within macrophages as well as loss of high multiplicity cytotoxicity, while mutants defective in PC synthesis could be complemented either by reintroduction of PcsA or by overproduction of PmtA. The lowered yields and reduced cytotoxicity in mutants with defective PC biosynthesis were due to three related defects. First, there was a poorly functioning Dot/Icm apparatus, which delivers substrates required for intracellular growth into the cytosol of infected cells. Secondly, there was reduced bacterial binding to macrophages, possibly due to loss of PC or a PC derivative on the bacterium that is recognized by the host cell. Finally, strains lacking PC had low steady state levels of flagellin protein, a deficit that had been previously associated with the phenotypes of lowered cytotoxicity and poor cellular adhesion.

INTRODUCTION

Legionella pneumophila is a Gram-negative respiratory pathogen that causes severe pneumonia in immunocompromised individuals as well as occasional community-wide epidemic outbreaks resulting from single-source aerosols (Chow and Yu, 1998; Fraser *et al.*, 1977). After deposition in human lungs, the organism grows within alveolar macrophages, which fail to degrade the bacterium, and instead allow the formation of a membrane-bound replication vacuole (Nash *et al.*, 1984; Horwitz, 1983). A remarkable aspect of the natural history of the disease is that growth in host macrophages appears to mimic the strategy used by the microorganism to replicate within amoebae in the environment (Rowbotham, 1980). The bacterium probably has intimate interaction with unicellular eukaryotes during extracellular growth in the environment, as *L. pneumophila* primarily inhabits complex biofilms, such as those that develop in plumbing sources prior to aerosol contact with

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humans (Rogers *et al.*, 1994). Therefore, in addition to being an intracellular parasite, the bacterium comes into contact with unique metabolites provided by eukaryotic hosts in its environmental reservoir.

Intracellular growth and formation of the replication vacuole requires the Dot/Icm secretion system, a multiprotein complex in the bacterial envelope that allows translocation of macromolecules across host cell membranes (Sexton and Vogel, 2002; Segal and Shuman, 1999; Vogel *et al.*, 1998). *L. pneumophila* strains that properly assemble this complex recruit membranous material derived from host cell early secretory compartments, and this contributes to the formation of the replication vacuole containing the bacterium (Derre and Isberg, 2004; Kagan *et al.*, 2004; Kagan and Roy, 2002; Swanson and Isberg, 1995). A number of bacterial proteins have been identified that are translocated by the Dot/Icm system across the vacuolar membrane, presumably manipulating host cell signaling and survival pathways (Chen *et al.*, 2004; Conover *et al.*, 2003; Luo and Isberg, 2004; Nagai *et al.*, 2002; Shohdy *et al.*, 2005) (Gao and Abu Kwaik, 1999).

The absence of certain components of the Dot/Icm apparatus (DotL, DotM and DotN) results in lowered plating efficiency of bacteria on solid medium, probably because a partially assembled Dot/Icm lacking these components is misregulated (Buscher *et al.*, 2005; Conover *et al.*, 2003). To identify proteins that may regulate the function or assembly of the Dot/Icm system, a genetic screen was performed to isolate insertion mutations that had phenotypes similar to *dotL* mutations. As seen with *dotL* mutations, strains isolated from this mutant hunt all showed lowered viability in the presence of overproduced DotA, a component of the apparatus, but showed normal viability in strains making either no or low levels of DotA (Conover *et al.*, 2003). One of the mutants that had reduced plating efficiency in the presence of DotA overproduction had an insertion in the *lidK* gene (lpg1584), predicted to encode phosphatidylcholine synthase (Pcs), a recently discovered enzyme that allows the synthesis of phosphatidylcholine (PC) lipids in some bacterial species (Sohlenkamp *et al.*, 2000; de Rudder *et al.*, 1999). In a separate study, *lidK* (or *pcsA* to denote its activity) was shown to be one of a number of orfs from a variety of bacterial species that can promote the production of PC when overexpressed in *E. coli*, consistent with *lidK/pcsA* encoding a Pcs (Martinez-Morales *et al.*, 2003).

PC is a major essential component of eukaryote membranes, but only a fraction of bacterial species synthesize this phospholipids (Wilderman, *et al.*, 2002). Many of the bacterial species that produce PC are found intimately associated with eukaryotic hosts (Sohlenkamp *et al.*, 2003). Those bacteria that synthesize PC do so via one or more enzymatic pathways, and the enzymatic activities for two of these pathways have been identified in *L. pneumophila* by genomic searches (Fig. 1A; (Martinez-Morales *et al.*, 2003)). The first pathway uses a strategy also found in eukaryotes, in which phosphatidylethanolamine is subjected to a series of methylation reactions catalyzed by the phospholipid *N*-methyltransferase enzyme (PmtA; (Arondel *et al.*, 1993)). The second pathway is promoted by the PcsA enzyme unique to bacteria, which condenses exogenous choline and CDP-diacylglyceride to produce PC in a single step (Fig. 1A; (de Rudder *et al.*, 1999)). Pcs requires that the microorganism be in contact with eukaryotic hosts, or have access to eukaryotic metabolites, because choline is not a biosynthetic product of prokaryotes. Although it has been shown that *L. pneumophila* Orfs corresponding to both pathways are enzymatically active when expressed in *E. coli*, it is not clear if either contributes to PC production in *L. pneumophila* (Martinez-Morales *et al.*, 2003).

There is little information regarding why some bacteria species have PC in their envelopes. The most striking phenotype for PC-deficient mutants is in the plant pathogenic *Agrobacterium tumefaciens*, in which the lack of PC has the unexplained effect of reducing

transcription of genes encoding components of the T DNA conjugative apparatus (Wessel *et al.*, 2006). Mutations causing a PC deficit in other microorganisms that interact with host cells similarly cause alterations in host cell relationships. For instance, *Bradyrhizobium japonicum* PC mutants show inefficient symbiosis with the soybean host plant (Minder *et al.*, 2001), while *Brucella* mutants unable to synthesize PC have lowered virulence in a mouse model and less efficient replication vacuole formation (Comerci *et al.*, 2006; Conde-Alvarez *et al.*, 2006)).

In this work, we demonstrate that the presence of PC in *L. pneumophila* is important for both adhesion to host cells as well as for function of the Dot/Icm system. The loss of PC results in the absence of the flagellin protein, a posttranscriptional defect that may be the major cause of altered host cell interactions observed in PC-defective mutants.

RESULTS

Mutations in *lidK/pcsA* gene reduce the plating efficiency of *L. pneumophila* in the presence of high-level expression of the *dotA* gene (Conover *et al.*, 2003). Therefore, for most experiments presented here, bacterial strains were used that produced low levels of the DotA protein encoded by derivatives of the plasmid pGC36. Such strains grow within macrophages, although there is some reduction in the efficiency of effector translocation relative to wild type strains. Strains expressing low levels of DotA show normal viability on bacteriological media in the presence of a *lidK/pcsA* mutation (Conover *et al.*, 2003).

Mutations in the *L. pneumophila lidK/pcsA* gene result in reduced phosphatidylcholine content

It has been reported that phosphatidylcholine (PC) represents approximately 30% of the *L. pneumophila* phospholipid (Hindahl and Iglewski, 1984). To determine if the demonstrated phosphatidylcholine synthase activity of the *lidK/pcsA* gene product (Martinez-Morales *et al.*, 2003) contributes to the steady state level of this phospholipid, bacteria were grown in broth to post-exponential phase ($A_{600}=3.5$), labeled with $[1-^{14}\text{C}]$ acetate, and total lipids were extracted and separated by 2-dimensional thin layer chromatography (Fig. 1B-E; Experimental Procedures). In cells having an intact *lidK/pcsA* gene, chromatograms revealed four major compounds corresponding to PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Fig. 1B,C), with >30% of the total phospholipid being PC (Fig. 1D; GL233), in agreement with previous results (Hindahl and Iglewski, 1984). The relative levels of the four lipids was almost identical to those found in the fully Dot/Icm⁺ parent strain Lp02 or in JR32, another *L. pneumophila* strain that is commonly used (Supplemental Data). In contrast, the *lidK::Tn10* mutant showed low amounts of PC in the membrane (Fig. 1D), with approximately 6% of the total phospholipids being PC in bacteria grown to post-exponential phase (Fig. 1D). The deficit of PC in this mutant could be completely complemented in *trans* by a single open reading frame encoding *lidK/pcsA* (Fig. 1D; GL266). As the product of *lidK/pcsA* was demonstrated to produce PC dependent on the addition of exogenous choline (Martinez-Morales *et al.*, 2003), and its absence in *L. pneumophila* results in reduced PC synthesis, this gene will be exclusively referred to as *pcsA*.

Although PC represented only a small fraction of the phospholipid found in the *pcsA*⁻ mutant, this amount increased with storage at -80°C and subsequent recovery of CFU at 37°C. Therefore, a deletion of *pcsA* was constructed and analyzed soon after isolation. The amounts of PC in this strain were extremely low, indicating that continued storage selects for isolates with enhanced PC content (Fig. 1E; CCL24). As any residual PC was probably due to the presence of the phospholipid *N*-methyltransferase encoded by *pmtA* (orf lpg2158), we analyzed the effects of eliminating the second pathway of PC synthesis (Fig. 1A). In a

strain containing an intact *pcsA* gene, deletion of *pmtA* resulted in at least as much PC as in the wild type control both in post-exponential phase (Fig. 1E) or in cells extracted from other growth phases (data not shown). Therefore, the pathway that utilizes phosphatidylcholine synthase encoded by the *pcsA* gene is the major source of PC in *L. pneumophila*, and the activity of PmtA is likely to be only detected in the absence of the *pcsA* gene. The low level of PC in the absence of *lidK/pcsA* was not due to choline in the medium interfering with expression or activity of the *pmtA* gene, as growth of wild type strains in the absence of choline resulted in similarly low levels of PC (data not shown). This indicates that the amount of flux through the PmtA pathway is probably much lower than that through the PcsA path using most growth conditions.

As the *pcsA*⁻ mutants showed varying amounts of residual PC in membrane extracts, we isolated a strain lacking both *pmtA* and *pcsA*. Although isolation of the double mutant required a two-step procedure, in which the $\Delta pmtA$ allele was crossed into a $\Delta pcsA/ppcsA$ ⁺ heterozygote followed by curing of the wild type *pcsA*⁺ gene, strains lacking both enzymes in PC biosynthesis grew as well in bacteriological medium as did the parental $\Delta pcsA$ single mutant (data not shown). The resulting double mutant (*pcsA*::Gm^R $\Delta pmtA$) grown to postexponential phase showed no detectable levels of PC after chromatographic analysis of phospholipids (CCL26; Fig. 1E), and PC levels in this strain were returned to wild type levels by introduction of a wild type copy of *pcsA* in *trans* (CCL39; Fig. 1E). Interestingly, high PC levels could be restored in the double mutant by introduction of *pmtA* on a multicopy plasmid (CCL41; Fig. 1E).

***L. pneumophila* lacking PC results in reduced yields after infection of macrophages**

Strains lacking either one or both pathways of PC biosynthesis were analyzed for their ability to grow within mouse bone marrow-derived macrophages (Fig. 2A and B). Both the $\Delta pcsA$::Gm^R and the $\Delta pcsA$::Gm^R $\Delta pmtA$ mutants showed lower yields of bacteria after three days incubation than the parental strain (GL233) that has a normal content of PC. In contrast, the $\Delta pmtA$ mutant was indistinguishable from the parental control (Fig. 2B), consistent with its high levels of PC (Fig. 1E). Introduction of a plasmid expressing *pcsA*⁺ in *trans* restored intracellular growth of both the $\Delta pcsA$::Gm^R (Fig. 2A) and the $\Delta pcsA$::Gm^R $\Delta pmtA$ double mutant (Fig. 2B) to levels that were indistinguishable from the parental control, indicating that defective growth was due to lack of PC. Interestingly, the double mutant harboring *pmtA* on a plasmid also was able to grow as well as the wild type strain (Fig. 2B), consistent with the observation that overproduction of PmtA on a plasmid restores high level PC synthesis (Fig. 1E). To determine if the defect in intracellular growth was at the level of formation of replication vacuoles, the number of bacteria per vacuole was examined 18 h after the introduction of *L. pneumophila* onto macrophages (Fig. 2C). The *pcsA* mutant was defective for formation of large replication vacuoles, and macrophages harboring the mutant accumulated vacuoles with bacteria that failed to initiate replication.

The observed depression in intracellular growth caused by a deficit in PC synthesis may result from a poorly functioning Dot/Icm translocator, which would interfere with the ability of the microorganisms to direct formation of the morphologically distinct replication vacuole. Alternatively, the bacteria could proliferate slowly once a properly constructed vacuole is formed. These possibilities can be distinguished, because mutations that impair translocator function result in strains that traffic to the macrophage endocytic network. Furthermore, interference with Dot/Icm function should result in depressed efficiency of substrate translocation into target cells. To test for defective trafficking of the *L. pneumophila*-containing vacuole, macrophages incubated for one hour with bacteria were assayed for colocalization of this compartment with the endocytic marker LAMP-1 (Andrews *et al.*, 1998). About 2-3 times as many *pcsA*⁻ bacteria colocalized with LAMP-1 compared to the low DotA expressing control (Fig. 3A). These levels of colocalization

approached those of a strain having a defective Dot/Icm translocator (Fig. 3A; *dotA*⁻*pcsA*⁺). The defect that led to colocalization with LAMP-1 compartments was totally reversed by complementation with a plasmid that expressed *pcsA*⁺ (Fig. 3A)

The lack of PC also led to a reduction in the efficiency of effector translocation (Fig. 3B). Macrophages incubated for one hour with *L. pneumophila* strains were fixed and probed with antibody directed against SidC, a substrate of the Dot/Icm system that localizes on the cytoplasmic face of the vacuolar membrane (Experimental Procedures; (Luo and Isberg, 2004)) Although the efficiency of translocation of SidC was relatively low in the parental *pcsA*⁺ strain that has reduced amounts of DotA protein, the lack of PC resulted in levels of SidC translocation that were far lower than the wildtype (Fig. 3B). This defect was fully complemented by the presence of *pcsA* *in trans* ($P < 8.2 \times 10^{-4}$; Fig. 3B) indicating that the absence of PC clearly interfered with proper Dot/Icm function.

Defective adhesion to macrophages by PC mutants

From the growth curves (Fig. 2A) and examination of replication vacuoles (Figs. 2C and 3A), it was clear that there were two levels of defect caused by the lack of PC. First there was a depressed yield of bacteria after three days incubation with macrophages. Secondly, the number of bacteria internalized by macrophages appeared to be lower for the PC mutants. To compare internalization levels by target cells, *L. pneumophila* strains were incubated with macrophages for 1 hour, fixed and assayed for internalization by microscopy, using an antibody probing strategy (Experimental Procedures). Internalization of a *pcsA*⁻ mutant was approximately 3-5 fold less efficient than strains expressing either chromosomal and/or plasmid-encoded copies of *pcsA*⁺ (Fig. 4A; *pcsA*⁻). Uptake was fully restored by introducing a wild type copy of *pcsA*⁺ into the mutant background (Fig. 4A; *pcsA*⁻/*ppcsA*⁺), and the identical results were obtained using an alternative assay for internalization involving gentamicin protection (data not shown; (Lobo and Mandell, 1973)). The defect in uptake was due to reduced association of the mutant with macrophages rather than disrupted internalization of bound bacteria. Uptake of the macrophage-associated *pcsA*⁻ mutant was indistinguishable from a number of other *L. pneumophila* strains (Fig. 4B). We also found that the kinetics of cell association differed for the wild type and *pcsA*⁻ mutant. The maximal association of the mutant occurred immediately after its centrifugation onto macrophages, whereas the wild type showed increasing association over a one hour time period of incubation with macrophages (data not shown).

The absence of the *pmtA* gene caused an unexpected effect. Although the $\Delta pcsA \Delta pmtA$ double mutant was defective for uptake by macrophages, the simple loss of *pmtA* from *L. pneumophila* resulted in a modest stimulation of bacterial association with macrophages (Fig. 4C). This was observed either by analyzing a deletion (Fig. 4C; $\Delta pmtA$) or the double mutant complemented *in trans* with *pcsA*⁺ (Fig. 4C; $\Delta pcsA \Delta pmtA / ppcsA$ ⁺). It is possible that the mono- and dimethyl intermediates in the pathway promoted by the PmtA enzyme may interfere with *L. pneumophila* adhesion, and their absence in the *pmtA*⁻ mutant is stimulatory for uptake. Alternatively, since we observed enhanced PC content in both the *pmtA* mutant and the complemented clone relative to wild type (Fig. 1E), the increased efficiency of cell association may be directly related to the increased PC content of these strains.

The possibility that lipid derivatives could interfere with PC-dependent uptake was further pursued. Absence of bacterial PC could indirectly reduce uptake by interfering with the ability of the bacterium to export a ligand that is bound by a cellular receptor, or directly, by preventing the synthesis of a PC-derived molecule that is recognized by the macrophage. There is precedent for respiratory pathogens using choline-derivatives as ligands for recognition by cellular receptors, in which case adhesion is inhibited by small molecule PAF

receptor antagonists (PAF-Ra) (Swords *et al.*, 2000; Cundell *et al.*, 1995). When a PAF-Ra was added during a short incubation of bacteria with macrophages, it specifically interfered with uptake of the *pcsA*⁺ strains, and had no effect on association of the $\Delta pcsA \Delta pmtA$ mutant (Fig. 4D). We saw similar levels of inhibition when, instead of PAF-Ra, PC vesicles were added to the infections (data not shown). These results are consistent with the model that some bacterial PC derivative plays a direct role in microbial association with macrophages.

The absence of PC interferes with selected functions associated with bacterial transmission to macrophages

A number of *L. pneumophila* virulence-associated functions are preferentially induced during the post-exponential phase of growth, and the induction of these factors is correlated with efficient establishment of intracellular growth (Hammer *et al.*, 2002; Byrne and Swanson, 1998). These properties include the Dot/Icm-dependent induction of macrophage cell death after high multiplicity infection, enhanced bacterial motility, and the induction of expression of translocated substrates of the Dot/Icm system (Luo and Isberg, 2004). Each of these properties was analyzed.

Rapid cell death to macrophages resulting from bacterial contact-dependent high multiplicity infection has been attributed to the insertion of a bacterium-derived pore into host macrophages by the Dot/Icm system (Zink *et al.*, 2002; Kirby *et al.*, 1998). To assay for this function, macrophages were allowed to come into contact with bacteria for one hour (Fig. 5A-D). A *pcsA*⁻ mutant was defective for contact-dependent cytotoxicity (Fig. 5A-D). This result was observed at all MOI tested, with the levels of cytotoxicity only slightly higher than a *dot/icm*⁻ strain. An intact *pcsA*⁺ gene provided *in trans* completely restored cytotoxicity (Fig. 5E). To determine if the defect in association of bacteria with the macrophage (Fig. 4) was the cause of the defect in cytotoxicity, bacteria were introduced at various MOI in the cytotoxicity assay and then analyzed for efficiency of bacterial binding to macrophages. At an MOI = 1000, there was little cytotoxicity (Fig. 5E), although the *pcsA*⁻ mutant had large numbers of bacteria bound to macrophages (Fig. 5F). In contrast, when a similar number of *pcsA*⁺ bacteria were bound to cells (using a 10 fold lower MOI; Fig. 5F), there was large-scale cytotoxicity (Fig. 5E). Therefore, the defect in cytotoxicity is not due to a defect in bacterial association with host cells.

The defective cytotoxicity is attributed to either defective Dot/Icm assembly (Kirby *et al.*, 1998) or an inability of the bacterium to assume a state of heightened competence for intracellular growth that has been closely correlated with entry into postexponential phase of broth culture (Molofsky and Swanson, 2004). This latter property has been termed the "Transmission Phenotype" to denote that the postexponential phase allows the bacteria to be transmitted from an extracellular locale to an intracellular niche and initiate replication efficiently (Molofsky and Swanson, 2004). To test for properties that are similarly associated with the Transmission Phenotype, the PC-defective mutants were assayed for motility and growth phase-dependent expression of translocated substrates. Mutants lacking PcsA were highly defective for establishing a motile state in postexponential phase (Fig. 6A), with only a few bacteria showing some movement in very dense bacterial cultures. In contrast, strains competent for PC synthesis, including the $\Delta pmtA$ strain, and PC mutants expressing either *pcsA*⁺ or *pmtA*⁺ on a plasmid, were able to establish a high degree of motility (Fig. 6A).

Although two phenotypes (motility and cytotoxicity) associated with the Transmission Phenotype were defective in mutants lacking PC, the mutants were still able to respond to postexponential phase signals based on the levels of translocated Dot/Icm substrates associated with the bacteria. In postexponential phase cultures, bacteria accumulate the

translocated substrate VipD (Fig. 6B, “P;”), with little VipD expression during logarithmic growth (Fig. 6B, “E;”) (VanRheenen *et al.*, 2006; Shohdy *et al.*, 2005)). In all mutants tested, including the $\Delta pcsA \Delta pmtA$ double mutant, this growth phase response appeared identical to wild type (Fig. 6B; $pcsA^+ pmtA^+$). Similar results were observed with the substrate SidC (data not shown). Therefore, it is unlikely that the absence of PC prevents detection of nutrient conditions associated with entry into the Transmissive phase.

As the lack of motility was unlikely to be due to general misregulation of post-exponential phase transcription, we looked directly for a loss in the steady state levels of flagellin protein due the absence of PC (Fig. 6B). Mutants with defects in PC biosynthesis (either $\Delta pcsA$ or $\Delta pcsA \Delta pmtA$) were devoid of either cell-associated or culture-associated flagellin after growth to post-exponential phase, a defect that could be complemented by either plasmid borne $pcsA^+$ or $pmtA^+$ (Fig. 6B). Consistent with the lack of effects of these deletion mutations on postexponential phase expression, transcription of the flagellin (*flaA*) structural gene was highly induced when cultures approached stationary phase (Fig. 6C). Therefore, the lack of motility observed in PC-defective mutants can be attributed to a post-transcriptional defect in accumulation of flagellin protein. Furthermore, as both cell association and cytotoxicity of *L. pneumophila* have been previously reported to be highly reduced in *flaA*⁻ mutants (Molofsky *et al.*, 2005), the loss of assembled flagellin is at least part of the reason that defects in host cell interaction were observed in bacteria lacking PC.

In contrast to the lowered flagellin levels observed in the absence of PC, the expression and stability of individual components of the Dot/Icm complex appeared unaffected. Immunoprobings with antibody directed against inner membrane components DotF, DotG and DotO, the outer membrane protein DotH and the cytoplasmic IcmQ showed that steady state levels for each of these proteins were identical in wild type, $\Delta pcsA \Delta pmtA$, and $\Delta pcsA \Delta pmtA/ppcsA^+$ strains (Fig. 7). The lack of effect was observed in both exponential and post-exponential phase, and was identical to what was observed for the control cytoplasmic protein isocitrate dehydrogenase (Fig. 7). Therefore, although translocation rates of Dot/Icm substrates were reduced in the absence of PC, the cause of this defect was not due to global reduction of steady state levels of Dot/Icm components in mutants having lowered PC content.

DISCUSSION

As most bacterial species do not have the capacity to synthesize phosphatidylcholine (PC), the reason for the presence of PC in some bacterial species has remained mysterious. It was originally proposed that bacteria with large amounts of internal membranes, such as photosynthetic bacteria, have a requirement for this lipid (Hagen *et al.*, 1966). Interestingly, several species that interact intimately with host cells have significant PC content in their membranes, so this lipid may play an important function in allowing these microorganisms to interface with their hosts (Lopez-Lara and Geiger, 2001; Goldfine, 1984). There had been little information supporting this hypothesis, and each species having PC may synthesize the lipid for a different reason. The original observation supporting its importance was the demonstration that mutations depressing PC synthesis in the nitrogen fixing *Bradyrhizobium japonicum* cause reduced root nodule occupancy (Minder *et al.*, 2001). Similarly, *Brucella abortus* mutants defective for synthesis of PC show unexplained defects in cell interactions and virulence (Comerci *et al.*, 2006; Conde-Alvarez *et al.*, 2006). The clearest connection between a molecular defect and a phenotype is the case of *A. tumefaciens*, in which loss of virulence in PC-defective mutants results from lowered transcription of the genes encoding the T DNA transfer apparatus, a result very different from what we observed with the Dot/Icm complex (Fig. 7). There is no mechanism, however, that links membrane composition to transcription of this locus (Wessel *et al.*, 2006). The experiments described here are

consistent with an important role for PC in the virulence of an intracellular pathogen. Many of the defects observed could be ascribed to loss of the flagellin protein, which unlike the *A. tumefaciens* example, occurs at the post-transcriptional level.

The low yield of bacteria after infection of macrophages by PC- mutants appears to be the consequence of two related defects: poor initiation of intracellular replication and a defect in the ability of the bacteria to associate with macrophages. Once initiation of replication occurs, however, the rate of replication of these mutants did not appear strongly altered by the absence of PC. This small effect on replication rate is somewhat surprising given the increased association with LAMP-containing compartments for mutants relative to wild type. Having a lowered initial level of infection, however, could be the result of both an uptake defect as well as loss of viability of the improperly targeted bacteria. Therefore, the viable PC- mutants seen at early timepoints may be primarily those that have targeted properly and which will eventually replicate. Furthermore, different initial doses of infected macrophages results in nonidentical monolayer conditions that can greatly affect the rate of bacterial intracellular replication. For instance, we have observed that at low MOI, there is a detectable cytokine response that is a function of bacterial infectivity levels (M. Liu and R. Isberg, data not shown). This response, in which monolayers having lower numbers of viable bacteria result in less restriction of *L. pneumophila* growth, makes it difficult to directly correlate replication rates to targeting defects.

There is a large body of work indicating that *L. pneumophila* entry into post-exponential phase allows expression of functions required for productive growth in macrophages (Byrne and Swanson, 1998). We found that the loss of PC interfered with proper function of a subset of activities associated with this state, such as cytotoxicity, motility, cell association and intracellular growth (Molofsky and Swanson, 2004). These defects did not appear to be a result of an inability to respond to signals resulting from nutrient depletion, as cultures of wild type strains and PC- mutants showed identical induction of both translocated substrates and the flagellin structural gene after entry into post-exponential phase. The absence of PC, however, did result in the loss of flagellin protein even though the *flaA* structural gene transcript was strongly induced in post-exponential phase (Fig. 6). As lack of flagella on the bacterial cell surface reduces both bacterial binding to macrophages as well as cytotoxicity (Molofsky *et al.*, 2005), the aberrant cellular interactions observed in PC- mutants may largely result from an inability to maintain high steady state levels of the flagellin protein. The molecular basis for the absence of this protein is unclear. Presumably, lack of PC prevents post-exponential phase assembly of the flagellar complex, with the result that unassembled subunits are degraded. PC may be required for assembly of this complex in *L. pneumophila* because the events involved in production of flagella all occur in nondividing bacteria, potentially raising problems not observed during exponential phase assembly. PC has many characteristics that may alter protein interactions. For instance, it has been shown to antagonize folding of the LacY protein, indicating it may control folding rates of inner membrane proteins (Bogdanov *et al.*, 1999; Goldfine, 1984).

The second explanation for the role of PC is that the choline head group directly interacts with target cells. In particular, some mucosal pathogens are believed to use bacterial surface molecules that have been modified by phosphorylcholine as ligands for host cell receptors (Swords *et al.*, 2000; Cundell *et al.*, 1995). The PC head group could act as a source for such modifications. The primary evidence that bacterial phosphorylcholine is recognized by mammalian receptors are studies indicating that an antagonist of platelet activating factor receptor (PAF-Ra) interferes specifically with adhesion of phosphorylcholine-modified bacterial ligand (Swords *et al.*, 2000; Cundell *et al.*, 1995). As PAF has a phosphorylcholine group, it is attractive to speculate that mammalian receptors that recognize the PAF-Ra antagonist also recognize phosphorylcholine-modified bacterial cell surfaces. Similar to

results in *Streptococcus pneumoniae* and *Haemophilus influenzae*, we found that the PC-dependent cell adhesion of *L. pneumophila* could be blocked by the addition of a PAF-Ra, with adhesion of a mutant lacking PC being totally resistant to the action of the antagonist (Fig. 4). Mammalian cells may directly recognize an *L. pneumophila* ligand that contains either the phosphocholine headgroup or a derivative of PC. Consistent with this model, we found that the efficiency of bacterial association with macrophages was a direct function of the PC concentration, as derivatives that had higher PC levels than wild type also associated with macrophages more efficiently than wild type, as if a PC derivative were limiting for cell association.

The clear preference of *L. pneumophila* for using PcsA, rather than the PmtA pathway must be explained. One possibility is that the products of the PmtA pathway negatively impact either bacterial viability or the ability of the organism to interact with the host. Unlike PcsA, which only produces PC as a product, the PmtA pathway results in low-level synthesis of the monomethylated and dimethylated derivatives of phosphatidylethanolamine, which have uncertain roles in membrane biology. These lipids could interfere with either adhesion of the cells or assembly of membrane proteins. A second explanation for the predominance of the PcsA pathway is that the synthase is a direct sensor of environmental conditions, using choline availability as an indicator of the status of the locale in which the bacterium is found. Limiting choline concentrations may be a signal for the bacterium to either slow its growth or make compensatory changes in lipid side chain composition to accommodate environments in which choline is limiting. The presence of large pools of choline could be a signal for the bacterium that host cells are available for intracellular replication, allowing the proper assembly of envelope components critical for host cell interaction.

Although it may be counterintuitive that the bacterium relies on an extracellular source of precursor rather than utilize its own biosynthetic pools, the fact that interaction with hosts is a central aspect of the biology of *L. pneumophila* must be an important determinant of how this preference for PcsA arose. PC synthesis appears to be a critical sensor of the nature of the environment surrounding the microorganism. Biosynthetic precursors found in lung tissue, therefore, can modulate virulence of this organism by controlling the ability of the organism to incorporate PC into its cell envelope. Investigating how the environment controls the PC content in cells and how this phospholipid modulates host cell interaction are of central importance for understanding the pathogenesis of *L. pneumophila*.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, cell culture and media

The bacterial strains and plasmids used in this study are listed with their relevant characteristics in Table 1. The *L. pneumophila* strains used are derivatives of the *L. pneumophila* Philadelphia 1 strain Lp02 (*thyA* Δ [*hsdR-lvh*] *rpsL*) (Berger and Isberg, 1993), and were grown on casamino acids-yeast extract (CYE) solid medium or ACES-yeast extract (AYE) broth (Berger and Isberg, 1993). For *L. pneumophila*, antibiotics were used at the following concentrations: chloramphenicol, 5 μ g/ml; kanamycin, 40 μ g/ml; streptomycin, 50 μ g/ml; and gentamicin, 50 μ g/ml. The growth media were supplemented with thymidine at 100 μ g/ml or 5% sucrose when appropriate. The *L. pneumophila* strains used in all assays were grown to post-exponential phase ($OD_{600} \approx 3.2 - 3.5$) unless stated otherwise. Antibiotics were used at the following concentrations with *E. coli* strains: kanamycin, 40 μ g/ml; ampicillin 100 μ g/ml and chloramphenicol, 30 μ g/ml.

All DNA manipulations were as described previously (Conover *et al.*, 2003).

Primary bone marrow-derived macrophages were isolated from the femurs of female A/J mice and maintained as described (Swanson and Isberg, 1995). Typically, 1×10^5 macrophages were plated per well for immunofluorescence studies, and $4-5 \times 10^5$ macrophages per well were used to monitor bacterial growth.

Construction of the *pcsA* and *pmtA* deletion mutants

The *pcsA(lidk)::miniTn10* insertion was isolated previously (Conover *et al.*, 2003). The $\Delta pmtA$ deletion on the *L. pneumophila* chromosome was isolated by first constructing an internal deletion in *pmtA* using PCR generated fragments, leaving 6 amino acids at the amino terminus and 3 amino acids at the carboxyl terminus (Supplemental Data). These were then cloned on the suicide plasmid pSR47s (*kan^RsacB*) to generate the plasmid pGD51. The plasmid was introduced into appropriate *L. pneumophila* strains as described (Conover *et al.*, 2003). After selecting for integration by plating on kanamycin-containing medium, transconjugants were purified and plated onto 5% sucrose CYET plates to select for sucrose resistant recombinants that had lost the plasmid. Construction of the $\Delta pcsA::Gm^R$ mutant used the same strategy, with a *Gm^R* fragment inserted into a total deletion of the *pcsA (lidK)* gene in the suicide plasmid pSR47s (Supplemental Data). Construction of the $\Delta pcsA::Gm^R \Delta pmtA$ double mutation in a strain harboring the plasmid pGC41 $td^+ pcsA^+$ (Thy⁺) is described in the text (Fig. 2). The double mutant was isolated in the LP03 strain background, which has a chromosomal *thyA*⁻ mutation. This allowed a selection for curing (loss) of the pGC41 plasmid, which has the phage T4 *td* gene enabling growth in the absence of thymidine (Berger and Isberg, 1993), but also rendering the strain trimethoprim sensitive. In the presence of medium containing trimethoprim and thymidine, bacteria harboring pGC41 are unable to grow, while strains that have lost pGC41 are resistant to trimethoprim, as described (Berger and Isberg, 1993).

Construction of complementation plasmids

The two complementing plasmids encoding the complete ORFs of *pcsA* (pGC41) and *pmtA* (pTC5) were constructed in the plasmid pGC36 (Conover *et al.*, 2003) by placing each gene under the control of *P_{tac}* in an operon with *td*?*i*. The complementing genes containing sufficient upstream DNA to allow efficient translation were amplified by PCR from chromosomal DNA using primers with the indicated engineered restriction sites.

Lipid profiles of *L. pneumophila* PC mutants

L. pneumophila cultures (1 ml) were grown in AYE at 37°C to an optical density of $A_{600}=3.5$, labeled for 40 min with 5 μ l of [$1-^{14}C$]acetate (60 mCi/mmol; 200 μ Ci/ml) and subsequently harvested. Lipids were then extracted (Bligh and Dyer, 1959) and analyzed by 2-D thin layer chromatography (TLC) (de Rudder *et al.*, 1997). The TLC plates were exposed to Kodak X-Omat film, lipid spots were scraped, and quantified by liquid scintillation counter. In addition to the results reported, strains growing in exponential phase were also analyzed, using an identical approach.

LAMP-1 targeting assay

L. pneumophila strains were grown and tested in the LAMP-1 targeting assay exactly as previously described (Conover *et al.*, 2003). The number of LAMP-1-positive phagosomes per bacterial strain was quantified by counting cell-associated bacteria per strain ($N = 312$). The average and standard deviation reported here were calculated from three independent infections per strain.

Intracellular growth assays

Intracellular growth curves were performed in bone marrow-derived macrophages from the A/J mouse as described, incubating macrophages with bacteria at an MOI \approx 0.05 or as indicated (Conover *et al.*, 2003). Each experiment was performed in triplicate.

To examine microscopically the intracellular growth of *L. pneumophila* inside macrophages, mammalian cells were plated at density of 1×10^5 per coverslip and infected with the indicated strains at MOI \approx 1, fixed in PLP-sucrose (McLean and Nakane, 1974) and analyzed as described previously (Conover *et al.*, 2003). The number of intracellular bacteria in each phagosome in separate macrophages was recorded by visual inspection. Data shown represent the distribution of phagosomes containing 1 to 3, 4 to 9 and more than 10 bacteria. These data were calculated by averaging triplicate samples of 104 scored phagosomes per coverslip.

Cytotoxicity assay

Contact dependent cytotoxicity assays were performed in macrophages plated on coverslips 24 hours prior to infection, as described performing 1 hour infections at 37°C in 5% CO₂ (Kirby *et al.*, 1998). Pore-forming activity was measured as the percentage of total cells that stained positive for ethidium bromide relative to acridine orange stained cells.

Immunofluorescence assay of bacterial uptake and SidC translocation

Mammalian cells were plated one day prior to infection with the indicated post-exponentially grown motile *L. pneumophila*. Unless otherwise indicated, the infected macrophages were spun for 5 minutes and the infection proceeded at 37°C for the times indicated, until stopped by three washes with RPMI medium. Samples were fixed in PLP-sucrose and stained for extracellular bacteria with rabbit anti-*L. pneumophila* serum and Texas Red-anti rabbit IgG. The monolayers were then methanol permeabilized (Conover *et al.*, 2003) and stained with rabbit anti-*L. pneumophila* serum and FITC-anti rabbit IgG to detect total bacteria. Percentage of internalized/bound bacteria was scored as fraction of bacteria resisting staining with Texas Red-IgG but which stain with FITC-IgG. The data reported in each experiment is the average of triplicate infections scoring 104 macrophages per coverslip.

Translocation of the Dot/Icm substrate SidC was performed as described (Luo and Isberg, 2004; Conover *et al.*, 2003). Macrophages were incubated with noted bacterial strains for one hour, and fixed samples were then probed with affinity purified rabbit anti-SidC and rat anti-*L. pneumophila*, followed by detection with fluorescent secondary antibodies. 104 phagosomes per coverslip were scored, and data were expressed as mean percentage of phagosomes showing staining for SidC \pm S.D.

Assay for bacterial uptake in platelet activating factor receptor antagonist (PAF-Ra) treated macrophages

Platelet Activating Factor-16 Antagonist (PAF-Ra, Calbiochem, La Jolla, CA, catalog # 511082) was dissolved in EtOH following manufacturer specifications. The compound was serially diluted in RPMI to obtain the appropriate concentration. Macrophages were pretreated at 37°C with 100 nM PAF-Ra for 30 minutes prior to infection as described (Swords *et al.*, 2000). Once the drug was added to the mammalian cells, they were immediately infected with post-exponential motile *L. pneumophila* at MOI \approx 10 for 10 minutes. The infection was stopped by extensive washes and the samples were processed for immunofluorescence as described above.

Immunoprobings of *L. pneumophila* lysates for post-exponential phase expressed proteins

For analysis of growth-stage specific expression of proteins, *L. pneumophila* strains were grown at 37° C in AYE broth. The equivalent of 0.5 OD units of each strain were harvested at both exponential ($A_{600} = 1.0-1.5$) and post-exponential ($A_{600} = 3.5-4.0$) phases of growth, centrifuged at $16,000 \times g$ to separate bacterial cells from culture supernatants and the resulting supernatants were frozen at -80° C for later analysis. Bacterial cell pellets were then resuspended in 150 μ l SDS-PAGE sample buffer and boiled for 5 minutes. 5 μ l of each sample was resolved by SDS-PAGE and analyzed by Western blotting using various antisera. For the analysis of flagellin in culture supernatants, frozen supernatants were thawed on ice and trichloroacetic acid was added to a final concentration of 10%. Samples were vortexed for 1 min and incubated on ice for 20 min. Samples were pelleted at $16,000 \times g$ for 10 min and resulting precipitates were washed twice with ice-cold ethanol and air dried for 15 min at 37° C. Dried pellets were then resuspended with 30 μ l SDS-PAGE sample buffer and boiled for 5 min. 10 μ l of each sample was resolved by SDS-PAGE and analyzed by Western blotting using rabbit antisera against the noted *L. pneumophila* proteins. Serum specific for *B. subtilis* isocitrate dehydrogenase (ICDH) was provided by A. L. Sonenshein (Tufts University School of Medicine, Boston MA). Serum specific for flagellin was provided by W. F. Dietrich (Harvard Medical School, Boston MA), antisera directed against *L. pneumophila* proteins were raised previously in our laboratory. Affinity purified serum directed against VipD has been previously described (VanRheenen *et al.*, 2006).

RNA extraction, reverse transcription, and real-time PCR

To determine the amounts of *flaA* transcript in *L. pneumophila* strains by using real time quantitative PCR (qPCR), *L. pneumophila* was grown in AYE medium at 37°C and samples were removed in either late exponential phase ($A_{600} = 1.0$) or postexponential phase ($A_{600} = 3.5$) to prepare RNA. Total RNA was then isolated using RNeasy (QIAGEN). Reverse transcription was performed on the samples using 1 μ g of RNA with Superscript II reverse transcriptase (Invitrogen) and random decamers (Ambion). qPCR was performed in real time in a Clontech quantitative thermocycling spectrophotometer using the SYBR green PCR master mix (Applied Biosystems) with primers specific for *flaA* (*flaA* forward: 5' CCATCAGCAGCAGTGAGTGT 3', *flaA* reverse: 5'TCAATGGCGTCAGTAACCAA 3') and 16S rRNA (16S forward: 5' CTAAGGAGACTGCCGGTGGTGAC, 16S reverse: 5'CGTAAGGGCCATGATGACTT 3'). The transcript levels of *flaA* were normalized to 16S rRNA values for each condition and displayed in graphical format.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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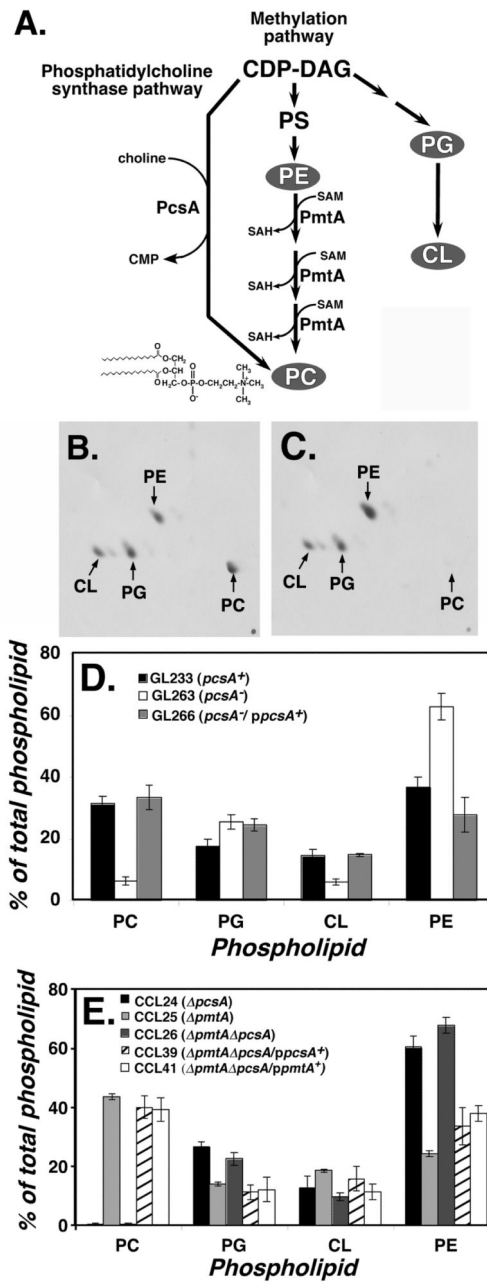


Figure 1. Knockout of *pcsA* causes drastic loss of PC content in *L. pneumophila* membranes (A). Pathways for phosphatidylcholine biosynthesis in bacteria. Shown are the two major pathways for synthesis of phosphatidylcholine (PC) in bacteria, catalyzed by phosphatidylcholine synthase (PcsA) and phospholipid *N*-methyltransferase (PmtA). *L. pneumophila* Philadelphia 1 ORFs encoding both of these enzymes have the predicted activities when expressed in *E. coli* (Martinez-Morales *et al.*, 2003). CDP-DAG: cytidine diphospho-diacylglyceride. PS: phosphatidylserine. PG: phosphatidylglycerol. CL: cardiolipin. SAM: *S*-adenosylmethionine. SAH: *S*-adenosylhomoserine. PE: phosphatidylethanolamine. In gray are the three major lipids found in bacterial membranes in addition to PC (Lopez-Lara and Geiger, 2001). (B). Phospholipid content of *L. pneumophila* labeled with [1-¹⁴C]acetate was determined by thin layer chromatography

(TLC) of membrane extracts (Experimental Procedures). Shown is two-dimensional TLC fractionation of GL233 (*pcsA*⁺) lipids. (C). Phospholipids from GL263 (*pcsA*⁻) prepared as above and subjected to two-dimensional TLC fractionation. (D). Complementation of PC biosynthesis defect by cloned *pcsA*⁺ gene. Displayed are relative percentages of phospholipids in various *L. pneumophila* strains having either an intact *pcsA*⁺ gene or the *pcsA*⁻ mutation. Data shown are mean of triplicate samples. (E). Total absence of PC in $\Delta pcsA \Delta pmtA$ strain and its complementation. Phospholipid profiles are displayed as in (D) for strains noted in legend. Data shown are mean of triplicate samples. PC: phosphatidylcholine. PE: phosphatidylethanolamine. PG: phosphatidylglycerol. CL: cardiolipin.

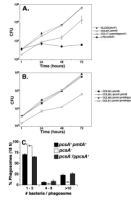


Figure 2. Defective intracellular growth in strains lacking PC

(A). Defective growth of $\Delta pcsA::Gm^R$ in bone marrow-derived macrophages. Assays for viable counts over time performed as described (Experimental Procedures). Strains are: GL233, $pcsA^+pmtA^+$; CCL24, $\Delta pcsA$; CCL17, $\Delta pcsA/ppcsA^+$; LP03, $dotA^-$. Data shown are means \pm S.D. of triplicate incubations performed in parallel. (B). Loss of $pmtA$ is not sufficient to impair *L. pneumophila* intracellular growth. Strains are: CCL25, $\Delta pmtA$; CCL26, $\Delta pmtA \Delta pcsA$; CCL39, $\Delta pmtA \Delta pcsA/ppcsA^+$; CCL41, $\Delta pmtA \Delta pcsA/ppmtA^+$. Data shown are means \pm S.D. of triplicate incubations performed in parallel. (C) Absence of PcsA results in defective replication vacuole formation. Macrophages plated on coverslips were incubated with $pcsA^+$ (GL233), $pcsA^-$ (GL263) and $pcsA^-/ppcsA^+$ (GL266) for 18 hours and processed for immunofluorescence (Experimental procedures). The number of bacteria per macrophages was recorded for 104 phagosomes in triplicate samples for each strain. Shown are typical experiments. All experiments were repeated at least twice.

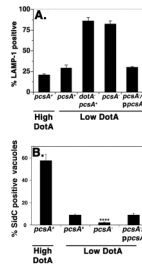


Figure 3. The function of the Dot/Icm system is impaired by loss of PC

(A). Loss of PC results in defective targeting of the *Legionella*-containing vacuole. Bone marrow-derived macrophages were incubated with noted *L. pneumophila* strains for 25 min and colocalization of the late endosome/lysosome glycoprotein marker LAMP-1 with bacteria was scored by immunofluorescence microscopy (Experimental Procedures). (B). The absence of PC results in defective translocation of a Dot/Icm substrate. Bacteria were incubated with bone marrow-derived macrophages for one hour and scored for the colocalization of SidC by immunofluorescence microscopy (Experimental Procedures). High DotA: strains contain wild type levels of DotA protein. Low DotA: Strains express attenuated amounts of DotA (Conover *et al.*, 2003). High DotA strains used: *pcsA*⁺, LP02. Low DotA strains used: *pcsA*⁺, GL233; *pcsA*⁻; GL263; *pcsA*⁻/*ppcsA*⁺, GL266; *dotA*⁻, GL83. ****; $P < 8.2 \times 10^{-4}$. Data shown are the mean \pm standard deviation of 3 independent experiments in which 104 phagosomes were quantified for each strain.

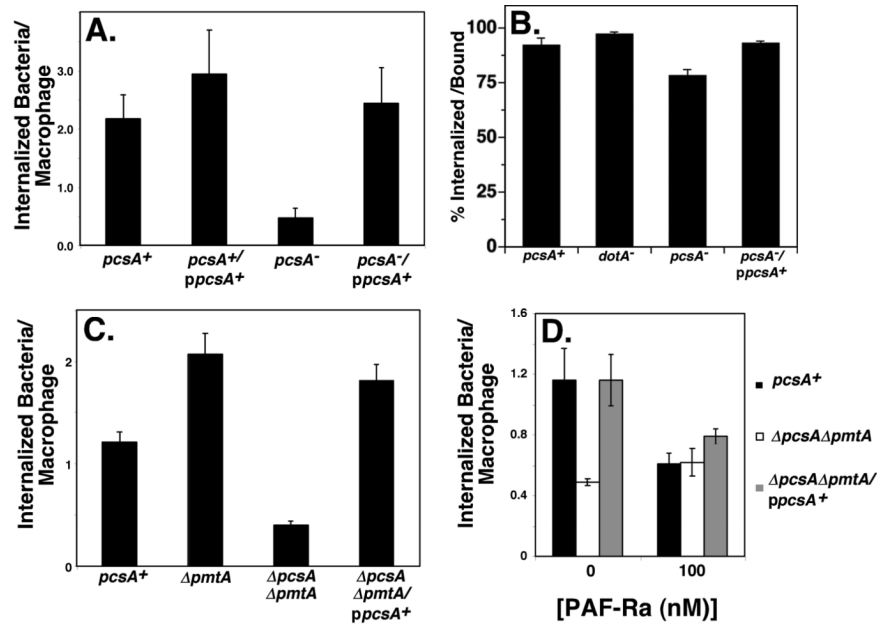


Figure 4. Absence of PC results in defective association with target macrophages

(A). Efficient cell association requires the activity of PcsA. Macrophages were infected at MOI \approx 10, after synchronizing uptake with a 5 min centrifugation at room temperature followed by 10 min incubation at 37°C. The number of internalized bacteria per macrophage was scored by double indirect immunofluorescence microscopy. (B). Uptake efficiency of bound bacteria. Uptake efficiency was determined by antibody probing as in panel A. This measurement is defined as the percentage of cell associated bacteria that are internalized. (C). Bacterial association with macrophages is altered by the absence of PmtA. Internalization was determined as above. (D). PAF-Ra inhibits PC-dependent uptake. Macrophages were pretreated with PAF-R antagonist (1-O-hexadecyl-2-acetyl-*sn*-glycerol-3-phospho-(N,N,N-trimethyl)-hexanolamine; PAF-Ra) for 30 min., bacteria were allowed to bind for 10 minutes at 37°C, and attachment was assayed as above. Data shown are the mean \pm standard deviations of triplicate determinations. Experiment was performed 4 times, and displayed is a typical experiment. Strains used were: *pcsA*⁺, GL233; *pcsA*⁺/*ppcsA*⁺, CCL29; *pcsA*⁻, GL263; *pcsA*⁻/*ppcsA*⁺, GL266; *dotA*⁻, GL83; Δ *pmtA*, CCL25; Δ *pmtA* Δ *pcsA*, CCL26; Δ *pmtA* Δ *pcsA*/*ppcsA*, CCL39.

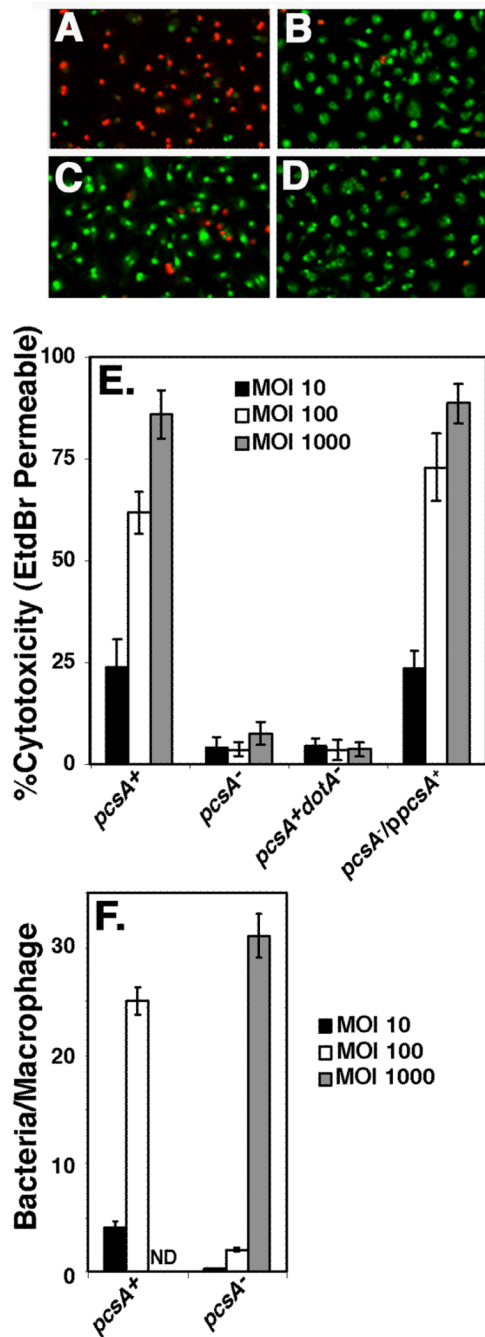


Figure 5. The defect in Dot/Icm-dependent cytotoxicity caused by the absence of PC is not the result of lack of adhesiveness

Bacteria were allowed to incubate with macrophages for 55 min at 37°C at the indicated series of MOI with the following strains: *pcsA*⁺, GL84; *pcsA*⁻, GL263; *pcsA*⁺*dotA*⁻, GL83; *pcsA*⁻/*ppcsA*⁺, GL266. Merged images of 10X magnifications of incubations with bone marrow macrophages incubated at an MOI = 100 are displayed for: (A) *dotA*⁺*pcsA*⁺, (B) *dotA*⁻*pcsA*⁺, (C) *pcsA*⁻*dotA*⁺, and (D) *lidC* *dotA*⁺ (Conover et al., 2003). Green cells are those staining with acridine orange, while red cells are those that are dead and permeable to EtdBr. (E). Defective cytotoxicity in a *pcsA*⁻ mutant is independent of bacterial dose. Cytotoxicity was measured by identifying cells permeable to ethidium bromide (EtdBr),

which was added after removing the medium immediately after the infection period. % cytotoxicity was determined as the percentage of cells observed to be EtdBr-permeable in the rhodamine fluorescence channel relative to the total number of acridine orange stained cells observed in the FITC channel, using grabbed images (Experimental Procedures). (F) Incubation of the *pcsA*⁻ mutant at high multiplicity allows adhesion of bacteria to macrophages. In parallel to the cytotoxicity assay, incubations were fixed, bacteria were identified with an anti-*L. pneumophil* antibody and the number of bacteria bound per macrophage was determined at the noted MOI. Strains are same as in panel (E). Quantitation of binding at MOI = 1000 for the *pcsA*⁺ strain was not determined (ND). All experiments were repeated twice.

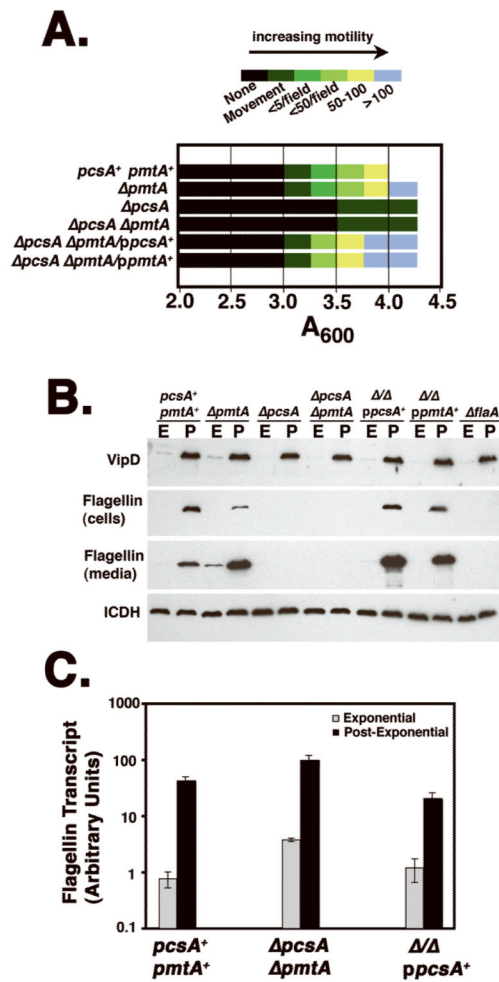


Figure 6. Selective defects in *L. pneumophila* factors associated with high efficiency growth in host cells

(A) A deficit in PC results in a block in induction of motility in postexponential phase. Bacteria were grown in culture in AYE broth and at noted A₆₀₀ readings, a drop of culture was placed on a coverslip and several fields using a 40X lens were analyzed for the presence of motile bacteria. The degree of motility was noted using the color-coded scale in the figure, with each color representing the number of bacteria observed moving per field. If no bacteria were showing overt motility, but there was occasional movement of tethered bacteria, these cultures were scored as “movement,” which was the first sign that some degree of motility was beginning to occur in a culture. Experiment was repeated three times. (B) The absence of PC causes a reduction in steady state flagellin production. The noted bacterial strains were grown in AYE medium (Experimental Procedures) to either exponential (A₆₀₀= 1.4-1.8; “E”) or postexponential phase (A₆₀₀= 3.5-4.0; “P”). The cells were adjusted to the identical densities, pelleted, subjected to SDS-PAGE on a 10% gel, and either cell pellet (cells) or culture supernatant (media) were immunoprobed with antibody to VipD, *L. pneumophila* flagellin or to isocitrate dehydrogenase (ICDH), to control for identical loading conditions. Strains: *pcsA⁺pmtA⁺*, GL233; *ΔpmtA*, CCL25; *ΔpcsA*, CCL24; *ΔpmtAΔpcsA*, CCL26; *ΔΔ/ppcsA⁺*, CCL39; *ΔΔ/ppmtA⁺*, CCL41. (C) The defect in flagellin production occurs at the post-transcriptional level. Bacteria from exponential (E) or post-exponential (P) phase were prepared and subjected to qPCR, using flagellin structural

gene (*flaA*)-specific primers. Amount transcript was then determined as described (Experimental Procedures). Experiment was performed twice.

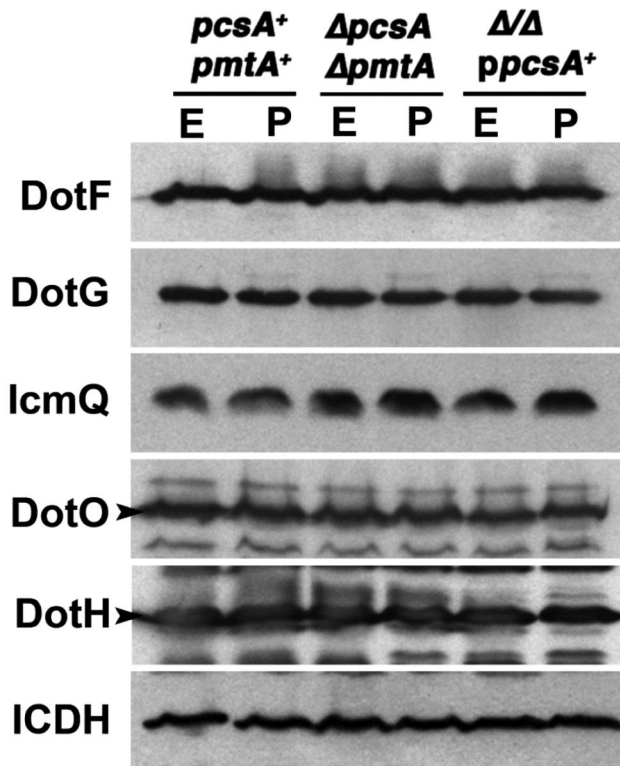


Figure 7. Absence of PC does not affect the steady state levels of a subset of Dot/Icm proteins
 The noted bacterial strains were grown in AYE medium (Experimental Procedures) to either exponential (A_{600} = 1.4-1.8; “E”) or post-exponential phase (A_{600} =3.5-4.0; “P”). The cells were adjusted to identical densities, pelleted, lysed in sample buffer and resolved by SDS-PAGE. Proteins were blotted to PVDF membranes and immunoprobed with rabbit antibodies to DotF, DotG, DotO, DotH, or IcmQ. Samples were also immunoprobed with antibodies to isocitrate dehydrogenase (ICDH) to control for identical loading conditions. Strains: *pcsA*⁺ *pmtA*⁺, GL233; Δ *pcsA* Δ *pmtA*, CCL26; Δ *pcsA* Δ *pmtA*/ *ppcsA*⁺, CCL41.

Table 1

Bacterial strains and plasmids used in this work.

Plasmid / Strain	Genotype/ Relevant Characteristics	Reference
PLASMIDS		
pDOT1	pMMB66EH <i>tdAi bla⁺ dotA⁺</i>	(Berger and Isberg, 1993)
pSR47s	Kan ^R <i>sacB</i> suicide vector	(Merriam <i>et al.</i> , 1997)
pGC36	pKB5 <i>dotA⁺</i>	(Conover <i>et al.</i> , 2003)
pGC41	pGC36 <i>pcsA⁺</i>	This study
pTC5	pGC36 <i>pmtA⁺</i>	This study
pTC6	pSR47s Δ <i>pcsA::Gm^R</i>	This study
pGC51	pSR47s Δ <i>pmtA</i>	This study
pKB5	pMMB66EH ori RSF1010 <i>tdAi bla⁺</i>	(Berger and Isberg, 1993)
STRAINS ¹		
LP02	Philadelphia-1 <i>rpsL</i> Δ [<i>hsdR-lvh</i>] <i>thyA</i> -	(Berger and Isberg, 1993)
LP03	LP02 <i>dotA03- thyA</i> -	(Berger and Isberg, 1993)
JR32	Philadelphia-1 <i>hsdR</i>	(Sadosky <i>et al.</i> , 1993)
GL83	LP02 <i>dotA</i> / pKB5	(Conover <i>et al.</i> , 2003)
GL84	LP02 <i>dotA</i> / pDOT1	(Conover <i>et al.</i> , 2003)
GL233	LP02 <i>dotA</i> / pGC36	(Conover <i>et al.</i> , 2003)
GL263	LP02 <i>pcsA::miniTn10kan dotA</i> /pGC36	This study, NT
GL266	LP02 <i>pcsA::miniTn10kan dot</i> /pGC41	This study, NT
GL301	LP02 Δ <i>pmtA dotA</i> -	This study
GL302	LP02 Δ <i>pmtA dotA</i> / pGC36	This study
GL303	LP02 Δ <i>pmtA thyA</i> -	This study
GC197	LP02 <i>pcsA::miniTn10kan dotA</i>	(Conover <i>et al.</i> , 2003)
CCL1	LP02 Δ <i>pmtA dotA thyA</i> -	This study
CCL17	LP02 Δ <i>pcsA::Gm^R dotA</i> -/ pGC41	This study
CCL21	LP02 Δ <i>pmtA</i> Δ <i>pcsA::Gm^R dotA</i> -	This study
CCL24	LP02 Δ <i>pcsA::Gm^R dotA</i> -/ pGC36	This study
CCL25	LP02 Δ <i>pmtA dotA</i> -/ pGC36	This study
CCL26	LP02 Δ <i>pmtA</i> Δ <i>pcsA::Gm^R dotA</i> - / pGC36	This study
CCL29	LP02/pGC41	This study
CCL39	LP02 Δ <i>pmtA</i> Δ <i>pcsA::Gm^R dotA</i> -/ pGC41(<i>dotA⁺ pcsA⁺</i>)	This study
CCL41	LP02 Δ <i>pmtA</i> Δ <i>pcsA::Gm^R dotA</i> -/ pTC5(<i>dotA⁺ pmt⁺</i>)	This study

¹ All strains are derivatives of *L. pneumophila* Philadelphia 1