A conserved OmpA-like protein in *Legionella pneumophila* required for efficient intracellular replication

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One sentence summary: CmpA is an OmpA-like protein in *Legionella pneumophila* that is required for efficient intracellular replication in both primary macrophages and in the environmental host *Acanthamoeba castellanii*.

Editor: Akio Nakane

ABSTRACT

The OmpA-like protein domain has been associated with peptidoglycan-binding proteins, and is often found in virulence factors of bacterial pathogens. The intracellular pathogen *Legionella pneumophila* encodes for six proteins that contain the OmpA-like domain, among them the highly conserved uncharacterized protein we named CmpA. Here we set out to characterize the CmpA protein and determine its contribution to intracellular survival of *L. pneumophila*. Secondary structure analysis suggests that CmpA is an inner membrane protein with a peptidoglycan-binding domain at the C-terminus. A *cmpA* mutant was able to replicate normally in broth, but failed to compete with an isogenic wild-type strain in an intracellular growth competition assay. The *cmpA* mutant also displayed significant intracellular growth defects in both the protozoan host *Acanthamoeba castellanii* and in primary bone marrow-derived macrophages, where uptake into the cells was also impaired. The *cmpA* phenotypes were completely restored upon expression of CmpA in trans. The data presented here establish CmpA as a novel virulence factor of *L. pneumophila* that is required for efficient intracellular replication in both mammalian and protozoan hosts.

Keywords: *Legionella*; OmpA-Like; Dot/Icm; secretion system; intracellular pathogen; vacuolar pathogen

INTRODUCTION

*Legionella pneumophila* is the bacterial pathogen that causes Legionnaires’ disease, a severe pneumonia that if left untreated can lead to death (Fraser et al. 1977; McDade et al. 1977). *L. pneumophila* is a Gram-negative bacterium, which is found ubiquitously in the environment as a parasite of protozoa (Fields 1996). It is considered an accidental opportunistic human pathogen, because it can cause disease in immunocompromised individuals, but cannot be transmitted between individuals (Yu et al. 1983; Fields 1996; Shin 2012). One of the major virulence factors of *L. pneumophila* is a type IVB secretion system, called Dot/Icm, which delivers a large number of bacterial proteins into the host cell (Marra et al. 1992; Berger and Isberg 1993; Segal, Purcell and Shuman 1998; Vogel et al. 1998). The delivered proteins, termed effectors, target specific cellular processes in the host cell, resulting in altered trafficking of the *L. pneumophila*-containing vacuole, and promoting the survival and replication of the bacterium inside the modified vacuole (Horwitz 1983; Isberg, O’Connor and Heidtman 2009).
The transport mechanism of the Dot/Icm secretion system is not well understood, with limited structural data available (Kubori et al. 2014), and largely undefined substrate selectivity (Nagai et al. 2005; Burstein et al. 2009). One of the Dot/Icm components, DotK/IcmN, has been implicated in playing a role as a cell-wall anchor of the Dot/Icm secretion system through the binding to peptidoglycan (Nagai and Kubori 2011). The DotK/IcmN protein is one of six L. pneumophila proteins containing the domain called OmpA-like, or OmpA-C-like, annotated as Pfam F00691 (Finn et al. 2013). The OmpA-like protein domain denotes homology to the C-terminal portion of the canonical Escherichia coli outer membrane porin OmpA. Proteins containing an OmpA-like domain are predicted to bind to peptidoglycan at the cell wall, and are important for the virulence of a number of intracellular pathogens, including Francisella tularensis (Mahawar et al. 2012), Leptospira interrogans (Ristow et al. 2007) and Coxiella burnetii (Martinez et al. 2014). Of the six L. pneumophila proteins containing an OmpA-like domain, the best studied is DotK/IcmN, which is encoded within one of the two dot/icm loci. The dotK/icmN gene is dispensable for intracellular replication within human macrophages, but is partially required during replication in the protozoan host Acanthamoeba castellanii (Segal and Shuman 1999). Three other OmpA-like proteins are predicted to function as structural components of the flagella: MotB, MotD and Lpg2962. The lipoprotein Plp/PAL is a surface exposed protein (Ludwig et al. 1991; Hoffman, Ripley and Weeratna 1992), and has been explored as a possible diagnostic tool, as it can be detected in the urine of patients with Legionnaires’ disease (Kim et al. 2003; Gholipour et al. 2014). Finally, the OmpA-like protein that is encoded by open reading frame Lpg0657 is a highly conserved protein within the Legionella genus, but has not been studied to date. Here we set out to characterize the potential role of the Lpg0657 OmpA-like protein in the pathogenesis of L. pneumophila. We find that the Lpg0657 protein, which we renamed CmpA, is required for efficient intracellular replication of L. pneumophila in both the protozoan host A. castellanii and in primary murine macrophages, but not in broth. The importance of the CmpA protein may be in part due to its contribution to bacterial uptake into host cells.

**MATERIALS AND METHODS**

**Strains and media**

All bacterial strains, plasmids and oligonucleotide primers used in this study are listed in Table 1. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA. Bacto-agar, tryptone and yeast extract were purchased from Difco, Franklin Lakes, NJ, USA. L. pneumophila strains used in this study were grown on charcoal-yeast-extract (CYE) plates as described previously (Merriam et al. 1997; Roy and Isberg 1997). When needed, chloramphenicol was added to the medium at a concentration of 10 μg mL⁻¹, and kanamycin at 20 μg mL⁻¹.

**Cell culture**

Primary cells were cultured at 37°C in 5% CO₂. Bone marrow-derived macrophages were cultured from female A/J mice as described previously (Celada et al. 2014). Acanthamoeba castellanii (ATCC strain 30234) was cultured routinely at room temperature in ATCC medium 712 (PYG). One hour before and after infection, A. castellanii cultures were maintained at 37°C in a 5% CO₂ atmosphere in PYG medium without glucose, proteose peptone and yeast extract.

**Intracellular growth assays**

Intracellular growth assays were conducted in A. castellanii (ATCC strain 30234) or in bone marrow-derived murine macrophages, as described previously (Coers et al. 2000; Ninio et al. 2005). For complementation studies, 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to all wells. Intracellular competition was performed in the host A. castellanii in a protocol modified from (Rothmeier et al. 2013). In detail, A. castellanii was plated in 24-well plates at a density of 5 × 10⁴ and infected with a 1:1 mixture of the competing strains at an overall multiplicity of infection (MOI) of 0.25. After 2 h, all wells were washed with warm phosphate-buffered saline (PBS), and incubated at 37°C for 3 days. Initial uptake was determined by processing wells immediately following the wash step to...

Table 1. Strains, plasmids and primers.

| Legionella pneumophila strains | Genotype | Reference |
|-------------------------------|----------|-----------|
| CR39 | L. pneumophila serogroup 1, strain Lp01 rpsL | Berger and Isberg (1993) |
| CR38 | Lp01 rpsL dotA | Zuckman, Hung and Roy (1999) |
| SN272 | Lp01 rpsL cmpA262-7e, Kan’ | This study |
| SN316 | Lp01 rpsL cmpA262-7e, Kan’ (pIG1) | This study |
| SN315 | Lp01 rpsL (pIG1) | This study |

| Plasmids | Important properties | Reference |
|---------|---------------------|-----------|
| pJB1806 | L. pneumophila expression vector | Bardill, Miller and Vogel (2005) |
| pSN84 | Cloning vector derived from pJB1806, N-terminal Flag tag | This study |
| pIG1 | pSN84 encoding for a Flag-CmpA fusion protein | This study |

| Primers | Sequence | Restriction site |
|---------|----------|-----------------|
| SN242 | AGCCTTTAAAGAGATAGGAG | N/A |
| SN243 | GTTTCAAGCCGCTTGGAG | N/A |
| SN264 | GGGGATCTTTTTGGGAAAAATTTAATGAGATGCT | BamHI |
| SN280 | GGCGTCGACGTTATTTTACATAAGGCATTTG | PstI |
determine the number of colony-forming units (CFU). Every 3 days supernatant was collected and combined with cell lysate from the same well. Bacteria were then plated for CFU enumeration, and diluted 1:1000 and used to re-infect a fresh batch of cells. For differential enumeration of the two strains, bacteria were plated on both plain CYE and CYE-containing kanamycin.

**Plasmid construction**

For the construction of *L. pneumophila* expression vector pSN84, the 3 x Flag tag sequence was amplified using primers SN137 and SN138 and the resulting PCR fragment was digested with BglII and BamHI and ligated with pLB1806 that was linearized with BamHI and treated with calf intestinal alkaline phosphatase. The result is a *L. pneumophila* expression vector that can be used to generate N-terminal Flag-tag fusion proteins under the control of the Ptacl IPTG-inducible promoter. For the generation of plasmid pLC1, primers SN264 and SN280 were used to amplify the cmpA reading frame. The product was digested with BamHI and PstI, and ligated with plasmid pSN84 that was digested with the same enzymes. The resulting plasmid encodes for the Flag-CmpA fusion protein under an IPTG-inducible promoter for expression in *L. pneumophila*.

**Immunoblot analysis**

*L. pneumophila* strain SN316 was grown on either plain CYE or CYE containing 0.5 mM IPTG for 2 days. Bacterial cells were collected from the plates, adjusted for OD_{600nm} and precipitated using ‘trichloroacetic acid’. Proteins were separated by SDS-PAGE and transferred to Immobilon-Membranes (Millipore, Billerica, MA, USA). The membrane was blocked and probed with monoclonal FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:3000 in blocking solution. After incubation with primary antibody, blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:5000. SuperSignal West Dura Extended Duration Substrate (ThermoScientific, Waltham, MA, USA) was used for antibody detection, and the blot was imaged using ImageQuant LAS4000 (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

**Gentamicin protection assay to measure uptake**

Gentamicin protection assay was performed as described in Hibi, Segal and Shuman (2001). In brief, bone marrow-derived macrophages from A/J mice were plated at a density of 1 x 10^5 per well in 24-well plates. Indicated strains of *L. pneumophila* were used to infect macrophages at an MOI of 100. The plates were spun and incubated at 37 °C for 10 min. Immediately after, all wells were washed with PBS containing 100 μg mL^-1 gentamicin. Media containing the same concentration of gentamicin was added to all wells and incubated for an additional 45 min. Cells were lysed with H_2O, and the lysate was collected, diluted and plated on CYE to determine the number of CFUs.

**Salt sensitivity**

For salt sensitivity assays, bacteria were grown on CYE plates for approximately 48 h, and then scraped, resuspended in H_2O and adjusting for OD_{600nm}. Ten-fold serial dilutions of the bacterial suspensions were spotted on CYE plates made with and without the addition of 100 mM NaCl. The plates were incubated at 37 °C for 3 to 4 days before imaging. The experiment was repeated three times and a representative experiment is shown.

**RESULTS**

**Lpg0657 is a highly conserved uncharacterized OmpA-like protein in *L. pneumophila***

The protein encoded by open reading frame Lpg0657 from *L. pneumophila* strain Philadelphia1 is highly conserved among all 10 sequenced *L. pneumophila* strains, with over 97% sequence identity to homologs from strains Lens, ATCC 43290, LPE509, Thunder-Bay, Lorraine, Paris, Alcoy, Corby and HL06041035. Homology also extends to a large number of other Legionella species with over 66% identity to orthologs from *L. shakespearei* and *L. moravica*, and around 50% identity to orthologs from nine other Legionella species.

The lpg0657 gene encodes for a protein of 249 amino acids with several predicted domains, illustrated in Fig. 1A. Secondary structure analysis of the protein sequence, using both the SMART database (Letunic, Doerks and Bork 2012) and the TMpred server (Hofmann and Stoffel 1993), strongly suggests the presence of two inner-membrane protein domains, as well as the predicted soluble OmpA-like domain. OmpA-like domain resembles the C-terminal peptidoglycan-binding domain of Escherichia coli OmpA, but has no homology to the portion of the protein associated with the outer membrane. Indeed, the OmpA-like domain can be found in a number of other peptidoglycan-binding proteins. Open reading frame Lpg0657 was originally annotated as OmpA, but since the protein not predicted to be an outer membrane protein we named the protein CmpA, to denote homology to the C-terminus domain of *E. coli* OmpA.

CmpA is one of six *L. pneumophila* proteins that contain the OmpA-like domain. Protein sequence alignment of all six OmpA-like proteins reveals that beyond the OmpA-like domain, no significant homology can be detected between these proteins (Fig. 1B). The SMART database predicts the presence of an additional protein domain, the zinc finger, Pfam PF13488, overlapping one of the predicted transmembrane helices in CmpA (Fig. 1A). Zinc finger domains are common sequence motifs found in membrane proteins (Kim et al. 2005). There are several examples of zinc finger domains mediating interactions between transmembrane helices and contributing to packing and oligomerization of membrane proteins (Kim, Chamberlain and Bowie 2004; Arbelly et al. 2006; Elbaz, Salomon and Schuldiner 2008). Interestingly, no other *L. pneumophila* protein is predicted to have the same domain architecture as CmpA, and proteins of the same architecture in other organisms remain uncharacterized.

**Generating a cmpA mutant in *L. pneumophila***

To characterize the cmpA gene, we attempted to generate a clean deletion mutant using well-established methods of allelic exchange (Merriam et al. 1997). Efforts to generate a deletion mutant that lacks the entire coding sequence of the cmpA gene have repeatedly and consistently resulted in 100% of the intermediate merodiploid clones resolving back into the wild-type genotype, rather than a knockout genotype. We opted instead to use a transposon mutant from a partially sequenced *mariner* mini transposon (Murata et al. 2006) mutant library. Sequencing showed that the cmpA gene was interrupted at nucleotide 262 downstream of the start site of the gene (illustrated in Fig. 2A).
Figure 1. CmpA belongs to the C-OmpA-like family of L. pneumophila proteins. (A) Schematic representation of predicted protein domains found in the CmpA protein sequence. The cmpA gene encodes for a protein 249 amino acids in length. Two putative transmembrane domains are depicted as gray rectangles marked TM1 and TM2, predicted using both TMpred (Hofmann and Stoffel 1993) and the SMART database (Letunic, Doerks and Bork 2012). TM1 ranges between amino acids 29 and 47, and TM2 ranges between amino acids 76 and 98. The SMART database also predicts the presence of two additional domains, defined by the Pfam database (Finn et al. 2013). A glycine zipper domain, PfamPF13488, is predicted at amino acid range 61–103 and portrayed by a white rectangle encompassing TM2. The OmpA-like domain, Pfam PF00691, ranges between amino acids 132 and 228 and is represented by a black rectangle. The length and location of the different domains are shown to scale, with the ruler at the bottom marking residue numbers. (B) Multiple sequence alignment of all six C-OmpA-like proteins found in L. pneumophila. Sequence alignment was performed using the T-Coffee server (Notredame, Higgins and Heringa 2000; Wallace et al. 2006) and color coded for degree of homology with pink denoting strong homology, as shown in the key. The C-OmpA-like region is highly conserved among all six proteins.

The transposon mutant was transferred to a fresh background using methods previously described (Murata et al. 2006), and the genotype was confirmed by PCR (Fig. 2B), using primers that flank the transposon insertion site (Fig. 2A). A complementation strain was constructed by introducing an expression vector into the cmpA mutant, where expression of the Flag-CmpA fusion protein is driven by an IPTG-inducible promoter. Immunoblot analysis of the complemented strain shows that the expression of the Flag-CmpA fusion protein is strongly regulated by IPTG, with no signal detected in samples from cells that were not induced by IPTG (Fig. 2C).

CmpA is important for efficient intracellular infection

The initial difficulty in obtaining the cmpA mutant has prompted us to test whether the gene is important for housekeeping functions. To test this, we examined the ability of the cmpA mutant to grow in broth. Our results show that cmpA can replicate as well as an isogenic wild-type strain in liquid growth media, showing similar growth kinetics as the wild type (Fig. S1, Supporting Information). To examine the effect of a mutation in cmpA on the ability of L. pneumophila to infect host cells, and replicate within the cells, a quantitative intracellular growth assay was performed. The results clearly demonstrate that the cmpA mutant has an intracellular growth defect. In the protozoan host A. castellanii, growth at 72 h after infection was more than 10 times lower than that observed in the wild-type strain (Fig. 3A). Similar results were obtained using primary bone marrow-derived murine macrophages, where 72 h after infection the mutant reached CFU numbers that were roughly two orders of magnitude below those of the wild type (Fig. 3B). In both hosts tested, the dotA mutant, lacking an essential component of the Dot/Icm secretion system, serves as a negative control. The dotA mutant failed to replicate in both macrophages and protozoa, demonstrating that at the conditions used in the assay, host cells are able to successfully restrict bacterial replication, and
Figure 2. Genotypic characterization of the cmpA mutant. The cmpA mutant was analyzed to verify the location of the transposon insertion within the cmpA gene. (A) A schematic representation of the Lpg0657 locus encoding the cmpA gene. The location of the transposon insertion at nucleotide 262 is marked with a large triangle, and the primers used to characterize the strain are marked by arrows, FWD indicating primer SN242, and REV indicating primer SN243. The two primers span the transposon insertion site, and are designed to generate a 400 base pair (bp) fragment in a PCR with the LP01 wild-type strain CR39, and a 1800 bp fragment in the isogenic cmpA transposon insertion mutant SN272. (B) Gel electrophoresis of genotyping PCRs using genomic DNA as template with primer pair SN242 and SN243. Lanes are marked on the top to indicate the strain tested, WT refers to the LP01 strain CR39, and cmpA indicates the cmpA transposon mutant SN272. (C) Image showing an immunoblot probed with a monoclonal anti-Flag antibody for detection of the Flag-CmpA fusion protein expressed off the pIG1 plasmid. Whole cell lysates from L. pneumophila cmpA mutant harboring the pCmpA plasmid pIG1 (strain SN316) were analyzed. A specific band corresponding to a protein with a molecular weight slightly above 36 kDa was detected in whole-cell lysates from cells that have been induced with IPTG (+ IPTG). The anti-Flag-reactive product was not detected in lysates isolated from cells that were not induced with IPTG (no IPTG). The positions of molecular weight standards (kDa) are indicated.

replication is only observed in strains that have a functional Dot/Icm virulence system (Fig. 3A and B). Importantly, the defect observed in the cmpA mutant was fully complemented by adding the cmpA gene in trans using a plasmid encoding for a Flag-CmpA fusion protein under the control of the IPTG-inducible promoter Ptac. Complementation was observed in both protozoa and mammalian cells (Fig. 3A and B). The complementation data demonstrates that the defect observed in the cmpA mutant is a result of the interruption of the cmpA gene, and not a result of a potential polar effect, or another mutation elsewhere in the genome. To further evaluate the impact of cmpA on the fitness of L. pneumophila during infection of host cells, a competition assay between the cmpA mutant and the isogenic wild-type strain was conducted. The results show that when the two strains were mixed at a ratio of 1:1 and used to initiate an infection in the protozoa A. castellanii, the wild type quickly outcompeted the cmpA strain and 3 days after the initiation of the infection, wild type is the only strain recovered from the infected protozoan culture (Fig. 3C). These results indicate that the cmpA mutant has a significant disadvantage during an intracellular infection.

**Bacterial envelope integrity is not compromised in a cmpA mutant**

As a potential peptidoglycan-binding protein, CmpA could play a role in the maintenance of the bacterial envelope, as was described for the L. pneumophila protein EnhC (Liu, Conover and Isberg 2008). To test this, we used a well-established salt sensitivity plating assay, typically used to detect salt-resistant mutants of Legionella, like dotA and other Dot/Icm mutants (Vogel, Roy and Isberg 1996). The salt sensitivity tests reveal that the cmpA mutant does not display heightened sensitivity to sodium, as compared to wild type (Fig. 4), suggesting that the integrity of the bacterial envelope is not compromised in this mutant. Interestingly, we found that the cmpA mutant was slightly more resistant than the wild type when grown in the presence of sodium, but not as resistant as the dotA strain (Fig. 4).
Figure 3. The CmpA protein is required for efficient intracellular replication in both protozoan and mammalian hosts. *L. pneumophila* growth rates were determined in the protozoan host *A. castellanii* (A), and in mouse bone marrow-derived macrophages (B). Intracellular growth kinetics of *L. pneumophila* strain SN272 (cmpA, triangles) was compared to that of wild-type *L. pneumophila* strain CR39 (WT, closed circles), and ΔdotA mutant strain CR58 (ΔdotA, squares). Growth was significantly attenuated in the cmpA mutant, and this defect was fully complemented in the cmpA mutant expressing CmpA off a plasmid (cmpA pCmpA, inverted triangles). No difference in growth rate was detected in the wild-type strain overexpressing CmpA (WT pCmpA, open circles). Each time point represents the mean number of viable bacteria recovered from triplicate wells ± standard error of the mean. Error bars are obscured by data symbols in data points with very low error. Statistical significance was determined using a Student’s t-test (***P ≤ 0.001, ****P ≤ 0.0001 vs WT). (C) Wild-type strain CR39 (WT, circles) and the isogenic mutant strain SN272 (cmpA, triangles) were mixed at a 1:1 ratio and used at an MOI of 0.25 in an intracellular competition assay in the protozoan host *A. castellanii*, as outlined in Materials and Methods. All experiments were repeated three times, and a representative experiment of each is shown.
HAS been strongly correlated with mutations in the cmpA gene (P < 0.001). The mutant to compete against the wild-type is presented here. The CmpA protein is one of six proteins that contain an OmpA-like homology domain strongly suggests that CmpA may be directly or indirectly involved in processes that facilitate uptake into host cells. Together, the results presented here establish CmpA as a novel virulence factor of L. pneumophila that is important for infection in both protozoan and mammalian hosts.

To begin probing the mechanism by which CmpA contributes to intracellular infection, uptake into cells was examined. Using a gentamicin protection assay, uptake into primary macrophages was quantified. The results show that the cmpA mutant has a defect in entering macrophages, and the defect can be complemented by expression of CmpA in trans (Fig. 5). This suggests that CmpA may be directly or indirectly involved in processes that facilitate uptake into host cells. Together, the results presented here establish CmpA as a novel virulence factor of L. pneumophila that is important for infection in both protozoan and mammalian hosts.

DISCUSSION

The characterization of CmpA, a novel virulence factor of L. pneumophila, is presented here. The CmpA protein is one of six L. pneumophila proteins that contain an OmpA-like homology domain, often associated with virulence. CmpA is highly conserved within L. pneumophila. Nevertheless, a cmpA mutant replicates as well as wild type in broth; therefore, the CmpA protein is unlikely to serve a housekeeping function under these conditions. The strong conservation of the CmpA protein, together with the inability of the cmpA mutant to compete against the wild-type strain in an intracellular growth competition, highlights the evolutionary importance of the cmpA gene as it pertains to intracellular survival.

Secondary structure analysis of the CmpA protein sequence suggests that it is an inner-membrane protein, with two transmembrane helices. The presence of a predicted OmpA-like domain strongly suggests that CmpA has the capacity to bind to teptidoglycan at the cell wall. None of the other L. pneumophila proteins containing the OmpA-like domain are predicted to span the inner membrane twice; therefore, CmpA is likely to have a structure and a function that are distinct from those of the other L. pneumophila OmpA-like proteins.

Salt resistance of the cmpA mutant was assayed in order to determine if CmpA could play a role in the maintenance of the bacterial envelope. Interestingly, we found that not only does the cmpA mutant not display a defect growing in the presence of salt, but growth of this mutant was actually better than that of the wild type under high-salt conditions (Fig. 4). Salt resistance in L. pneumophila has been strongly correlated with mutations in the Dot/Icm secretion system (Vogel, Roy and Isberg 1996), presumably due to decreased leakiness of the Dot/Icm system in these mutants. The cmpA mutant shows an intermediate salt resistance phenotype, with resistance that is higher than that seen in the wild type, but lower than that observed for the dotA mutant. This data suggest that the CmpA protein may interact with the Dot/Icm secretion system, or with another transport apparatus, contributing to the overall flow through that system. For example, CmpA could function as part of a larger complex, and may help anchor that complex to the cell wall, similar to Escherichia coli MotB (DeRosier 1998), which also contains an OmpA-like domain and several transmembrane helices.

Obtaining a clean deletion mutant of cmpA could not be achieved, possibly due to a polar effect that such a deletion would have on neighboring genes. A polar effect could result in deregulation of another gene causing either reduced expression or overexpression of that gene, and suggests that the cmpA gene may be encoded as part of an operon. To exclude the possibility of a polar effect in the transposon mutant studied here, a com-
plementation vector was used for the expression of CmpA under the control of the PtaC-inducible promoter. Both the intracellular growth defects and the uptake defect seen with the cmpA transposon mutant were completely restored upon complementation in trans. These results demonstrate that the phenotypes observed for the cmpA mutant are a result of the disruption of the cmpA gene, and not a result of a polar effect on other genes, or the effect of another mutation elsewhere in the genome.

As is the case for other insertion inactivation mutants, the cmpA mutant may still have a residual portion of the gene expressed, up to the insertion point. The implications of this in terms of the interpretation of our results is that we may be underestimating the contribution of cmpA to the pathogenesis of Legionella. It is possible that a stronger phenotype may have been observed if a complete clean deletion could be generated.

The mechanism by which CmpA contributes to intracellular survival is yet to be determined. Our data suggest that the intracellular defect of the cmpA mutant may in part be due to reduced uptake into host cells. The results presented here demonstrate the potential importance of OmpA-like proteins for the virulence of pathogenic bacteria.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

FUNDING
This work was supported by the Drexel Graduate Student Association (graduate fellowship to I.P.G.), and by the Pennsylvania Department of Health, Commonwealth Universal Research Enhancement Program (CURE grant to S.N).

Conflict of interest. None declared.

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