FEMS Microbiology Letters, 363, 2016, fnw173

doi: 10.1093/femsle/fnw173
Advance Access Publication Date: 14 July 2016
Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

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

Ian P. Goodwin[1], Ogan K. Kumova[1] and Shira Ninio[1,2,*]

[1]Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA 19129, USA and [2]The Yigal Allon Kinneret Limnological Laboratory, Israel Oceanographic and Limnological Research, Migdal 14950, Israel

*Corresponding author: The Yigal Allon Kinneret Limnological Laboratory, Israel Oceanographic and Limnological Research, PO Box 447, Migdal 14950, Israel. Tel: +972 (4) 661-8924; E-mail: shira.ninio@ocean.org.il

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-teminus. 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).

Received: 11 May 2016; **Accepted:** 11 July 2016

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 Ppl/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 media 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% CO_2. Bone marrow-derived macrophages were cultured from female A/J mice as described previously (Celada *et al.* 1984). *Acanthamoeba castellanii* (ATCC 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% CO_2 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 5×10^4 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**

Name	Genotype	Reference
CR39	*L. pneumophila* serogroup 1, strain Lp01 *rpsL*	Berger and Isberg (1993)
CR58	Lp01 *rpsL dotA*	Zuckman, Hung and Roy (1999)
SN272	Lp01 *rpsL cmpA*$^{262::Tn}$, Kan^r	This study
SN316	Lp01 *rpsL cmpA*$^{262::Tn}$, Kan^r (pIG1)	This study
SN315	Lp01 *rpsL* (pIG1)	This study

Plasmids

Name	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

Name	Sequence	Restriction site
SN242	AGCTGTTAAGAGAGTAGGAG	N/A
SN243	GTTTCAGCTTGCGCTTGAG	N/A
SN264	GCGGGATCCTTTTGCGAAATTTAATGAGATGCT	BamHI
SN280	GCGCTGCAGTTATTTTACATAAGCCATTTGTG	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 × Flag tag sequence was amplified using primers SN137 and SN138 and the resulting PCR fragment was digested with BglII and BamHI and ligated with pJB1806 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 Ptac IPTG-inducible promoter. For the generation of plasmid pIG1, 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-P 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 ImgeQuant LAS4000 (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Gentamicin protection assay to measure uptake

Gentamicin protection assay was performed as described in Hilbi, Segal and Shuman (2001). In brief, bone marrow-derived macrophages from A/J mice were plated at a density of 1×10^5 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 Pfam PF00691. The 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 glycine zipper, Pfam PF13488, overlapping one of the predicted transmembrane helices in CmpA (Fig. 1A). Glycine zipper domains are common sequence motifs found in membrane proteins (Kim *et al.* 2005). There are several examples of glycine zipper domains mediating interaction between transmembrane helices and contributing to packing and oligomerization of membrane proteins (Kim, Chamberlain and Bowie 2004; Arbely *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* minitransposon (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).

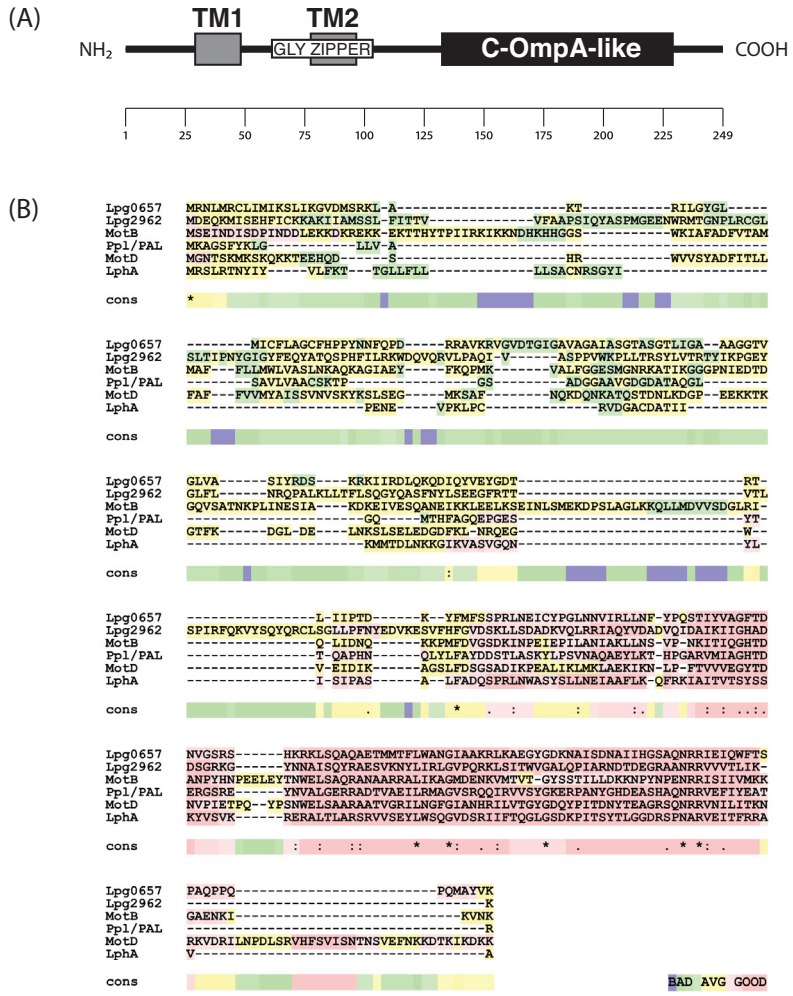

Figure 1. CmpA belongs to the C-OmpA-like family of *L. pneumophila proteins*. (**A**) Schematic representation of predicted protein domains found in the CmpA protein sequence. The *cmpA* gene encodes for a protein 249 amino acids in length. Two putative transmembrane domains are depicted as gray rectangles 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, Pfam PF13488, 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

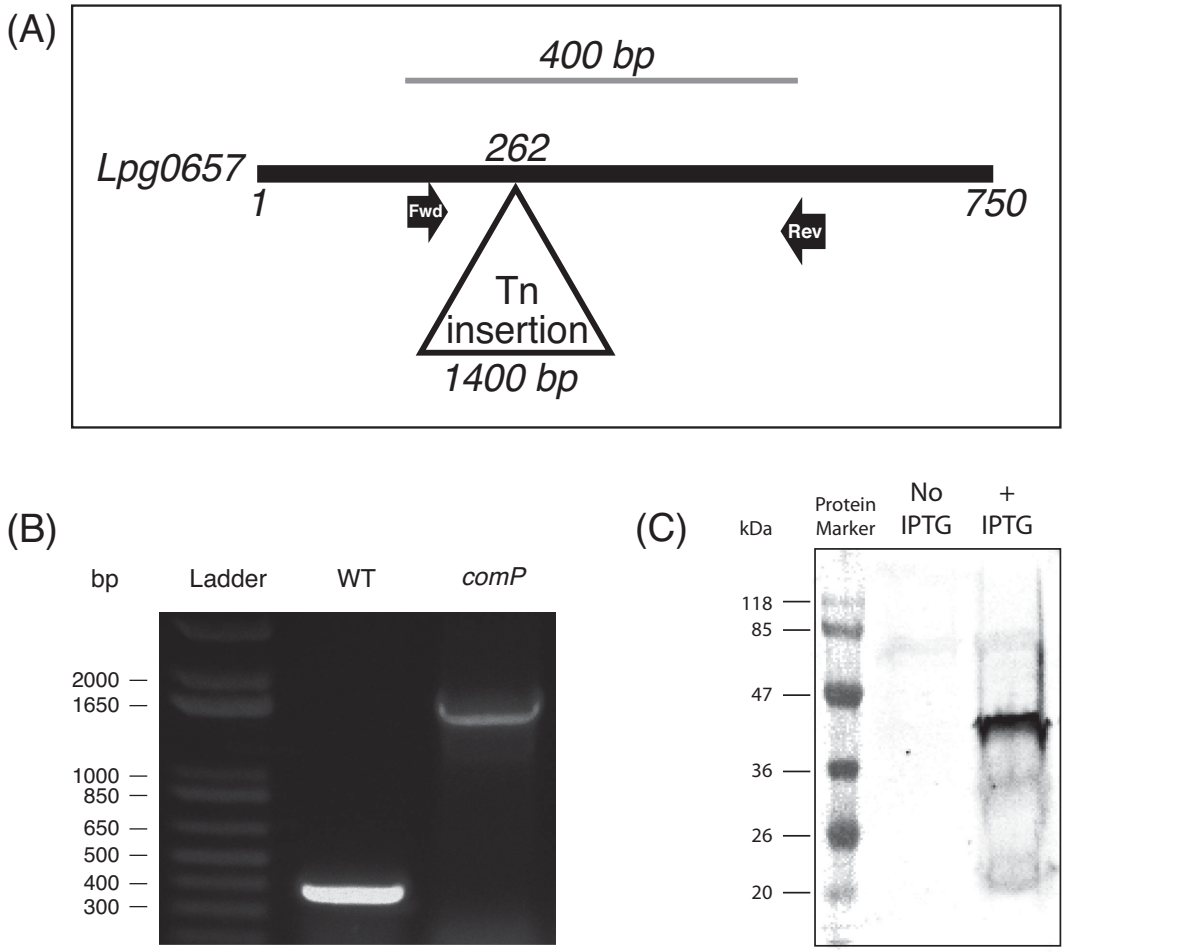

Figure 2. Genotypic characterization of the *cmpA* mutant. The *cmpA* mutant was analyzed to verify the location of the transposon insertion within the *cmpA* gene. (**A**) A schematic representation of the Lpg0657 locus encoding the *cmpA* gene. The location of the transposon insertion at nucleotide 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 LP01strain 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 envelop 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).

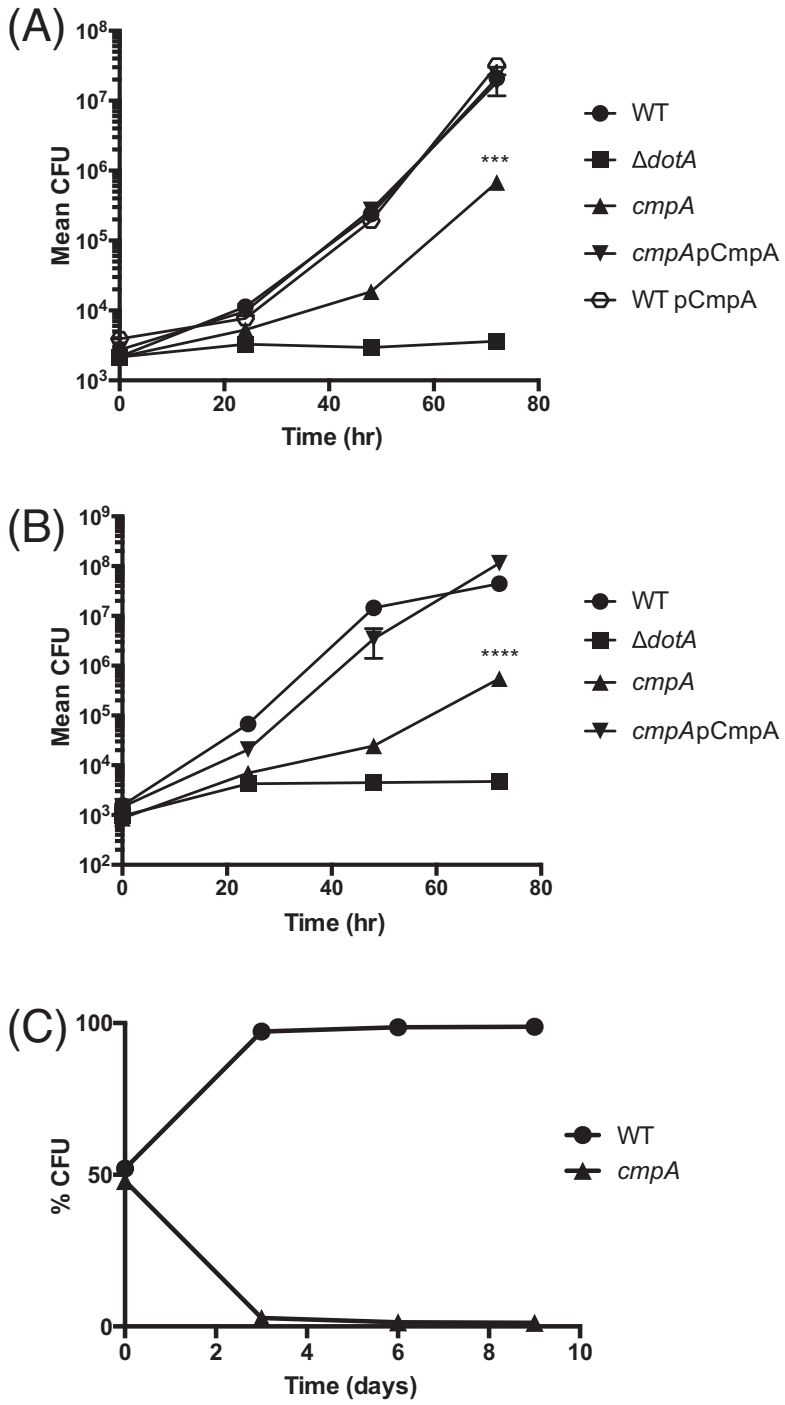

Figure 3. The CmpA protein is required for efficient intracellular replication in both protozoan and mammalian hosts. *L. pneumophila* growth rates were determined in the protozoan host *A. castellanii* (**A**), and in mouse bone marrow-derived macrophages (**B**). Intracellular growth kinetics of *L. pneumophila* strain SN272 (*cmpA*, triangles) was compared to that of wild-type *L. pneumophila* strain CR39 (WT, closed circles), and Δ*dotA* mutant strain CR58 (Δ*dotA*, 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 (***$P \leq 0.001$, ****$P \leq 0.0001$ 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.

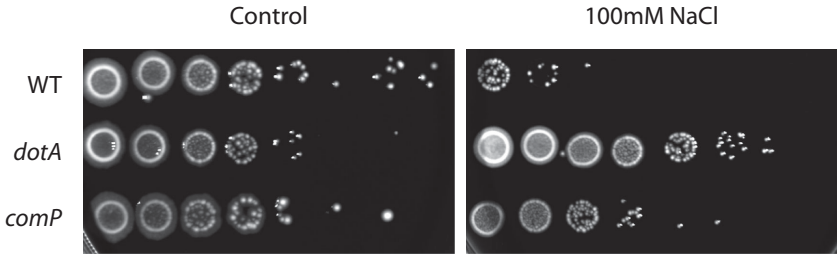

Figure 4. The cmpA mutant has an intermediate salt resistance phenotype. The ability of the different strains to grow in the presence of 100 mM NaCl was assayed by spotting bacteria on plain CYE plates (control) or CYE plates supplemented with 100 mM NaCl. Plate grown cells were scraped, resuspended in H_2O and adjusted for $OD_{600\ nm}$. Ten-fold serial dilutions of the wild-type strain CR39 (WT), mutant strain SN272 (*cmpA*) and *dotA*-deficient strain CR58 (Δ*dotA*) were plated and allowed to grow at 37°C for 3 days. A representative experiment out of three repeats is shown.

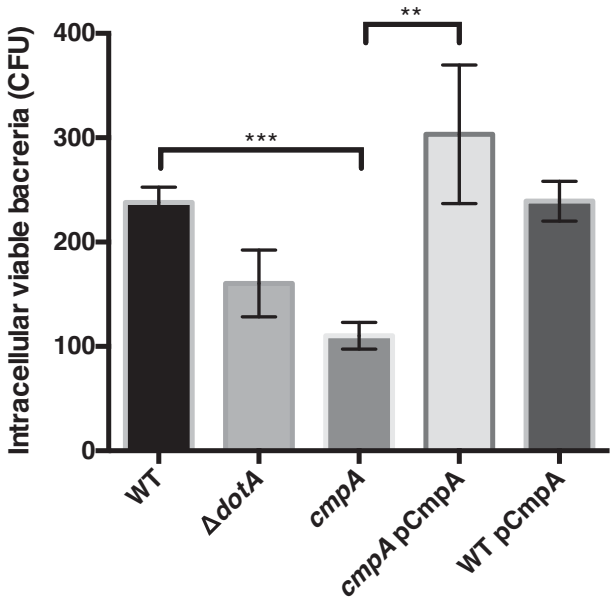

Figure 5. Uptake into mouse macrophages is reduced in the *cmpA* mutant. The efficiency of uptake into bone marrow-derived A/J mouse macrophages was tested using a gentamicin protection assay. *L. pneumophila* strains were used to infect macrophages at an MOI of 100, uptake was allowed to proceed for 10 min before the cells were treated with gentamicin to eliminate extracellular bacteria and cell lysates plated for CFU. CFU recovered from the cells infected with wild-type strain CR39 (WT), mutant strain SN272 (*cmpA*), *dotA*-deficient strain CR58 (Δ*dotA*) and the wild type and *cmpA* strains harboring plasmid pIG1 (WT pCmpA and *cmpA* pCmpA, respectively) are plotted. Uptake of the *cmpA* strain is reduced by roughly 50%, and is restored to wild-type levels in the complemented strain. Values represent the mean number of viable bacteria recovered from triplicate wells ± standard error of the mean. Statistical significance was determined using a Student's t-test (**$P \leq 0.01$, ***$P \leq 0.001$).

CmpA has a role in uptake into host cells

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 peptidoglycan 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 vacuolar pathogens.

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.

REFERENCES

Arbely E, Granot Z, Kass I *et al.* A trimerizing GxxxG motif is uniquely inserted in the severe acute respiratory syndrome (SARS) coronavirus spike protein transmembrane domain. *Biochemistry* 2006;**45**:11349–56.

Bardill JP, Miller JL, Vogel JP. IcmS-dependent translocation of SdeA into macrophages by the *Legionella pneumophila* type IV secretion system. *Mol Microbiol* 2005;**56**:90–103.

Berger KH, Isberg RR. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol Microbiol* 1993;**7**:7–19.

Burstein D, Zusman T, Degtyar E *et al.* Genome-scale identification of *Legionella pneumophila* effectors using a machine learning approach. *PLoS Pathog* 2009;**5**:e1000508.

Celada A, Gray PW, Rinderknecht E *et al.* Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. *J Exp Med* 1984;**160**:55–74.

Coers J, Kagan JC, Matthews M *et al.* Identification of Icm protein complexes that play distinct roles in the biogenesis of an organelle permissive for *Legionella pneumophila* intracellular growth. *Mol Microbiol* 2000;**38**:719–36.

DeRosier DJ. The turn of the screw: the bacterial flagellar motor. *Cell* 1998;**93**:17–20.

Elbaz Y, Salomon T, Schuldiner S. Identification of a glycine motif required for packing in EmrE, a multidrug transporter from *Escherichia coli*. *J Biol Chem* 2008;**283**:12276–83.

Fields BS. The molecular ecology of *legionellae*. *Trends Microbiol* 1996;**4**:286–90.

Finn RD, Bateman A, Clements J *et al.* Pfam: the protein families database. *Nucleic Acids Res* 2013;**42**:D222–30.

Fraser DW, Tsai TR, Orenstein W *et al.* Legionnaires' disease: description of an epidemic of pneumonia. *New Engl J Med* 1977;**297**:1189–97.

Gholipour A, Moosavian M, Makvandi M *et al.* Development of an indirect sandwich ELISA for detection of urinary antigen, using *Legionella pneumophila* PAL protein. *World J Microb Biot* 2014;**30**:1463–71.

Hilbi H, Segal G, Shuman HA. Icm/dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Mol Microbiol* 2001;**42**:603–17.

Hoffman PS, Ripley M, Weeratna R. Cloning and nucleotide sequence of a gene (ompS) encoding the major outer membrane protein of *Legionella pneumophila*. *J Bacteriol* 1992;**174**:914–20.

Hofmann K, Stoffel W. TMBASE - a database of membrane spanning protein segments. *Biol Chem H-S* 1993;**374**:166.

Horwitz MA. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J Exp Med* 1983;**158**:2108–26.

Isberg RR, O'Connor TJ, Heidtman M. The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. *Nat Rev Microbiol* 2009;**7**:13–24.

Kim MJ, Sohn JW, Park DW *et al.* Characterization of a lipoprotein common to *Legionella* species as a urinary broad-spectrum antigen for diagnosis of Legionnaires' disease. *J Clin Microbiol* 2003;**41**:2974–9.

Kim S, Chamberlain AK, Bowie JU. Membrane channel structure of *Helicobacter pylori* vacuolating toxin: role of multiple GXXXG motifs in cylindrical channels. *P Natl Acad Sci USA* 2004;**101**:5988–91.

Kim S, Jeon T-J, Oberai A *et al.* Transmembrane glycine zippers: physiological and pathological roles in membrane proteins. *P Natl Acad Sci USA* 2005;**102**:14278–83.

Kubori T, Koike M, Bui XT *et al.* Native structure of a type IV secretion system core complex essential for *Legionella pathogenesis*. *P Natl Acad Sci USA* 2014;**111**:11804–9.

Letunic I, Doerks T, Bork P. SMART 7: recent updates to the protein domain annotation resource. *Nucleic Acids Res* 2012;**40**:D302–5.

Liu M, Conover GM, Isberg RR. *Legionella pneumophila* EnhC is required for efficient replication in tumour necrosis factor alpha-stimulated macrophages. *Cell Microbiol* 2008;**10**:1906–23.

Ludwig B, Schmid A, Marre R *et al.* Cloning, genetic analysis, and nucleotide sequence of a determinant coding for a 19-kilodalton peptidoglycan-associated protein (Ppl) of *Legionella pneumophila*. *Infect Immun* 1991;**59**:2515–21.

McDade JE, Shepard CC, Fraser DW *et al.* Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *New Engl J Med* 1977;**297**:1197–203.

Mahawar M, Atianand MK, Dotson RJ *et al.* Identification of a novel *Francisella tularensis* factor required for intramacrophage survival and subversion of innate immune response. *J Biol Chem* 2012;**287**:25216–29.

Marra A, Blander SJ, Horwitz MA *et al.* Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. *P Natl Acad Sci USA* 1992;**89**:9607–11.

Martinez E, Cantet F, Fava L *et al.* Identification of OmpA, a *Coxiella burnetii* protein involved in host cell invasion, by multi-phenotypic high-content screening. *PLoS Pathog* 2014;**10**:e1004013.

Merriam JJ, Mathur R, Maxfield-Boumil R *et al*. Analysis of the *Legionella pneumophila fliI* gene: intracellular growth of a defined mutant defective for flagellum biosynthesis. *Infect Immun* 1997;**65**:2497–501.

Murata T, Delprato A, Ingmundson A *et al*. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. *Nat Cell Biol* 2006;**8**:971–7.

Nagai H, Cambronne ED, Kagan JC *et al*. A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *P Natl Acad Sci USA* 2005;**102**:826–31.

Nagai H, Kubori T. Type IVB secretion systems of *Legionella* and other gram-negative bacteria. *Front Microbiol* 2011; **2**:136.

Ninio S, Zuckman-Cholon DM, Cambronne ED *et al*. The *Legionella* IcmS-IcmW protein complex is important for Dot/Icm-mediated protein translocation. *Mol Microbiol* 2005;**55**:912–26.

Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 2000;**302**:205–17.

Ristow P, Bourhy P, da Cruz McBride FW *et al*. The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog* 2007;**3**:e97.

Rothmeier E, Pfaffinger G, Hoffmann C *et al*. Activation of Ran GTPase by a *Legionella* effector promotes microtubule polymerization, pathogen vacuole motility and infection. *PLoS Pathog* 2013;**9**:e1003598.

Roy CR, Isberg RR. Topology of *Legionella pneumophila* DotA: an inner membrane protein required for replication in macrophages. *Infect Immun* 1997;**65**:571–8.

Segal G, Purcell M, Shuman HA. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *P Natl Acad Sci USA* 1998;**95**:1669–74.

Segal G, Shuman HA. *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. *Infect Immun* 1999;**67**:2117–24.

Shin S. Innate immunity to intracellular pathogens: lessons learned from *Legionella pneumophila*. *Adv Appl Microbiol* 2012;**79**:43–71.

Vogel JP, Andrews HL, Wong SK *et al*. Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* 1998;**279**:873–6.

Vogel JP, Roy C, Isberg RR. Use of salt to isolate *Legionella pneumophila* mutants unable to replicate in macrophages. *Ann N Y Acad Sci* 1996;**797**:271–2.

Wallace IM, O'Sullivan O, Higgins DG *et al*. M-Coffee: combining multiple sequence alignment methods with T-Coffee. *Nucleic Acids Res* 2006;**34**:1692–9.

Yu VL, Zuravleff JJ, Gavlik L *et al*. Lack of evidence for person-to-person transmission of Legionnaires' disease. *J Infect Dis* 1983;**147**:362.

Zuckman DM, Hung JB, Roy CR. Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. *Mol Microbiol* 1999;**32**:990–1001.