RESEARCH LETTER

The metal efflux island of *Legionella pneumophila* is not required for survival in macrophages and amoebas

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Abstract

Legionella pneumophila is an intracellular pathogen causing pneumonia-like disease in humans. A 43-kb putative heavy metal efflux gene island was found on the *L. pneumophila* genome. Large *Legionella* deletion strains of the metal efflux genes were tested in human THP-1-derived macrophages and amoebal *Acanthamoeba castellanii* cells and were able to survive and replicate similar to the wild type, suggesting that they do not play a significant role within the intracellular environment. Examination of the sequence of this genomic island revealed that some genes were not accurately annotated and there were no known metal-responsive regulators encoded in this region. Therefore, functional roles of these metal resistance genes were tested by conducting metal resistance assays. Individual genes were cloned in an expression vector and expressed in an appropriate metal-sensitive *Escherichia coli* background with varying concentrations of the tested metal. Of the 11 efflux systems, a role was determined only for one. A Cu(I)-translocating P_{IB}-type ATPase was shown to be encoded by *lpg1024*. This gene, termed *copA*, complemented a copper-sensitive (Δ*copA*) *E. coli* strain *in trans* and was able to confer copper resistance.

Introduction

Legionella pneumophila is an intracellular pathogen that is able to survive and replicate within human and amoebal hosts by prevention of the phago-lysosome fusion. It is ubiquitous in freshwater and man-made aquatic habitats, thriving in warm environments freely, within free-living amoebae, or associated in biofilms (Atlas, 1999). *Legionella pneumophila* is the causative agent of legionellosis (McDade *et al.*, 1977), a pneumonia-like disease that is attributed to approximately 4–20% of cases of community-acquired pneumonia (Rusin *et al.*, 1997).

Survival within host human macrophages is a multifaceted process involving the expression of a variety of virulence factors. Environmental cues, such as inorganic ion concentrations, have been found to modulate expression of virulence factors (Mekalanos, 1992). Metal-induced up-regulated genes have been identified in the host intracellular environment. Examples include the PhoP/PhoQ two-component regulatory system of *Salmonella*, which is regulated by the low magnesium concentration within the host phagosome (Garcia Vescovi *et al.*, 1996), and the *helABC* cluster of *Legionella*, a tripartite efflux pump that appears to detoxify a metal-rich environment. The *helABC* cluster resides in a 43-kb heavy-metal efflux gene island, encoding three putative RND-type metal efflux systems and five P-type ATPases. Expression of these genes may be involved in metal homeostasis and subsequent survival within the host human macrophage.

For intracellular pathogens, metal ion concentrations and regulation processes within the phagosome are not well understood. Importantly, these metal ions play crucial roles for a wide array of biological processes for these microorganisms. Consequently, the link between necessary metals and the elemental characterization of the niche within the vacuole must be further investigated. Recent work has shed some light on the types and quantities of metals within the mycobacterial vacuole (Wagner *et al.*, 2005). The concentration of certain metals such as copper and zinc within the phagosome occupied with pathogenic *Mycobacterium* was at

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higher levels than nonpathogenic species. These data suggest that metal concentrations within the phagosomal compartment vary depending on the expression of specific virulence factors. However, it is not understood how metals and the phagosomal niche are entwined.

To determine whether genes present in the heavy-metal efflux gene island are necessary for intracellular survival and replication, cell growth was measured by green fluorescent protein (GFP) expression in knockouts of specific regions. Moreover, we aimed to determine whether these genes played a role in metal homeostasis by functionally characterizing these genes via measurement of bacterial growth in the presence of the appropriate metal in a relevant metal-sensitive *Escherichia coli* background.

Materials and methods

Bacterial strains and plasmids

The strains used in this study are listed in Supporting Information, Table S1. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth at 37 °C. To study metal inhibition of growth, strains were grown overnight from a single colony in LB and diluted 1 : 50 the following day into LB supplemented with $AgNO_3$ (12.5, 25, 37.5, or 50 μM), $CaCl_2$ (25, 50, or 100 mM), $CdCl_2$ (10, 20, 30, 50 or 100 μM), $CuCl_2$ (l.5, 2.0, 2.5, 3.0, or 3.5 mM), or $ZnSO_4$ (0.25, 0.5, 0.75, or 1.0 mM). Antibiotic concentrations used were as follows: chloramphenicol, 25 μg mL^{-1} and kanamycin, 50 μg mL^{-1}. After 16 h incubation period at 37 °C, cell growth was measured at $OD_{600\,nm}$. All experiments were performed at least three times, and the data shown are from one representative experiment performed with triplicate cultures, averaged, and SD ($n = 3$).

Legionella pneumophila strains used are derivatives of strain JR32, a restriction-deficient, constitutively competent streptomycin-resistant derivative of the Philadelphia-1 isolate (de Felipe *et al*., 2008). *Legionella pneumophila* strains were grown in liquid media, *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract, or on solid media, ACES-buffered charcoal yeast extract plates. Chloramphenicol and kanamycin were used at 5 and 50 μg mL^{-1}, respectively. Deletion mutants of the efflux-pump genes in JR32 were constructed by allelic exchange (Segal & Shuman, 1997). Deletions were then moved to a clean genetic background by natural transformation of the KS79 strain with genomic DNA carrying the deletions. The obtained strains were transformed with plasmid pXDC31 and used to measure intracellular growth by GFP fluorescence.

Plasmids for expression of the putative efflux system were obtained by cloning the corresponding genes under control of P*tac* in the polylinker of pMMB207C. The operon carrying the three genes encoding RND pumps (about 6 kb) was cloned in as two 3-kb inserts by introducing silent restriction sites. Primer and plasmid sequences are available upon request.

Intracellular growth in THP-1-derived human macrophages and *Acanthamoeba castellanii*

Human THP-1 cells were routinely maintained in RPMI 1640, 2 mM glutamine, 10% fetal calf serum (FCS) at 37 °C under CO_2 (5%). For *Legionella* infection, THP-1 cells were incubated overnight in a 96-well microplate (1×10^5 cells per well) in media containing 10 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA). After the overnight stimulation, cells were washed and allowed to stabilize in medium without PMA for 2 days. Before the infection, the medium was replaced with 100 μL per well of warm CO_2-independent tissue culture media (Gibco Invitrogen Corporation, Cat. no. 18045) containing 10% FCS, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 5 μg mL^{-1} chloramphenicol. Cells were then infected with *L. pneumophila* strains harboring the GFP-expressing plasmid pXDC31 grown on charcoal–yeast extract plates containing 0.5 mM IPTG. *Legionella pneumophila* were resuspended at 1×10^8 cells mL^{-1} in CO_2-independent medium and 10 μL of the bacterial suspension was added per well (a multiplicity of infection of 10). Infections were synchronized by spinning the bacteria at 650 ***g*** for 10 min onto the adhered host cells.

Infection of *A. castellanii* (ATCC 30234) proceeded similarly. *Acanthamoeba castellanii* was grown as adherent cells in proteose peptone–yeast extract–glucose medium (PYG) at 30 °C. For *Legionella* infection, *A. castellanii* were resuspended in PYG and seeded in a 96-well microplate (1×10^5 cells per well) and incubated at 30 °C for 1 h or until cells were adherent. The PYG was aspirated and replaced with warm (30 °C) AC buffer containing 0.5 mM IPTG and 5 μg mL^{-1} chloramphenicol. *Legionella pneumophila*, resuspended in AC buffer, was added to the wells at a multiplicity of infection of 10. Infections were synchronized by spinning the bacteria at 650 ***g*** for 10 min onto the adhered host cells.

Intracellular multiplication of *Legionella* in THP-1 cells or *A. castellanii* was monitored automatically by measuring GFP fluorescence at an excitation of 485 nm and emission of 520 nm in a Tecan Infinite M200 plate reader every hour for 72 h. Fluorescence data were subjected to background subtractions (noninfected sample), and time *x*/time 0 calculations (to produce normalized relative fluorescence values). All intracellular multiplication experiments were performed at least three times, and the data shown are from one representative experiment performed in triplicate wells and averaged.

Results and discussion

The putative metal efflux gene island is not required for survival in human macrophages and amoebas

Analysis of the *L. pneumophila* genome for candidate genes encoding heavy-metal efflux systems revealed the presence of a genomic region rich in genes for putative efflux systems. The region from *lpg1006* to *lpg1035* carries 14 genes possibly encoding three RND-type systems and five P-type ATPases (Fig. 1). An additional P-type ATPase gene and three genes encoding an additional RND-type system are located 50 kb away from this region. Moreover, some of these genes were shown to be upregulated during infection in human macrophages (Rankin *et al.*, 2002).

To study the role of these efflux systems in the biology of *L. pneumophila*, large deletions to remove all or part of the efflux system-encoding genes were constructed (Fig. 1). The deletion strains did not show any growth defect in rich medium (data not shown), suggesting that this genomic region is not required for viability. Little is known about the content of the intracellular eukaryotic cell vacuole in which *L. pneumophila* resides and replicates, but it is possible that *L. pneumophila* uses efflux systems to resist the toxic effects of monovalent and divalent cations and metals present within the eukaryotic cell vacuole. To determine whether the efflux systems were required for survival of *L. pneumophila* in the replicative vacuole, we transformed the deletion strain with a GFP-expressing plasmid. The strains were used to infect macrophage-like PMA-differentiated human THP-1 monocyte cells. Bacterial survival and growth was monitored by fluorescence measurements during a 72-h time course. As expected, wild-type *L. pneumophila* showed a high level of growth (up to 50-fold increase in fluorescence intensity), while the type IV secretion mutant *dotA* failed to grow (Fig. 2). All of the genomic deletion strains were able to grow intracellularly, suggesting that the efflux systems and the other deleted genes are not required for intracellular growth in human macrophages.

Intracellular growth and survival of *L. pneumophila* in the amoebal cell *A. castellanii* was also tested. The wild-type *L. pneumophila* grew robustly, while the *dotA* mutant failed to grow (Fig. 2). The genomic deletion strains were capable of intracellular survival and growth. These data suggest that

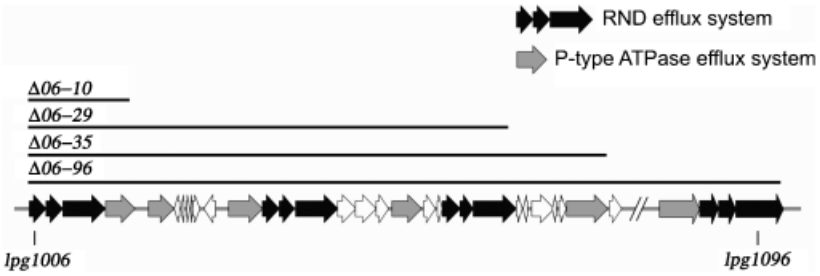

Fig. 1. Diagram representing the genomic region of the metal efflux gene island of *Legionella pneumophila* and the four deletions that were created (Δ06-10, Δ06-29, Δ06-35, and Δ06-96).

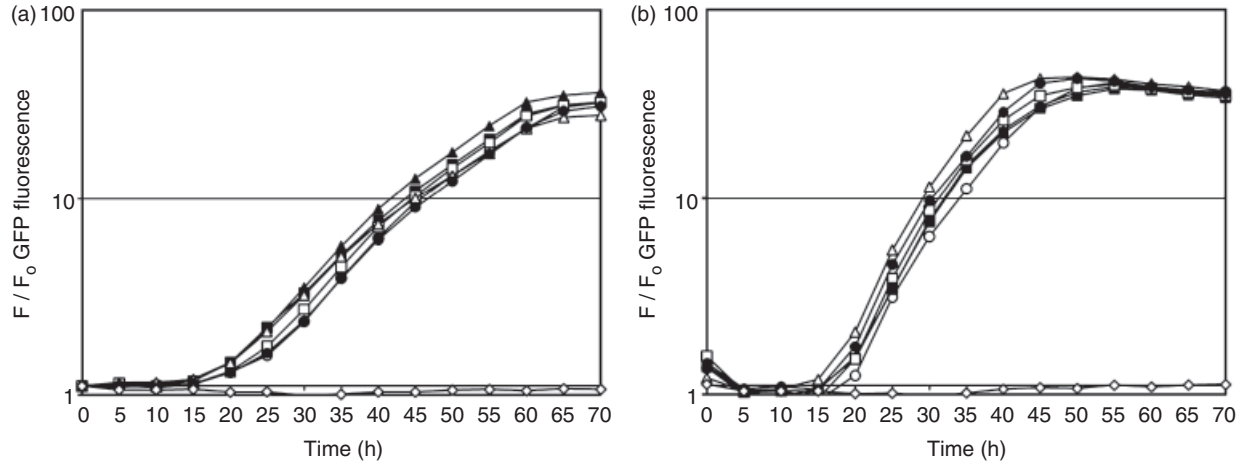

Fig. 2. Intracellular growth of *L. pneumophila* and deletion strains of the high-density gene efflux region in human THP-1 cells (a) and *A. castellanii* (b). Δ06-10 is Δ*lpg*1006–*lpg*1010, Δ06-029 is Δ*lpg*1006–*lpg* 1029, Δ06-35 is Δ*lpg*1009–*lpg*1035 and Δ06-96 is Δ*lpg*1006–*lpg*1096. ○, wild type; ■, Δ06-10; ▲, Δ06-29; □, Δ06-35; △, Δ06-68; ●, Δ06-96; ◇, Δ*dotA*.

under the conditions tested, the efflux pumps are not required for survival and growth in either amoebal or human macrophage cells.

Other genes encoding efflux systems are present in the *L. pneumophila* genome, for example the putative Cu(I)-P_{IB}-type ATPase (*lpg1626*), and might compensate for the loss of the efflux system genes present in the deleted region(s).

Some genes on the heavy-metal efflux gene island need to be reannotated

The sequence of the putative metal efflux gene island was thoroughly analyzed. First, we searched for possible metal-responsive regulators because previous research had shown that some of the genes in the metal efflux gene island were upregulated in macrophages (Rankin *et al.*, 2002). Usually, metal-responsive regulators are encoded in close vicinity of the structural genes they regulate. There are no known metal-responsive regulators encoded on the metal efflux gene island and only very few are present on the chromosome. In fact, the two regulators present on the *L. pneumophila* chromosome, ArsR-like (*lpg2723*) and MerR-like (*lpg0057*) transcriptional regulators, do not appear to regulate a metal transporter. Both precede an NADH-dependant flavin oxidoreductase and a glutaredoxin, respectively. It remains unknown how genes present on the heavy-metal efflux gene island are regulated.

Based on analysis of the gene sequence, several genes present on the heavy-metal efflux gene island were

Table 1. Functional analysis of heavy-metal efflux gene island

	Metal tested in relevant background			
Plasmid and putative function	Cu^{2+}	Ag^{2+}	Zn^{2+}	Cd^{2+}
pXDC1	Δ*cusCFBA*, Δ*cueO*	Δ*cusCFBA*, Δ*cueO*	Δ*zntA*	Δ*zntA*
RND-type transporter	No resistance	No resistance	No resistance	No resistance
lpg1006-1008				
pXDC2	Δ*copA*	NA	Δ*zntA*	Δ*zntA*
P-Type ATPase	No resistance		No resistance	No resistance
lpg1009				
pXDC3	Δ*copA*	NA	Δ*zntA*	Δ*zntA*
P-Type ATPase	No resistance		No resistance	No resistance
lpg1010				
pXDC4	NA	NA	Δ*zntA*	Δ*zntA*
P-Type ATPase			No resistance	No resistance
lpg1017				
pXDC5	Δ*cusCFBA*, Δ*cueO*	Δ*cusCFBA*, Δ*cueO*	Δ*zntA*	Δ*zntA*
RND-type transporter	No resistance	No resistance	No resistance	No resistance
lpg1018-1020				
pXDC6	Δ*copA*	Δ*copA*	NA	NA
P-Type ATPase	Resistance	No resistance		
lpg1024				
pXDC7	Δ*cusCFBA*, ΔcueO	Δ*cusCFBA*, Δ*cueO*	Δ*zntA*	Δ*zntA*
RND-type transporter	No resistance	No resistance	No resistance	No resistance
lpg1027-1029				
pXDC08	Δ*cusCFBA*, Δ*cueO*	NA	NA	NA
Multi-copper oxidase	No resistance			
lpg1035	Δ*copA*			
	No resistance			
pXDC9	Δ*copA*	NA	NA	Δ*zntA*
P-Type ATPase	No resistance			No resistance
lpg1093				
pXDC10	Δ*cusCFBA*, Δ*cueO*	Δ*cusCFBA*, Δ*cueO*	Δ*zntA*	NA
RND-type transporter	No resistance	No resistance	No resistance	
lpg1094-1096				
pXDC11	Δ*cusCFBA*, Δ*cueO*	Δ*cusCFBA*, Δ*cueO*	Δ*zntA*	Δ*zntA*
RND-type transporter	No resistance	No resistance	No resistance	No resistance
lpg2134-2136				

Resistance, minimum inhibitory concentrations were comparable to wild-type; no resistance, minimum inhibitory concentrations were similar to empty vector control; NA, not assayed.

incorrectly annotated. For example, *lpg1017* (*mgtA*) and *lpg1093* (*pacL*) were annotated as an Mg^{2+} ATPase and a cation ATPase, respectively. However, BLAST analysis suggests that both *mgtA* and *pacL* are very similar to each other and both are likely to encode a Ca^{2+}-translocating P-type ATPase. Furthermore, the stop codons of these genes encoding a P-type ATPase overlap with the start codon of the first gene of the downstream genes encoding an RND complex, indicating transcriptional and functional linkage. Unfortunately, it is difficult to determine the substrate(s) in cases where there is no apparent signature sequence. In addition, *lpg1035* has been annotated as a copper P_{IB}-type ATPase, but rather is more likely a multicopper oxidase and outer membrane protein.

lpg1024 encodes a Cu(I)-translocating P_{IB}-type ATPase $CopA_{LPG1}$

Functional roles of the genes from the putative metal efflux gene island were investigated by testing metal resistance in a relevant genetic *E. coli* background. Putative functions of each candidate gene are listed in Table 1. To determine whether these genes promoted resistance to their predicted cations, we conducted assays in which the cells containing the individual plasmids were tested for growth in media containing Cu^{2+}, Ag^{+}, Zn^{2+}, Cd^{2+}, or Ca^{2+}. None of these candidate genes appeared to confer resistance to the individual metals tested when compared with the empty vector control or the relevant metal-sensitive mutant background (Table 1), with the exception of *lpg1024*, which conferred resistance to Cu^{2+}.

It is possible that the candidate genes investigated in this study may confer metal resistance; however, complementing these genes *in trans* in an *E. coli* background may not be sufficient to detect a phenotype because of codon usage bias or because an *E. coli* background may not be a suitable environment for the function of *Legionella* proteins. Other RND systems such as CzcCBA from *Cupriavidus metallidurans* CH34 could also not confer metal resistance in *E. coli* strains tested (unpublished data).

BLAST analysis suggested *lpg1024* $CopA_{LPG1}$ to be a Cu^{2+}-P_{IB}-type ATPase (Axelsen & Palmgren, 1998). *Lpg1024* was able to promote resistance to copper when compared with the copper-sensitive (Δ*copA*) mutant and the empty vector control, Δ*copA* (pMMB207C) (Fig. 3).

$CopA_{LPG1}$ exhibits 45% amino acid sequence identity to $CopA_{EC}$ of *E. coli*. $CopA_{EC}$ has been shown to be a copper-translocating P_{IB}-type ATPase involved in copper resistance (Rensing *et al.*, 2000). P_{IB}-type ATPases generally contain one or more cytosolic metal-binding domains (MBD) that are characterized by a pair of N-terminal metal-coordinating $Cys(X)_2Cys$ sequences. For copper export, the metal is transferred to the ATPase MBD. In some cases, metallochaperones can directly transfer metal ions to the transmembrane MBD, circumventing the cytosolic MBD (Gonzalez-Guerrero & Arguello, 2008). Neither of the two N-terminal $Cys(X)_2Cys$ motifs is required for either copper resistance or transport as shown by site-directed mutagenesis (Fan *et al.*, 2001). Interestingly, $CopA_{LPG1}$ contains a derivative metal-coordinating $Cys(X)_2Cys$ sequence in which only one $Cys(X)_2Cys$ sequence is preceded by a $Cys(X)_2Glu$ sequence. It is possible that this divergence may play a role in differentiating metals for subsequent metal export.

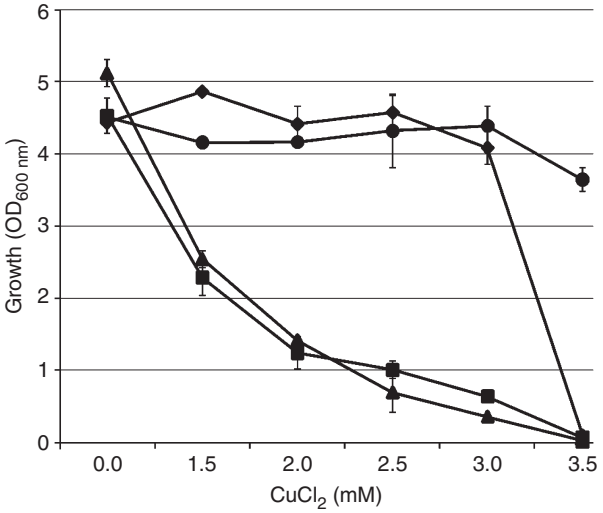

Fig. 3. Copper resistance of *Legionella lpg1024* gene in *Escherichia coli*. Overnight cultures were diluted 1 : 50 into fresh LB medium with the indicated concentrations of $CuCl_2$. Cell growth was monitored at $OD_{600\,nm}$ after 16 h incubation at 37 °C with shaking. *Escherichia coli* W3110 (●) wild type, (◆) Δ*copA* pXDC6, (■) Δ*copA*, and (▲) Δ*copA* pMMB207C. The bars of SD are shown ($n = 6$).

Conclusions

The *Legionella* intracellular lifestyle allows these microorganisms to gain a competitive advantage over other microorganisms by exquisitely manipulating and ultimately evading the host immune response. *Legionella pneumophila* resides within the phagosome of host human macrophages. The link between copper and metal efflux gene expression within the vacuole is not understood. It is possible that within the vacuole copper is delivered by host cell metal transporters to initiate the production of toxic hydroxyl radicals by the Fenton reaction. Consequently, the expression of copper-exporting P_{IB}-type ATPases allows for the extrusion of toxic copper ions, enabling *Legionella* survival and growth within host cell macrophages. MymT, a metallothionein of the intracellular pathogen *Mycobacterium tuberculosis*, has been shown to sequester copper (Gold *et al.*, 2008). MymT is sensed by the copper-responsive transcriptional repressor CsoR, which allows expression of the copper-exporting ATPase CtpV in macrophages (Liu

et al., 2007). The source of the copper is not known. Nonetheless, the involvement of copper and copper extrusion strategies within the phagosome of macrophages is significant and must be further characterized. Surprisingly, we did not observe any effect of a complete deletion of the putative metal efflux gene island on survival in human macrophages and amoebas, which would indicate that this region is of functional importance at another stage in the life cycle of *Legionella*. However, there are additional copies of these genes not present in the metal-efflux gene island but elsewhere on the chromosome that may play a role in intracellular survival. Clearly, at least one gene, probably more, encodes a metal efflux pump.

Legionella is able to survive in environments outside of host eukaryotic cells, for example warm water tanks. In fact, the presence of *Legionella* in an engineered water system such as hospitals poses a significant health threat. Ag/Cu ionization is a widely used method to eradicate *Legionella* from hospital water systems (Stout and Yu, 2003). The presence of these gene islands, which could potentially increase resistance to both copper and silver, might decrease the effectiveness of Ag/Cu ionization in the future.

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pathogen-induced microenvironments within the host cell's endosomal system. *J Immunol* **174**: 1491–1500.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Strains.

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