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Polyketide synthase (PKS) reduces fusion of *Legionella pneumophila*-containing vacuoles with lysosomes and contributes to bacterial competitiveness during infection

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Summary

L. pneumophila-containing vacuoles (LCVs) exclude endocytic and lysosomal markers in human macrophages and protozoa. We screened a *L. pneumophila* mini-Tn10 transposon library for mutants, which fail to inhibit the fusion of LCVs with lysosomes by loading of the lysosomal compartment with colloidal iron dextran, mechanical lysis of infected host cells, and magnetic isolation of LCVs that have fused with lysosomes. *In silico* analysis of the mutated genes, *D. discoideum* plaque assays and infection assays in protozoa and U937 macrophage-like cells identified well established as well as novel putative *L. pneumophila* virulence factors. Promising candidates were further analyzed for their co-localization with lysosomes in host cells using fluorescence microscopy. This approach corroborated that the O-methyltransferase, PilY1, TRP-containing protein and polyketide synthase (PKS) of *L. pneumophila* interfere with lysosomal degradation. Competitive infections in protozoa and macrophages revealed that the identified PKS contributes to the biological fitness of *pneumophila* strains and may explain their prevalence in the epidemiology of Legionnaires´ disease.

Introduction

L. pneumophila is an environmental, Gram-negative bacterium and the causative agent of Legionnaires' disease. The bacterium replicates intracellularly within a membrane-bound compartment of protozoan hosts and human macrophages, the *Legionella*-containing vacuole (LCV) (Horwitz, 1983; Roy, 2002). During intracellular infection the LCV actively recruits small GTPases (Arf1, Ran Rab1, Rab7, Rab8 and Rab14), modulates the host phosphoinositide metabolism, modifies the host endocytic pathway, intercepts vesicle trafficking and avoids fusion with lysosomes (Swanson and Isberg, 1995; Abu Kwaik, 1996; Roy *et al*., 1998, Tilney *et al*., 2001; Nagai *et al*., 2002; Roy and Tilney, 2002; Ingmundson *et al*., 2007; Urwyler *et al*., 2009; Ge and Shao, 2011; Hilbi and Haas, 2012; Rothmeier *et al*., 2013). Recently, it has been shown that *L. pneumophila* can inhibit autophagy by irreversibly inactivating the Atg8 lipidation pathway (Choy *et al*., 2012). The pathogen also uses the proteasome machinery to generate amino acids essential for bacterial growth within LCVs (Price *et al*., 2011). Both processes are mediated by effectors of the Dot/Icm type IVB secretion system (T4BSS), which also plays a central role in the inhibition of the fusion of LCVs with lysosomes. So far, more than 300 substrates of T4BSS have been experimentally verified (Isberg *et al*., 2009; Hubber and Roy, 2010; Ge and Shao, 2011; Gomez-Valero *et al*., 2011). Since the loss of a single effector of this translocation apparatus rarely causes a severe defect in intracellular growth, overlapping functions in the effector repertoire were suggested. Moreover, it was speculated that this large number of effectors may reflect a co-evolution with a wide range of protozoan hosts (Ge and Shao, 2011).

Although several factors involved in the inhibition of the fusion of LCVs with lysosomes have already been described, a comprehensive understanding of this hallmark of *L. pneumophila* pathogenesis is far from being understood. Outer membrane vesicles (OMVs), LPS of high molecular weight, and regulators of *L. pneumophila* flagellation all contribute to the avoidance of lysosomal fusion by T4BSS-independent mechanisms (Fernandez-Moreira *et al*., 2006; Byrne and Swanson, 1998; Molofsky *et al*., 2005; Seeger *et al*., 2010). Therefore, it is likely that further molecules that interfere with LCV-lysosome fusion are still unknown. This prompted us to screen a *L. pneumophila* mini-Tn10 transposon library for mutants that fail to avoid the fusion of their respective LCV with lysosomes. The applied protocol included the infection of *D. discoideum* cells with *L. pneumophila* mutants, loading of the lysosomal compartment with colloidal iron dextran, mechanical lysis of infected host cells, and magnetic isolation of LCV that have fused with lysosomes. The mutants obtained were analyzed for their ability to inhibit plaque formation by *D. discoideum* and to replicate in *D. discoideum* and macrophage-like U937 cells. We identified *L. pneumophila* mutants, which exhibit an increased co-localization with lysosomes. The range of mutants verified by this approach propose that interference with lysosomal degradation is multifactorial. *In silico* analysis including species comparisons and competitive infections in protozoa and macrophages suggest that the identified PKS cluster contributes to the biological fitness of *L. pneumophila* strains. The implication for the prevalence of *L. pneumophila* strains in the epidemiology of Legionnaires´ disease is discussed.

Results

Development and evaluation of a screening protocol for obtaining transposon mutants with defects in avoiding lysosomal fusion

L. pneumophila mutants defective in avoiding vacuolar maturation accumulate in the late endosomal and lysosomal compartment. Based on this premise, we developed a screen that facilitates the isolation of such mutants (Fig. 1A). *D. discoideum* cells were infected with pools of *L. pneumophila* Corby mini-Tn10 transposon mutants as detailed below. Subsequently, host cells were incubated with iron-dextran that was chased into lysosomes. Infected host cells were mechanically lysed, and the homogenates then applied onto MiniMACS separation columns in their magnetic holders. The flow-through fractions contained nuclei, mitochondria, ribosomes and other organelles, whereas the eluted fractions were enriched in lysosomes and bacteria that were enclosed in iron-dextran-containing vacuoles. These lysosomal fractions were lysed and plated onto BCYE agar plates supplemented with kanamycin. Bacterial colonies were scraped into liquid medium and the selection process was repeated four times.

The suitability of this protocol for enrichment of mutants unable to avoid lysosomal fusion was validated by comparing the *L. pneumophila* Corby wildtype strain and a *dotA*-negative mutant. LCVs

of *dot*A-negative *L. pneumophila* strains are known to fuse with lysosomes and early endosomes after entry into the host cell (Roy *et al*., 1998). Our results revealed a significantly higher bacterial association with lysosomes for *dot*A-negative bacteria (77%) compared to the *L. pneumophila* Corby wild-type strain (15%) (Fig 1B). Thus, we conclude that the screening protocol is applicable for the isolation of *L. pneumophila* mutants which are defective in arresting LCV-lysosome fusion.

Screening of transposon mutants and identification of transposon insertion sites

To identify novel *L. pneumophila* factors responsible for avoiding lysosomal degradation, a mini-Tn10 transposon mutant library consisting of 5960 mutants was constructed as previously described (Pope *et al*., 1994). The randomness and uniform insertion of the transposon into the chromosome was verified by Southern blot hybridization of 25 randomly chosen mutants. Given that the *L. pneumophila* Corby genome possesses about 3000 open reading frames, the transposon library represents a 2-fold coverage of the genome (Gomez-Valero *et al*., 2011). The mutant library was divided into six equal pools and each pool (A-F) was subjected to four rounds of the described screening protocol (see above). After the final selection, 48 randomly selected colonies from each pool were analyzed for the presence of genetically identical siblings by Southern blot hybridization (Fig.1C). In total, 54 mutants among fifteen sibling families with at least three members per family were selected. The strains obtained from the screen are summarized in Table 1. The sites of transposon insertions were identified by cloning the respective fragments containing the transposon into plasmid pUC19, sequencing and comparing with the *L. pneumophila* Corby genomic sequence. In three cases (D5, E37 and F33), identification of the exact position of insertion was aggravated due to the presence of multiple repeated regions (TP1-TP4) in the *L. pneumophila* Corby genome. To overcome this problem, we utilized the InFiRe method (Shevchuk *et al*., 2012). It should be noted that strains belonging to the same sibling family do not necessarily possess insertions at the same position. In several cases, mutants with insertions in the same gene, but in different positions, were selected from different pools.

In silico *analysis of selected genes*

Based on COG annotation (Clusters of Orthologous Groups of proteins), 11 genes identified in our study belong to the biosynthesis group involved in secondary metabolism, 24 are unclassified or were assigned as unknown or with predicted functions. The minor groups of genes belong to cell motility (5 genes), energy production and conversion (4 genes), signal transduction mechanisms (3 genes), cell wall and membrane biogenesis (1 gene), amino acid transport and metabolism (1 gene), lipid transport and metabolism (1 gene), inorganic ion transport and metabolism (1 gene) and replication recombination and repair (1 gene).

One third of all proteins was predicted to be localized in or associated with the cytoplasmic membrane. The gene product of mutant Lpc2235 (D3) is potentially localized in the bacterial outer membrane. The remaining proteins were either predicted to be cytoplasmic or their subcellular localization could not be assigned. Remarkably, the vast majority of affected genes are present exclusively in *L. pneumophila* strains, but not in environmental or relatively apathogenic isolates such as LLAP10, *L. hackeliae* or *L. micdadei*. This finding was confirmed using the nucleotide BLAST function which is integrated into the *Legionella* Genome browser (LGB). An additional analysis of gene products by the virulence prediction tool VirulentPred (Garg and Gupta, 2008) showed that most affected genes are potential virulence factors (Table 2).

Plaque formation and intracellular replication of selected mutants

The *D. discoideum* plaque assay reveals whether or not the respective *L. pneumophila* mutant displays virulence either by evading amoeboid killing or actively killing the host cell. Bacterial predation by *D. discoideum* was scored by plating amoebae on nutrient agar plates seeded with the respective mutants. Successful predation by the amoebae was visualized by the appearance of clear plaques. The absence of plaques reveals resistance to *D. discoideum* predation and may indicate a virulent phenotype (Shevchuk and Steinert, 2009; Tiaden *et al*., 2013). Based on this scoring system, we divided the mini-Tn10 mutants into four groups (see Table 2 and Material and Methods). Three mutants, Lpc0114 (B23), Lpc1579 (E8) and Lpc2666 (D1), were phenotypically similar to the *dot*A-negative mutant and produced amoebal plaques on more than 50 % of the bacterial lawn, 15 mutants produced amoebal plaques on 25-50 % of the bacterial lawn, nine mutants exhibited a low effect (10-25 %) and 23 mutants had no effect in the plaque assay.

To also analyze the mini-Tn10 transposon mutants for their ability to survive and replicate intracellularly within host cells, we performed infection assays of *D. discoideum* and U937 macrophage-like cells. The replication of the *L. pneumophila* Corby wild-type strain in *D. discoideum* typically results in a 1000-fold bacterial increase after day three post infection, whereas the *dot*A-negative mutant does not amplify during the same time period. We observed that most of the selected mini-Tn10 mutants were attenuated in their ability to multiply in *D. discoideum* cells. Eight mutants showed more than a 100-fold, five at least a 10-fold and 23 at least a 2-fold reduced intracellular replication rate (Table 2). The ability of the mini-Tn10 transposon mutants to inhibit plaque formation often correlated with their ability to replicate intracellularly within *D. discoideum* cells (Table 2).

We next determined whether intracellular replication was also possible in U937 macrophage-like cells. Only mutants which showed effects in plaque formation and (or) in *D. discoideum* infection assays and which revealed insertions in genes encoding for putative virulence factors were analyzed. Mutants which had been analyzed in previous studies (e.g. flagella, LPS, PlaB) were excluded from further investigations. Among the 10 analyzed mutants, only 2, Lpc2666 (D1) and Lpc0114 (B23), encoding a

cytosolic IMP_GMP specific 5'-nucleotidase, were attenuated in their ability to replicate intracellularly within U937 macrophage-like cells. Five mutants revealed a slight reduction in replication compared to the *L. pneumophila* Corby wild-type strain. The mutants Lpc1639 (C36, C37), Lpc1641 (D23) and Lpc3210 (B26) multiplied inside macrophages, as well as the *L. pneumophila* Corby wild-type strain (Table 2).

O-methyltransferase, TPR-containing protein, PilY1 and polyketide synthase (PKS) contribute to avoidance of lysosomal degradation

The aim of this work was to find novel factors involved in the modification of intracellular trafficking. As most of the genes selected in this screening were described and characterized previously, we focused on yet uncharacterized mutations within genes of putative novel virulence factors. For further characterization, we chose the O-methyltransferase (Lpc0263), the PilY1 (Lpc2666), the hypothetical protein containing a TPR motif (Lpc2669), and the putative PKS (Lpc1639)**.** The lysosome fusion phenotypes of the 4 mutants were analyzed by fluorescence microscopy. We labeled the respective *L. pneumophila* strains (wild-type, *dot*A-negative, heat inactivated *L. pneumophila* Corby, Mini-Tn*10* mutants) with rhodamine and measured their co-localization with mannose-6-sulphate (M-6-S), a marker for the *D. discoideum* lysosomal compartment (Neuhaus *et al.*, 1998). Statistical analysis of the confocal laser scanning microscopy images revealed that the co-localization ratios of all tested mutant strains were comparable to what was observed for the *dot*A-negative mutant. The percentage of wild-type LCVs positive for M-6-S after 4 hours of intracellular infection were significantly lower compared to LCVs of the *dot*A-negative *L. pneumophila* mutant. Heat-inactivated *L. pneumophila* Corby served as negative control and showed significantly higher co-localization with the lysosomal marker compared to the *dot*A-negative mutant (Fig. 2). Moreover, the mini-Tn10 transposon mutants revealed a significantly higher ratio compared to the *L. pneumophila* Corby wild-type strain. Interestingly, a direct correlation between the co-localization ratio and the ability to replicate intracellularly within macrophages or *D. discoideum* was not observed.

Analysis of the PKS synthase cluster

Although the cumulative size of PKSs and NRPSs (nonribosomal peptide synthesis) genes in *L. pneumophila* is higher than that of other γ-proteobacteria, the existence of this region has not yet received appropriate attention (Donadio *et al*., 2007). This and the fact that our genetic screen led to the identification of 2 independent PKS mutants prompted us to further analyze the gene products of the potential PKS cluster. Two of them (C36, C37) have an insertion in Lpc1639 and one (D23) in Lpc1641, encoding a sensory box sensor histidine kinase/response regulator, an element of a previously uncharacterized two-component system of *L. pneumophila* (Fig. 3A-B). We analyzed the

amino acid sequence of Lpc1639 with SBSPKS, a program for detection and analysis of PKS domains (Yadav *et al*., 2003, Anand *et al.*, 2010). The putative product of Lpc1639 is a multidomain protein with an acyl carrier protein (ACP), and ketosynthase (KS), acyl transferase (AT) and ketoreductase (KR) domains, most probably belonging to a type I PKS. Only two PKS genes, Lpc1639 and PksJ which were localized close to each other, were found in the *L. pneumophila* Corby genome (Fig. 3C). Orthologous gene clusters are also present in the closely related *L. pneumophila* strains Alcoy and Lens (Cazalet *et al*., 2004, D'Auria *et al*., 2010). Genes encoding for putative regulatory elements and genes for a two-component system were identified downstream of Lpc1639. The chemical structure of the polyketide could not be predicted for any of the *L. pneumophila* Corby PKSs. Furthermore, it is possible that additional genes outside of the PKS cluster contribute to the biosynthesis of the respective metabolite(s).

Role of the PKS cluster as fitness factor and distribution of the PKS cluster among diverse **Legionella** *species*

As mentioned above, we were not able to detect an effect of the KS domain of Lpc1639 (C36, C37) on intracellular replication in *D. discoideum*, *A. castellanii*, and U937 macrophage like cells (Tab. 2; Fig. 4A-C). Moreover, none of the analyzed PKS mutants revealed a reduced cytotoxicity on U937 macrophage-like cells after six days of co-incubation (Fig. 4D). Additionally, both Lpc1639 mutants were indistinguishable from wild-type *L. pneumophila* Corby with regard to growth in liquid culture and pigment production (data not shown).

Since the PKS cluster of *L. pneumophila* could be a non-essential fitness factor, which may only be relevant in competitive situations, we co-infected *D. discoideum* cells with the *L. pneumophila* Corby wild-type and the Lpc1639 strains. This approach revealed that Lpc1639 mutant became outcompeted 24 h post infection (Fig. 4E). Similar results in U937 cells corroborated the promoting effect of the PKS cluster in competitive co-infections (Fig. 4F).

KS domains are generally highly conserved and essential for the functionality of PKSs (Hertweck, 2009). To examine the distribution of PKSs among diverse *Legionella* species, we tested 19 *L. pneumophila* and nine non-*pneumophila* strains by Southern blot hybridization. The DNA of *Legionella* strains was restricted and hybridized with a probe specific for the KS domain of Lpc1639. This approach revealed that most of *L. pneumophila* strains possess the KS domain with different levels of homology (Fig. S1). In contrast, among the non-*pneumophila* species, only LLAP10 showed a low hybridization signal.

Discussion

To survive within phagocytic host cells, *L. pneumophila* has evolved a variety of strategies to avoid fusion with lysosomes (Hubber and Roy, 2010; Fernandez-Moreira *et al*., 2006). In this study we developed and applied a screen which facilitated the isolation of bacterial transposon mutants defective in the inhibition of lysosomal fusion. The selected 54 candidates were assigned to known virulence genes of *L. pneumophila*, genes described to be virulence-related in other pathogens, or to yet uncharacterized factors. These three classes of genes will be discussed in the following.

Among all mutants, 20 (37%) have been already described in previous studies (Table 1) as virulence genes of *L. pneumonia;* their predicted virulence is consistent with our experimental data (Table 2). For example, mutants B7, E33, A36 with insertions in an ABC transporter (Lpc1042) and E8 (Lpc1579), which encodes a sensory box/GGDEF family protein, were previously identified as virulence determinants for infection of amoeba (Aurass *et al*., 2009). Sensory box/GGDEF family proteins bind and hydrolyze the nucleotide second messenger cyclic diguanylate (c-di-GMP) and thereby regulate a wide variety of processes in bacteria, in particular transcriptional and posttranscriptional activity, enzymatic activity, and protein-protein interactions. These, in turn, modulate a wide range of infection-related cellular processes such as biofilm formation, flagella biosynthesis and induction of inflammatory mediators (Carlson *et al*., 2010, Abdul-Sater *et al*., 2012). The genome of *L. pneumophila* Philadelphia-1 encodes 22 predicted proteins containing domains related to c-di-GMP synthesis, hydrolysis, and recognition. Most of them are up-regulated in the transmissive phase during intracellular growth (Bruggemann *et al*., 2006). Deletion or overexpression of the Lpc1579 orthologue has no significant effect on the intracellular pool of c-di-GMP in *L. pneumophila* Philadelphia-1. Moreover, *L. pneumophila* strains lacking genes containing the individual GGDEF motif exhibit wild-type replication rates in host cells, reflecting the overlapping functions of these genes. In contrast, overexpression of the Lpc1579 (E8) orthologue significantly decreased the ability of the pathogen to replicate within *Acanthamoeba castellanii* and U937 macrophage-like cells and prevented the fusion with lysosomes (Levi *et al*., 2011). In our infection studies mutant Lpc1579 was strongly attenuated in its ability to replicate in *D. discoideum.* Interestingly, the replication of this mutant was only slightly reduced in U937 cells (Table 2). Another known regulator which was also identified in our study is Lpc1965 (E22). This factor has recently been characterized as responsive sensor kinase LqsT, which regulates the transcription of 105 genes, including 13 Dot/Icm T4BSS substrates, the macrophage infectivity potentiator (Mip), the *L. pneumophila* chitinase and flagellum components (Kessler *et al*., 2013). The mutants Lpc0756 (F13, F14; FliC) and Lpc0563 (F5; FlhB) are part of the flagellar regulon, which controls several virulence-associated transmissive traits, including the avoidance of lysosome fusion (Molofsky *et al*., 2005). Mutant Lpc1029 (F28; PlaB) carries an insertion in the phospholipase A, which is associated with the outer membrane. This factor is responsible for contact-dependent hemolytic activity and inactivation leads to impaired replication in the lung of infected guinea pigs (Bender *et al*., 2009, Schunder *et al*., 2010). Two transposon insertions in Lpc2522 (D8, D30) were found in the gene encoding for the

T4BSS effector PelB, which possesses a eukaryotic CaaX motif. This motiv was shown to be required for the recognition by the host-cell prenylation machinery. The prenylated effectors are anchored to host membranes and contribute modestly to the reduction of lysosomal fusion events with LCVs (Price *et al*., 2010b).

Further Dot/Icm T4BSS substrates identified in our screen are: Lpc2032 (A12, E12), which causes growth defects by modulation of the intracellular trafficking in *S. cerevisiae* (Heidtman *et al*., 2009, Gomez-Valero et al., 2011), and Lpc0335 (E38, E39), which together with SidA and Lpg1969 were reported to interact with IcmQ (Montminy, 2009). Lpc1466 (D36) and Lpc3210 (B26) are translocated substrates of the Dot/Icm system (Zusman et al., 2008, Huang et al., 2011).

Lpc2235 (D3; CopA2) appears to be a multicopper oxidase, which belongs to the metal efflux island of *L. pneumophila*. This island was hypothesized to be necessary for maintaining the concentration of certain metals within the phagosome, although its presence not required for intracellular growth (Kim *et al.*, 2009).

Most of the genes identified in this study have been shown to be up-regulated within human macrophages (Table S2) (Faucher *et al*., 2011). This is also the case for orthologues of the Lpc2662-Lpc2666 fimbrial synthesis gene cluster, which are only induced *in vivo* and seem to be co-regulated with the Dot/Icm T4BSS substrates SidA, SidE, Lpc0335 (E38, E39) and other virulence factors, such as the RpoE sigma factor and the TPR-containing protein-LidL (Bruggemann *et al.*, 2006; Newton *et al*., 2007). Moreover, since we have selected mutants 4 hours post infection in *D. discoideum*, it can also not be excluded that other factors contribute to the escape from lysosomes at earlier or later stages, or in other host cells. However, the diversity of identified factors indicates that multiple molecular mechanisms are involved in avoiding the lysosomal degradation pathway. A number of genes identified in this study encode for domains with homology to known virulence factors of other bacteria. Five mutations Lpc2666 (D1, F6 and F8; PilY1) and Lpc2669 (F10 and F41) are localized in a yet uncharacterized putative pili region. The thorough sequence analysis of this region showed that Lpc2666 encodes for PilY1, a protein with high homology to the PilY1 of *Pseudomonas aeruginosa*. This protein has multiple roles in virulence including swarming and twitching motility, secretion of secondary metabolites and in adhesion to differentiated human airway epithelial cells (Bohn *et al*., 2009, Heiniger *et al*., 2010, Kuchma *et al*., 2012). Lpc2669 encodes for a hypothetical protein conserved within the genus *Legionella*. The gene product is predicted to contain tetratricopeptide repeats (TPR), which are present in eukaryotes and prokaryotes and regulate diverse biological processes, such as organelle targeting and vesicle fusion (Blatch and Lassle, 1999; Zeytuni and Zarivach, 2012). Two of 10 *L. pneumophila* TPR proteins, LpnE and EnhC, are candidates for virulence factors that influence the translocation of certain T4BSS effectors (Bandyopadhyay *et al*., 2012). One TPR-containing protein, LidL, was identified in a screen for factors which influence the ability to synthesize and (or) translocate substrates of the T4BSS (Conover *et al*., 2003). Another mutant with a defect in the inhibition of LCV-lysosome fusion has an insertion in Lpc0263 (F30), a

gene encoding for an O-methyltransferase, which is conserved in *pneumophila* species. The O-methyltransferase of *L. pneumophila* Philadelphia-1, like the Dot/Icm T4BSS substrates Ceg7 and LegA12, belongs to the group of genes regulated by the arginine repressor ArgR, which, in turn, is required for optimal intracellular multiplication within the protozoan host *A. castellanii* (Hovel-Miner *et al*., 2010). The protein has the highest sequence similarity to the SAM-dependent methyltransferase, a virulence factor of *Francisella tularensis* (Champion, 2011).

Our analysis revealed significantly higher co-localizations of PilY1 (Lpc2666) and Lpc2669 mutants with lysosomes and a reduced replication rate in macrophages and protozoa. Remarkably, heat-inactivated, rhodamine-labeled *L. pneumophila* also showed a significantly higher co-localization with lysosomes in comparison to the *dot*A-negative strain. These observations suggest that *L. pneumophila* also uses T4BSS-independent mechanisms to avoid lysosomal degradation.

One of the most striking findings in our study is that a PKS is involved in avoidance of lysosomal fusion. The PKS mutant (Lpc1639), similarly to the *dot*A mutant, was incapable of preventing the fusion with lysosomes, though its survival and replication within macrophages and amoeba was not affected. The missing growth defect may be due to overlapping functions of *L. pneumophila* virulence factors. This is in line with the remarkable observation that the reduction of the *L. pneumophila* genome to 31% only leads to minimal attenuation within mouse macrophages (O' Connor *et al*., 2011). Thus, we conclude that synthesis of a polyketide metabolite is one of multiple mechanisms of *L. pneumophila* to escape the lysosome-endosome degradation pathway. The ability of PKS to reduce phagolysosomal fusion and macrophage-mediated killing was also described for *A. fumigatus* (Jahn *et al*., 2002, Thywissen *et al*., 2011). It has been speculated that HN-melanin produced by wild-type *A. fumigatus* leads to deregulation or even inhibition of the vATPase, similar to the *L. pneumophila* effector SidK (Xu *et al*., 2010, Thywissen *et al.*, 2011). Thus, it is possible that the product(s) of the *L. pneumophila* PKS region secrete, modify or neutralize intraphagosomal signals, which are necessary to avoid lysosomal digestion. One of these signals could be sensed by the two-component system of the PKS locus (Lpc1640), which is up-regulated in response to macrophage infection (Bruggemann *et al.*, 2006, Faucher *et al.*, 2011).

Interestingly, competitive co-infections of amoebae with PKS-positive and -negative strains revealed that PKS provides a selective advantage for *L. pneumophila*. This positive influence on competitiveness is remarkable since the distribution of KS domains is almost exclusively restricted to *pneumophila* strains, which are prevalent in the epidemiology of Legionnaires´ disease (Yu *et al*., 2002). The only exception among the analyzed non-*pneumophila* strains is LLAP-10, which also possesses a KS domain (Doleans *et al*., 2004). Since the LLAP-10 strain exhibits comparatively high replication rates in protozoa (Hagele *et al*., 2000), this exception also fits with our conclusion that PKS may be an important fitness factor for *L. pneumophila*.

Experimental procedures

Cultivation of bacteria and eukaryotic cells

L. pneumophila Corby (Jepras *et al*., 1985) was used to generate a random transposon library. The *L. pneumophila* Corby *dotA*::*Tn5* mutant was a gift from A. Flieger. All *L. pneumophila* strains and mutants were routinely cultured in buffered yeast extract (YEB) at 37 ^{o}C with agitation at 180 rpm or grown on buffered charcoal-yeast extract (BCYE) agar supplemented with 20 µg/ml kanamycin for 3-5 days. *K. aerogenes*, *E. coli* DH10β and DH5α were cultivated on LB medium at 37 ^{o}C overnight. *D. discoideum* AX2 was grown axenically at 24 °C in HL5 medium (Clarke *et al*., 1980) with agitation at 160 rpm for 3 days. U937 macrophage-like cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum at 37°C and 5 % CO_2 and differentiated with phorbol 12-myristate 13-acetate (Sigma) for 24 h before use.

Bacterial cytotoxicity on host cells was quantified by the MTT assay. In brief, 96-well tissue culture plates containing 1x10^5 differentiated U937 cells were infected with 10-fold serial dilutions of *L. pneumophila*, starting with 1 x 10^7 CFU per well. After 6 days of incubation, 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was added to each well. After 2 h of incubation, the MTT-culture medium was removed, and the resulting formazan dye was dissolved in 100 µl isopropanol and vigorously shaken at 900rpm. The optical density of each well was measured at 500 nm with the Thermo Scientific™ Varioskan™ Flash Multimode Reader. The number of bacteria required to kill 50% of the U937 cells (CT50) was determined by nonlinear regression analysis.

Transposon mutagenesis

To obtain random *L. pneumophila* Corby mutants, mini-Tn10 mutagenesis was applied (Pope *et* al., 1994). In brief, the delivery vector pCDP05a, which contains a kanamycin resistance (Km) gene, an altered target specificity transposase gene and a *sacB* gene for counter selection was electroporated into the strain *L. pneumophila* Corby. The settings employed were 25 mF capacitance at 2.3 kV and 100 Ohm. Transformed cells were mixed with 1 ml BYE medium and incubated at 37 °C for 16 h without agitation. Bacteria were then plated in 200 µl aliquots on BCYE agar with 25 µg/ml kanamycin and 5 % sucrose. 5960 individual mutant colonies were each transferred to 200 µl BYE medium, supplied with 20 µg/ml kanamycin and cultivated for 5 days. After cultivation, the transposon mutants were stored in 20 % glycerin at -20°C.

Preparation of iron-dextran particles

Colloidal iron particles coated with dextran with an average diameter of 8 nm were prepared as described (Rodriguez-Paris *et al*., 1993). In brief, 10 ml of 1.2 M $FeCl_2$ was mixed with 10 ml of 1.8 M $FeCl_3$ to which 10 ml of 25 % NH_3 was added while being vigorously agitated. The suspension was divided into six aliquots and placed onto a magnetic unit until the precipitate had gathered on the surface of the tube, than the supernatant was decanted. The precipitate was washed once with 5 % NH_3 and twice with deionized H_2O. The sediment was suspended in 80 ml of 0.3 M HCl and stirred for 30 min. Then 4 g of Dextran (64-76 kDa, Sigma) was added and stirred for a further 30 min. The resulting sample was dialyzed against cold deionized water for 2 days and filtered. The sterile solution with an approximate iron concentration of 10 mg/ml was stored at 4 °C for up to 3 months.

Iron particle-based screening of mutants

2.5 x 10^7 *D. discoideum* cells were seeded into 25 cm^2 cell culture flasks to a final cell density of 10^6 cells/ml and allowed to settle at 25.5 °C for 30 min. The *L. pneumophila* Corby transposon mutant library (5960 colonies) was divided into six equal pools (6 x 960). Five day-old plate cultures from each pool were suspended in 2 ml of sterile H_2O, mixed well and the cell density adjusted to 10^9 cells/ml. 250 µl of each of the prepared bacterial suspensions was added to each cell culture flask at a multiplicity of infection (MOI) of 10. Following an invasion period of 3.5 h, the colloidal iron particles were added to a final concentration of 1 mg/ml. After 4 h of incubation at 25.5 °C the *D. discoideum* cells were washed three times with 1 x Soerensen phosphate buffer, pH 6.0, and once in homogenization buffer (HB; 0.5 mM Na_2EGTA, 20 mM HEPES, 250 mM sucrose) supplemented with an EDTA-free protease inhibitor cocktail (Roche Diagnostic). Cells were resuspended in 2 ml of HB and then broken by 12 strokes in a Dura Grind stainless-steel homogenizer. The lysate was subjected to low-speed centrifugation (115 x g, 5 min at 4 °C) to remove nuclei and unbroken cells. The resulting post-nuclear supernatant was applied to a Miltenyi Biotec MiniMACS column in a magnetic separation unit (OctoMACS, MiltenyiBiotec). Then the column was washed twice with HB and bound material was eluted with 1 ml of 0.4 % Triton X100 in HB. A 100 µl aliquot of the eluate was immediately plated onto BCYE agar plates supplemented with kanamycin (20 µg/ml). The remaining eluate was frozen at -20 °C. After 5 days of culturing, the bacteria were harvested from the agar plates and the selection was repeated. In order to enrich the number of *Legionella* mutants defective in avoiding lysosomal fusion, 4 rounds of the selection procedure were performed. After the final selection, 48 colonies of each pool were picked from agar plates, cultivated in YEB medium supplemented with kanamycin (20 µg/ml) and DNA from each colony was processed for Southern blot hybridization.

Southern blot hybridization and identification of sibling families

Southern blot analysis was performed to confirm the randomness of the transposon library, to identify the families of siblings with identical genotypes, and to monitor *Legionella* species for the presence of the KS gene sequence. For that, an aliquot of genomic DNA (3-4 μg) of each mutant was extracted with a DNA isolation kit (Bio&Sell, Nexttec^{TM}) and digested overnight with the restriction enzymes EcoRI and PstI. The digested DNA was separated electrophoretically, blotted onto a nylon membrane (Hybond-N+, Amersham) and fixed by UV-crosslinking (UV-crosslinker, BioRad). A digoxigenin-11-dUTP (DIG)-labelled probe that was able to recognize the mini-Tn10 transposon was generated from the plasmid pCDP05a with the primer pair Km627f (5'-gcaatcaggtgcgacaatctatc-3') and Km627r (5'-aatgaaggagaaaactcaccgagg-3') specific for the kanamycin resistance gene. The PK probe was amplified from *L. pneumophila* Corby genomic DNA with the primer pair SB Lpc1639f (5'caattattggaatgagttgccgc-3') and SB Lpc1639r (5'-tctcgataggcagaagttaatgctt-3'). The incorporation of DIG into the PCR product was confirmed by electrophoresis. Hybridization and detection of digoxigenin-labeled kanamycin specific fragments were performed according to the manufacturer's recommendations (DIG nucleic acid detection kit; Roche).

Transposon insertion localization

For the localization of the transposon insertion, the genomic DNA from each mutant was digested with EcoRI and PstI and the obtained fragments were ligated into the plasmid pUC19. The plasmid was introduced into *E. coli* DH10β and selected on kanamycin to obtain vectors containing the DNA fragment with the inserted transposon. The plasmid was sequenced with the transposon-specific primer Tn4 or Tn5. For exact determination of transposon insertion sites, the obtained sequences were aligned against the NCBI database with BLASTn. The InFiRe method was applied for mutants for which the classical identification of transposon insertion was not successful (Shevchuk *et al*., 2012). Transposon insertions within all mutants were verified by comparison to the wild type strain via PCR shift analysis using gene-specific primers (Table S1).

In silico analysis of selected mutants

The genes selected in this study were analyzed using the *Legionella* Genome Browser. The database provides information concerning the reported or predicted function of a gene product, which belongs to a cluster of orthologous genes (COG) and enables to check for the presence of genes and the corresponding proteins in non-*pneumophila* species (LLAP10, *L. hackelie, L. micdadei*), which are not present in the NCBI database. The subcellular localization of the proteins was predicted by "ClubSub-P" (Paramasivam and Linke, 2011). Potential relations to virulence were predicted with VirulentPred (Garg and Gupta, 2008). Polyketide synthase domains were predicted with SBSPKS (Yadav *et al*., 2003, Anand *et al*., 2010).

D. discoideum plaque assay

To evaluate the potential virulence of transposon mutants in comparison to wild-type *L. pneumophila*, the plaque assay was performed as previously published (Shevchuk and Steinert, 2009, Tiaden *et al*., 2013). In brief, 10^3 *D. discoideum* cells were washed with 1 x Soerensen buffer. The amoebae were mixed with 10^7 *Klebsiella aerogenes* and 10^7 cells of a *L. pneumophila* transposon mutant in infection medium and were subsequently plated on SM agar in 12 well plates. After 3 days, the plates were examined for plaques formed by *D. discoideum* amoebae. The surface area of plaques formed by *D. discoideum* was determined by Adobe Photoshop CS2 and the ratio represents the percentage of the area covered by plaques to the whole area of the bacterial lawn.

Infection and competition assay

To examine the ability of selected *L. pneumophila* mini-Tn10 transposon mutants to grow within host cells, infection assays were performed in *D. discoideum* AX2, *A. castellanii* and U937 monocytes. For *D. discoideum,* 10^6/ml cells were seeded into 25-cm^2 cell culture flasks and infected with *Legionella* at an MOI of 0.02. At 0, 24, 48 and 96 h post inoculation, serial dilutions of cell lysates were plated on BCYE agar and the number of bacteria within the co-cultures was determined. U937 cells were seeded into 24-well tissue culture plates at a density of 5×10^5 cells/well and treated with 10^{-8} M phorbol 12-myristate 13-acetate (PMA) for 24 h. Differentiated U937 cells were infected with stationary phase *Legionella* at a multiplicity of infection (MOI) of 10. After 1 h of incubation, cells were treated with 100 µg/ml gentamycin for 1 h and washed with PBS to remove extracellular bacteria. At 1, 24 and 48h post infection, CFU/ml were determined by lysing the macrophages with sterile distilled water and plating the lysates on BCYE agar. For the competition assay, equal amounts of *L. pneumophila* Corby and the Lpc1639 (C36) Mini-Tn10 transposon mutant strain (kanamycin resistant) were used to co-infect the *D. discoideum* or U937 macrophage-like cells. Serial dilutions were plated on BCYE agar with and without antibiotic to determine the number of CFU/ml. To determine the number of wild-type bacteria, the CFU/ml on BCYE-kanamycin agar was subtracted from the CFU/ml on BCYE plates without kanamycin.

Fluorescence microscopy

The co-localization of *L. pneumophila* Corby mini-Tn10 transposon mutants with lysosomes was analyzed by immunofluorescence microscopy. Briefly, triplicate cultures of *D. discoideum* (5×10^6 /well) were seeded on glass cover slips in freshly mixed infection medium for 1 h at 25 °C, washed and infected with rhodamine-labeled *L. pneumophila* strains and mutants at a MOI of 100. The rhodamine labeling was performed as previously described (Unal and Steinert, 2006). The infection

was synchronized by centrifugation (300 x g, 5 min) and remaining bacteria were removed by washing with infection medium. After 4 h of incubation, the cells were rinsed 3 times with cold SorC buffer, fixed with methanol (-20 °C) for 30 min and washed again 3 times with cold SorC. After blocking with 2 % human AB serum in SorC for 30 min at room temperature, lysosomes were labeled with mAb 221-342-5 (1:1000), a monoclonal antibody against a common mannose-6-sulfate–containing carbohydrate epitope present on *D. discoideum* lysosomal enzymes (Neuhaus *et al.*, 1998). Alexa 488-conjugated goat-anti mouse IgG (Invitrogen) was used as a secondary antibody (1:1000) for 1 h at room temperature. Finally, DNA was stained with 1 µg/ml DAPI in PBS. Cover slips were mounted in Dako Fluorescence mounting medium and examined by confocal laser-scanning microscopy using a Leica DM6000 CS/ Leica TCS SP5 microscope. To quantify the fluorescence association of the lysosomal marker with the LCV, the images were loaded into Image J and transformed into 8-bit format (Image → Color → Split Channels). Then the red channel was activated and adjusted to the threshold (Image → Adjust → Threshold). The measurements were set (Analyze → Set measurements) by selecting "Integrated density", "Limit to Threshold", "Area" and "Redirect to green". A circle around the bacterium was drawn ("Wand tracing tool") and measured (Analyze → Measure). For estimation of lysosomal marker association integrated density values were divided by areas and the mean was plotted. Three independent experiments were performed for every mutant strain and for each experiment 100 bacteria were quantified.

Statistical analysis

Statistical significance was calculated with one-way ANOVA using InStat (GraphPad). Results with $p < 0.05$ were considered statistically significant.

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Figures

Fig. 1. Screening procedure for enrichment of mini-Tn10 transposon mutants with defects in avoiding LCV-lysosome fusion.

(A) Screening procedure. *D. discoideum* cells were infected with a pool of transposon-mutagenized *L. pneumophila* Corby (1) and incubated with iron-dextran that chases into lysosomes or late endosomes (2). After mechanical host cell lysis, iron-dextran-loaded lysosomes and associated LCVs were separated by MiniMACS columns (3) and cultivated on BCYE agar supplemented with antibiotic (4).

(B) Validation of screening. The *D. discoideum* cells were infected with the *L. pneumophila* Corby wild-type or the *dot*A mutant strain. The lysosomes were loaded with iron dextran 30 minutes before harvesting the cells. After 4 hours of infection the post nuclear supernatants were applied onto MiniMACS separation columns. Bound bacteria were eluted from the magnetic columns and plated on BCYE agar. The experiment was performed in triplicates.

(C) Selection of mutants. 5960 *L. pneumophila* Corby Mini-Tn10 transposon mutants were divided onto six equal pools (A-F). For each pool 4 rounds of selection were performed. After final selection, 48 colonies from each pool were analyzed for the presence of genetically identical siblings by Southern blot hybridization. In total, 54 mutants with defects in intracellular trafficking were selected.

Fig. 2. Co-localization of lysosomal marker and *L. pneumophila* Corby Mini-Tn*10* transposon mutants during infection of *D. discoideum*. *D. discoideum* cells were infected with rhodamine-labeled *L. pneumophila* mini-Tn10 transposon mutants, *L. pneumophila* Corby (WT), heat inactivated *L. pneumophila* Corby (Hi) and *dot*A-negative mutant strain (ΔdotA). After 4 h, cells were fixed with methanol, and lysosomes were labeled using mannose-6-sulfate (M-6-S) antibodies (green). (A) Representative confocal images demonstrating the locations of rhodamine-labeled wild-type and *dot*A bacteria and M-6-S-labeled lysosomes. (B) Calculated co-localization events.

Fig. 3. PKS modules of putative polyketide biosynthesis cluster.

(A) Organization of the putative PKS region of *L. pneumophila* Corby. The insertion of mini-Tn10 transposons are indicated by arrows. Three insertions were found in the Lpc1639 gene, encoding a putative PKS and one was found in the Lpc1641 gene, encoding a sensory box sensor histidine kinase/response regulator.

(B) Genes of the polyketide synthase region and their predicted functions.

(C) Domain organization of two *L. pneumophila* Corby PKSs genes. The analysis of DNA sequences were performed with SEARCHPKS. The acyltransferase (AT) domain selects the appropriate extender unit and transfers it to the acyl carrier protein (ACP) domain, where a thioester bond is formed. The ketosynthase (KS) domain is responsible for a decarboxylating condensation. The additional domains (ketoreductase (KR), methyl transferase (O-MT) and dehydratase (DH) are responsible for the modification of initial carbonyl groups. All domains in the same module are presented in the same color.

Fig. 4. *L. pneumophila* Lpc1639 mutant shows wild-type cytotoxicity and replication within different host cells, but exhibits reduced fitness in competitive infections.

(A-C) *D. discoideum* and *A. castellanii* were infected with *L. pneumophila* Corby, the *dot*A-negative mutant and transposon mutants with insertions in the Lpc1639 gene (C36, C37) at MOI 0.02. U937 macrophage–like cells were infected with same strains at MOI 10. Intracellular CFUs were measured by plating on BCYE agar plates at the indicated time points. Data represent the mean of 3 independent experiments (in triplicate) ±SD.

(D) Cytotoxicity of *L. pneumophila* Corby, the *dot*A-negative mutant and transposon mutants with insertions in the Lpc1639 gene (C36, C37) for U937 cells (MTT assay). Cytotoxicity is expressed as the bacterial dose that killed 50% of the cells (CT_{50}). The CT_{50} was calculated for each strain and plotted in this graph; error bars represent the SD from six independent replicates. Non-infected U937 cells were used as a baseline for the CT_{50} calculation. Both Lpc1639 mutants (C36, C37) and *L. pneumophila* Corby wild-type strain are cytotoxic for macrophages at low doses, whereas the *d*otA negative strain was not cytotoxic.

(E, F) Intracellular bacterial growth during the competitive infection of *D. discoideum* and U937 macrophage-like cells. Intracellular *L. pneumophila* Corby and Lpc1639 mutant C36 were determined by plating on BCYE agar plates at the indicated time points. Data represent the average of 3 independent experiments (in triplicate) ±SD.

805
806
807 **Tables**
808
809 **Table 1.** *L. pneumophila* mini-Tn10 transposon mutants with defects in avoiding the LCV-lysosome
810 fusion.

811

812 **Table 2**. Plaque and replication assay analyses of selected *Legionella* mini-Tn10 transposon mutants.

813

814 **Supporting information:**

815 **Fig. S1.** Southern blot analysis of genomic DNA from 19 strains of *L. pneumophila* and 10 non-
816 *pneumophila strains*. The genomic DNA was digested with *Eco* RV and probed with KS domain of
817 Lps1639. Lane (**1**) KS sequence (627bp), (**2**) *E. coli* DH5α (negative control), (**3**) *L. pneumophila*
818 Corby, (**4**) *L. pneumophila* Philadelphia-1 (patient isolate), (**5**) *L. pneumophila* Philadelphia-1, (**6**) *L.*
819 *pneumophila* Philadelphia-1 JR32, (**7**) *L. pneumophila* Paris, (**8**) *L. pneumophila* AA100/130b, (**9)**
820 *L. pneumophila* 640, (**10**) *L. pneumophila* 664, (**11**) *L. pneumophila* Los Angeles H, (**12**) *L.*
821 *pneumophila* Bloomingtion-2 E, (**13**) *L. pneumophila* Sg. 7, (**14**) *L. pneumophila* Sg. 8, (**15**) *L.*
822 *pneumophila* IN-23-G1-C2, (**16**) *L. pneumophila* Leiden 1, (**17**) *L. pneumophila* 797-PA-H, (**18**) *L.*
823 *pneumophila* 570-CO-H, (**19**) *L. pneumophila* 82A350, (**20**) *L. pneumophila* 1169-MN-H, (**21**) *L.*
824 *pneumophila* 2577 (**22**) *L. gormanii*, (**23**) *L. dumoffii*, (**24**) *L. oakridensis*, (**25**) *L. feeleii* Sg.1, (**26**) *L.*
825 *feelii* Sg.2, (**27**) *L. hackeliae* Sg.2, (**28**) *L. israelensis*, (**29**) LLAP-10, (**30**) *L. hackeliae* Sg.1, (**31**) *L.*
826 *lytica.*

827
828 **Table S1.** Primer for verification of transposon insertions.

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830 **Table S2.** Transcriptional changes of *L. pneumophila* during intracellular growth in THP-1 cells and
831 in *A. castellanii*.

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Table 1. *L.pneumophila* mini-Tn10 transposon mutants with defects in avoiding LCV-lysosome fusion.

Mutant	Name	Locus tag	Function	COG	Subcellular localization	Gene conservation	References
B23	NA	Lpc0114	cytosolic IMP-GMP specific 5'-nucleotidase	Unclassified	Cy	C,P,Ph, L, A,LLAP, H, M	
A12	polA	Lpc0118	DNA-polymerase I	Replication, recombination and repair	Cy	C,P,Ph, L, A LLAP, H, M	
F30	NA	Lpc0263	O-methyltransferase	Secondary metabolites biosynthesis, transport and catabolism	Cy	C,Ph, P, L, A	
A26, B9, D24	NA	Lpc0297	hypothetical protein	Function unknown	Cy	C,P,Ph, L, A, Ln	
B9	NA	Lpc0310	hypothetical protein	Unclassified	Cy	C, A,	
E38, E39	NA	Lpc0335	hypothetical protein	Unclassified	CM	C,P,Ph, L, A	(Bruggemann et al., 2006)
F5	flhB	Lpc0563	flagellar protein FlhB	Intracellular trafficking and secretion	U	C,P,Ph, L, A	
F13, F14	fliC	Lpc0756	flagellin	Cell motility	Ex	C,P,Ph, L, A,LLAP, H, M	
F28	plaB	Lpc1029	phospholipase	Unclassified	Cy	C,P,Ph, L, A, Ln, LLAP, H	(Bender et al., 2009, Schunder et al., 2010)
B7, E33, A36	abcT3	Lpc1042	hypothetical protein, ABC transporter predicted	Secondary metabolites biosynthesis, transport and catabolism	CM	C,P,Ph, L, H, M, Ln	(Aurass et al., 2009)
A45	NA	Lpc1055	coenzyme F390 synthetaseFtsA	Coenzyme transport and metabolism	Cy	C, P, Ph, L, A, Ln	
E23	astA	Lpc1135	arginine N-succinyltransferase, beta chain	Amino acid transport and metabolism	Cy	C, Ph, P, L, Ln, LLAP10, H, M,	
C29	NA	Lpc1235	oxidoreductase (L-gululonolactone oxidase)	Energy production and conversion	CM	C, Ph, P, L, A, Ln	
D36	NA	Lpc1466	hypothetical; 3-hydroxy-3-methylglutaryl coenzyme A reductase-like	Unclassified	Cy	C, Ph, P, L, A	(Price et al., 2010a)
E8	NA	Lpc1579	sensory box/GGDEF family protein	Signal transduction mechanisms	CM	C ,Ph, P, L, A	(Bruggemann et al., 2006, Aurass et al., 2009)
C36, C37	NA	Lpc1639	hypothetical protein, polyketide synthases (PKSs) domain	Secondary metabolites biosynthesis, transport and catabolism	CM	C,L, A, LLAP10	
D23	NA	Lpc1641	sensory box sensor histidine kinase/response regulator	Signal transduction mechanisms	CM	C, P, Ph, L	M. Lomma, 2009
F33	NA	Lpc1649-51	TP2 repeat region				
A20, A45	NA	Lpc1723	conserved hypothetical protein	Unclassified	Cy	C, P, Ph, L, A, Ln, LLAP	
D44, D47	imxF	Lpc1958	RND multidrug efflux transporter MexF	Secondary metabolites biosynthesis, transport and catabolism	CM	C,Ph, P, L, A, Ln	
E22	lqsT	Lpc1965	sensor histidine kinase/response regulator	Signal transduction mechanisms	CM	C,Ph, P, L, Ln	(Kessler et al., 2013)
D43	hydG-2	Lpc2009	hydrogenase/sulfur reductase gamma subunit	Energy production and conversion/Coenzyme transport and metabolism	U	C,Ph, P, L, A, Ln	
E12	mav1	Lpc2032	hypothetical protein, T4SS effector	Unclassified	Cy	C,Ph, P, L, A	(Heidtman et al., 2009, Gomez-Valero et al., 2011)
F38	NA	Lpc2077	putative secreted esterase	Cell wall/membrane biogenesis	Cy	C, L, P, Ph, A	
A30, A41	NA/NA	Lpc2104/05	putative nitroreductase MJ1384/hypothetical protein	Energy production and conversion/unknown	U	C/C	
A25	phaB	Lpc2215	acetylCoareductasepha B	Secondary metabolites biosynthesis, transport and catabolism,	Cy	C, P , Ph, L, A, LLAP10, A	
D3	copA2	Lpc2235	copA2, copper efflux ATPase	Secondary metabolites biosynthesis, transport and catabolism	OM	C, P, Ph, A	(Kim et al., 2009)
F7	NA/aroB	Lpc2356	DamX-related protein	Unclassified	CM	C, P, Ph, L, A	
D46	NA	Lpc2492	acyl CoA dehydrogenase, short chain specific	Lipid transport and metabolism	Cy	C ,P, Ph, L, A	
D30, D8	PelB	Lpc2522	PelB	Unclassified	Cy	C, P, Ph, L	(Luneberg et al., 2000), (Price et al., 2010b)
F6, F8, D1	NA	Lpc2666	type IV fimbrial biogenesis PilY1-related	Cell motility	U	C ,Ph, P, L, A	(Bruggemann et al., 2006)
F10, F41	NA	Lpc2669	hypothetical protein conserved within Legionellae	General function prediction only	Cy	C ,Ph, P, L	
D5, E37	NA	Lpc2762-3	TP3 repeat region				
B1	osmY	Lpc2961	osmotically inducible protein Y	General function prediction only	CM	C, P, Ph, L, A	
B26	mavW	Lpc3210	hypothetical protein	Unclassified	Cy	C, P, Ph, L, A	(Huang et al., 2011)
D40	NA	Lpc3236/37	conserved hypothetical protein/hypothetical protein	Function unknown	Cy/CM	C, P, Ph, L, A/C, P, L, A, M, H, LLAP10, Ln	
E16	NA	Lpc3313	sulfate transporter	Inorganic ion transport and metabolism	Cy	C,Ph, P, L, A	M. Lomma, 2009

The sub-cellular localization of the proteins was predicted by "ClubSub-P". Cy, cytoplasmic; CM, cytoplasmic membrane; Ex, extracellular; U, uncertain. Gene conservation in sequenced *Legionella* species: C, Corby; P, Paris; Ph, Philadelphia; L, lens; A, alchoi; LLAP10, *Legionella*-like amoebal pathogen; H, *hackeliae*; M, *micdadei*. The grey colour indicates low level of homology (70-95%).

Table 2. Plaque and replication assay analyses of selected *Legionella* mini-Tn10 transposon mutants.

Gene	Plaque assay	Replication assay *D. discoideum* (72h)	U937 (48h)	Virulence prediction with score	
Lpc0114	+++	+++	++	Virulent	0.5093
Lpc0118	-	-		Non-Virulent	-0.436
Lpc0263	++	++	+	Virulent	0.9805
Lpc0297	+	-		Virulent	0.9478
Lpc0310	+	-		Virulent	0.9952
Lpc0335	-	-		Virulent	0.4238
Lpc0563	-	++		Virulent	0.9978
Lpc0756	++	+++		Virulent	1.0086
Lpc1029	++	+++		Virulent	1.1651
Lpc1042	+	+	+	Non-Virulent	-0.802
Lpc1055	+	+		Virulent	1.0332
Lpc1135	-	+		Virulent	0.3014
Lpc1235	++	+		Virulent	0.6783
Lpc1466	-	+		Virulent	0.3410
Lpc1579	+++	+++	+	Virulent	1.0729
Lpc1639	-	-	-	Non-Virulent	-0.704
Lpc1641	-	+	-	Virulent	0.9912
Lpc1649-51	++	+++		-	-
Lpc1723	+	-		Virulent	1.0462
Lpc1958	-	-		Non-Virulent	-0.655
Lpc1965	-	-		Non-Virulent	-0.735
Lpc2009	-	-		Non-Virulent	-0.781
Lpc2032	-	+		Virulent	1.0279
Lpc2077	++	+++		Virulent	0.1042
Lpc2104/2105	-	-		Virulent	0.9873/1.0154
Lpc2215	-	+		Virulent	0.9916
Lpc2235	++	++		Virulent	0.9360
Lpc2356	-	+		Non-Virulent	-0.772
Lpc2492	-	+		Virulent	0.6855
Lpc2522	-	+		Virulent	0.9497
Lpc2666 (D1)	+++	+++	++	Virulent	0.9924
Lpc2666 (F6, F8)	+	+	+	Virulent	0.9924
Lpc2669	++	+	+	Virulent	0.3086
Lpc2762-3	++	++		-	-
Lpc2961	-	-		Virulent	0.0720
Lpc3210	++	+++	-	Virulent	0.7341
Lpc3236/3237	-	+		Virulent	1.0738/ 1.0196
Lpc3313	+	+		Virulent	0.7888
L.pn Corby	-	4 log (-)			
L.pn Corby dotA-	+++	0(+++)			

Plaque assay: 0-10% plaques (-); 10-25% plaques (+); 25-50% plaques (++); more than 50% plaques (+++).

Replication assay in *D. discoideum* AX2 and U937 macrophage-like cells: no attenuation (-), slightly attenuated (+); attenuated (++), defect in intracellular replication (+++).

843

844 Table S1. Primers for verification of transposon insertions.

Gene	Primer Forward 5'-3' (sense)	Primer Reverse 5'-3' (antisense)	Size of PCR fragment in WT *L. pneumophila*
Lpc0114	CTCAAAAATGGATACGCACAAGG	CGATCCCGGTACCTGATTAATAGA	1416
Lpc0262-0263	AGAAGCGGTCAATCTATGCTGG	CATGTACTGGAAACAGTGGATTGG	1413
Lpc0296-0297	CATAGGGGTCACCTCCAAAGTTAA	AGATACCCTGTTTTATGGCGCG	1145
Lpc 0310	CATCCCTTCTTGGGTTAATTCTATCC	ATTATGGTCAATGCGCTTGTGG	941
Lpc 0335	GCTGGTAATTTGACTGTGAGCATTT	TCAGGGATCGCAGTCACCAATT	935
Lpc 0563	GCCACAAACCTTCGTTTATTGG	TTATGGATGCCTTTGCGTGTTT	358
Lpc0756	CGACATCCTATCGACCTAACAATGA	TGAGGAGACTAAAACTATGGCTCAAGT	1451
Lpc 1029	CAGAACTGCCAAGTCAGTGAATTTT	AATAGACACCATTGGCAGTGCAA	1143
Lpc1042	CTGCTCTTGCTTGACTGACATTACTT	GGTAGTGCATCATGACTAATCGTTTACC	1817
Lpc 1055	TCCATTGGGGTAATTCGCAA	CCCCGGAAAAATTAAAGACTCATC	1134
Lpc 1135	ATGATGTTATTTCGTAGCGCTCG	CAATGGTGGAGTATCTTCTGTACGTATG	1041
Lpc1235	CTGATATGCGCAGCAAAAGAATC	CCCATTTCCTCTGCATCATTTTATG	1316
Lpc 1466	GTGGAGGGAGTGAAAATGCTAAATAAG	TAGTCCAGTAAGGCCATCATGCGA	1214
Lpc 1579/1580	CTCACTTTCCTCCAAATTAGCGTT	TGGTTTTCTTCTGGTCTTACCTGAA	1815
Lpc 1638/1639	CGACTTGCAGGAGTCCCTAAGTTA	AAAGCCAGGTTGCAGAATTGG	1453
Lpc 1641/1642	GGAGGAAATCAATGTCGCAACA	TCAAAAGATAGCCCCGTACGTG	1446
Lpc 1723/1724	ACAGGCCCAAGTCTCTGTATGGTT	AACAATTCAGATTTGCCGGGTT	1132
Lpc 1965	AATTAAGGAATCATGCAAAGGTTAAAA	ATCAATTTTGGGGAATTTTAAAGTGAA	1272
Lpc 1958	GTGAACAGATTGACATAAGAAGAGCCT	GCTCTTTGAAGCAGGAATTTTGG	1248
Lpc 2009	CCTCAGGCACTTTAAAATCCAGATT	CGAGTCCAATGCAAGGAAAATAGA	883
Lpc2077/2078	GCCCATTCACTGTAAATTTGGTG	TGTCTCCTTCTTTATCTCAACAGGTTC	3241
Lpc2104/2105	TCGGATTTCATATTACCTCCATGC	CCAGGAGAAAAACGGTACAAAA	1377
Lpc2522 RR	GCTGCAGTTCTGGATCAAATAGATAA	CTGATCCAGAGAGCGAATTGCAT	978
Lpc 2669	TTTAGCAAGAGTGCTTGTAGCTCTACC	CGTTTTTATAACCCGATTTCACCAG	1115
Lpc 2666	AATCGGTTTTCTCCTAACAAATCACTTG	TTGGGTTTATTACATGTCGCCAC	1419
Lpc 2762 RR	GCCTTACTCTCTTTAAAACTTCTTGGTG	TAATATGCCGAAGCCAGCCTCAT	1834
Lpc 2961	CAATTTCTTCTGGATGGGTCAAAT	GTGTATGCATTTTATTTTGGCTACTGC	973
Lpc 3210	GTGATAACCCCTTTCACCAAATTATG	TAGTTGCACGGATTTAGAAGGCA	1257
Lpc 3237	CCAAATACAATATCGGTGATCTGGT	GTGGAAGGAGTGAATTAATGGAAACT	548
Lpc3312	CGGAGGAAAAACCTAAATAGAGTAACTG	CATGAAACCTGAAATATGGCAAATG	1040
Lpc 2032	CAGACTATTGGTATGGCTTTTATCAGAC	AGGAAGCTCAGTATGGGGTTATCA	561
Lpc 0414	CCTGTCTGCTATACTTAAACAAGGAAAA	AGAAGGTTGTGTCATCCATTTCCTAT	1195
Lpc0263	AAATTCCCGCAAAGCTTTAGTG	AATCCTCCACCAGGGAAGAAA	861

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