Large-scale identification of *Legionella pneumophila* Dot/Icm substrates that modulate host cell vesicle trafficking pathways

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**Summary**

The bacterial pathogen *Legionella pneumophila* replicates in a specialized vacuole within host cells. Establishment of the replication vacuole depends on the Dot/Icm translocation system that delivers a large number of protein substrates into the host cell. The functions of most substrates are unknown. Here, we analysed a defined set of 127 confirmed or candidate Dot/Icm substrates for their effect on host cell processes using yeast as a model system. Expression of 79 candidates caused significant yeast growth defects, indicating that these proteins impact essential host cell pathways. Notably, a group of 21 candidates interfered with the trafficking of secretory proteins to the yeast vacuole. Three candidates that caused yeast secretory defects (SetA, Ceg19 and Ceg9) were investigated further. These proteins impinged upon vesicle trafficking at distinct stages and had signals that allowed translocation into host cells by the Dot/Icm system. Ectopically produced SetA, Ceg19 and Ceg9 localized to secretory organelles in mammalian cells, consistent with a role for these proteins in modulating host cell vesicle trafficking. Interestingly, the ability of SetA to cause yeast phenotypes was dependent upon a functional glycosyltransferase domain. We hypothesize that SetA may glycosylate a component of the host cell vesicle trafficking machinery during *L. pneumophila* infection.

**Introduction**

Many bacterial pathogens cause disease by growing inside eukaryotic cells. One strategy for successful intracellular replication involves growth within a membrane-bound vacuole. Such pathogens interact intimately with host cell vesicle trafficking pathways to direct the biogenesis of a membranous vacuole permissive for growth (Salcedo and Holden, 2005). Establishment of a membrane-bound compartment is thought to provide pathogens with a protected niche by allowing evasion of host cell innate immune mechanisms and access to a source of nutrients (Roy and Mocarski, 2007).

*Legionella pneumophila*, the causative agent of Legionnaire’s pneumonia, is one example of a bacterial pathogen that grows within a membrane-bound vacuole inside eukaryotic cells. *L. pneumophila* is a Gram-negative bacterium ubiquitously found in freshwater environments as an intracellular parasite of unicellular protozoans and in biofilms (Steinert *et al.*, 2002). *L. pneumophila* causes disease in humans by growing within alveolar macrophages after the inhalation of contaminated aerosols (McDade *et al.*, 1977; Horwitz and Silverstein, 1980). A striking feature of the bacterium’s growth within both protozoan and mammalian host cells is the entry of the *Legionella*-containing vacuole (LCV) into a unique membrane trafficking pathway. After uptake into host cells, the LCV evade trafficking into the antimicrobial lysosomal network (Horwitz, 1983a; Roy *et al.*, 1998). Instead, ER-derived vesicles and mitochondria are rapidly directed to the surface of the LCV, where the microorganism proceeds to grow (Horwitz, 1983b; Tilney *et al.*, 2001; Kagan and Roy, 2002).

Central to the ability of *L. pneumophila* to grow within both mammalian and protozoan cells is the Dot/Icm type IV secretion system that injects a large number of protein substrates into the host cell cytosol (Segal *et al.*, 1998; Vogel *et al.*, 1998). These translocated substrates are believed to be essential for biogenesis of the LCV and intracellular replication of *L. pneumophila* (Shin and Roy, 2008). In support of this hypothesis, mutations in the *dot/icm* genes prevent remodeling of the LCV into an ER-like compartment and result in routing into the...
Dot/Icm substrates modulate vesicle trafficking

Recent work from several groups has identified over 80 protein substrates of the Dot/Icm translocation system (Conover et al., 2003; Luo and Isberg, 2004; Campodonico et al., 2005; Shohdy et al., 2005; Ninio and Roy, 2007; Zusman et al., 2007; Altman and Segal, 2008; de Felipe et al., 2008). Consistent with the observed ability of *L. pneumophila* to remodel the LCV, a group of these proteins modulate host cell vesicle trafficking pathways (Nagai et al., 2002; Derre and Isberg, 2005; Shohdy et al., 2005; Machner and Isberg, 2006; 2007; Murata et al., 2006; Ingrumson et al., 2007; de Felipe et al., 2008; Pan et al., 2008). However, a complicating issue in understanding the precise functions of *L. pneumophila* Dot/Icm substrates arises from the fact that individual deletions of most substrate genes have little or no impact on intracellular growth of the bacterium. One explanation for this phenomenon may be that considerable functional redundancy exists among different substrates towards host cell targets. In addition, genetic studies indicate that multiple vesicle trafficking pathways are directed towards the LCV, suggesting that redundancy extends to the host as well as the bacterium (Dorer et al., 2006). Therefore, alternative systems for generating effector-dependent phenotypes will be important for a complete understanding of their functions during infection.

Ectopic expression in the budding yeast *Saccharomyces cerevisiae* has recently emerged as a powerful tool for both the identification and characterization of secreted bacterial effector proteins (Lesser and Miller, 2001; Valdivia, 2004; Kumar et al., 2006; Sisko et al., 2006; Kramer et al., 2007). Importantly, the generation of yeast phenotypes by bacterial effector proteins is specific, as non-translocated bacterial proteins rarely cause yeast phenotypes (Slagowski et al., 2008). Yeast expression has been utilized to identify and characterize *L. pneumophila* Dot/Icm substrates (Campodonico et al., 2005; Shohdy et al., 2005; de Felipe et al., 2008). However, these studies screened a limited number of substrates or used libraries constructed from randomly digested *L. pneumophila* genomic DNA, raising the possibility that these screens were not saturating. Thus, we hypothesized that more comprehensive screens will identify additional Dot/Icm substrates that modulate host cell vesicle trafficking or other essential host cell signalling pathways. In this report, we analysed a defined set of 127 confirmed or candidate Dot/Icm substrates for their ability to generate yeast phenotypes. This directed approach allowed for the identification of a large group of proteins that interfere with cell growth and disrupt protein secretion. Interestingly, the ability of one of these proteins, SetA, to cause growth and secretary phenotypes in yeast was dependent upon a functional glycosyltransferase domain.

**Results**

**Construction and characterization of a L. pneumophila yeast expression library**

We targeted *L. pneumophila* ORFs for yeast expression based on several overlapping criteria: (i) ORFs that encode known Dot/Icm substrates for which little or no functional data were available; (ii) ORFs predicted to encode proteins with eukaryotic-like domains (Cazan et al., 2004; de Felipe et al., 2005); (iii) ORFs that encode likely Dot/Icm substrates identified from a large-scale immunofluorescence-based screen currently ongoing in our lab (L. Huang et al., in preparation). As positive controls, we included the ORFs encoding the Dot/Icm substrates LidA, RalF, YllA/LegC7 and VipD in the library as these proteins have previously been shown to generate phenotypes in yeast (Campodonico et al., 2005; Derre and Isberg, 2005; Shohdy et al., 2005; VanRheenen et al., 2006). In total, we targeted 140 ORFs for yeast expression and successfully amplified 133 of these by PCR. From this group, 127 were successfully cloned into a yeast expression plasmid by homologous recombination (Experimental procedures). Of note, we routinely failed to clone six ORFs (*lgt1*, *legC5/lgt3*, *legC8/lgt2*, *sdeA*, *lpg1489* and *lpg2504*), as they were extremely toxic to yeast even at low expression levels (Table 1). To control protein expression in yeast, ORFs were cloned downstream of the inducible GAL1 promoter (Fig. 1A), which is repressed by glucose and induced by galactose (Giniger et al., 1985). ORFs were cloned as fusions to an N-terminal Xpress epitope tag to facilitate detection of expressed proteins while an N-terminal 6xHis tag was included to enable biochemical studies. We assessed the production of each *L. pneumophila* protein in yeast by immunoblot analysis with monoclonal anti-Xpress antibodies (Fig. 1B). Production of full-length protein was confirmed for 117 out of 127 candidates. Interestingly, for some proteins we observed the presence of higher molecular weight species (Fig. 1B, lower panel, see Lpg1978, Lpg0160 and Lpg1752 as examples), suggesting the presence of post-translational modifications or formation of higher-order oligomers.

**Production of L. pneumophila proteins inhibit yeast cell growth**

Translocated bacterial effector proteins often target conserved host cell processes essential for cell growth (Valdivia, 2004). Therefore, we tested if any of the *L. pneumophila* proteins in our expression bank caused yeast growth defects. The growth of strains producing *L. pneumophila* proteins was compared with empty vector control strains by spotting a series of 10-fold dilutions to selective media containing glucose or galactose. Strik-
ingly, 79 of the 127 L. pneumophila proteins in our bank negatively impacted yeast cell growth. Some of these growth defects were intermediate in nature, with strains growing at a reduced rate compared with the control strain (Fig. 2A, see Ceg7, Ceg19, Ceg9, LegS2 and SidA as examples), while another group exhibited severe growth defects with the strains growing extremely slowly or not at all (Fig. 2A, see SdbA, SidG, VpdA, Lpg0969 and Lpg1978 as examples). These results suggest that these proteins impact processes essential for yeast cell growth. In total, we found that 43 proteins caused an intermediate growth defect, 36 severely impacted growth, while 48 did not impact growth in a discernible manner (Fig. 2C and Table 1). Of the 48 that did not cause a growth phenotype, 41 of these were produced as assessed by immunoblot, indicating that the lack of phenotype was not due to lack of protein synthesis (Table S1). Determination of colony size provided quantitative support for the phenotypic growth defect categorization of strains (Fig. 2B).

Identification of L. pneumophila proteins that delay trafficking of host cell secretory proteins to the yeast vacuole

We next tested if any of the L. pneumophila proteins included in the expression bank impinged upon the

Table 1. Summary of Legionella proteins found to cause growth defects in S. cerevisiae in this study.

| Lpg No. | Alias          | Growth defect a | Lpg No. | Alias          | Growth defect a |
|---------|----------------|-----------------|---------|----------------|-----------------|
| 0012    | –              | +               | 1683    | –              | +               |
| 0030    | –              | +               | 1687    | –              | +               |
| 0059    | Ceg2           | +               | 1701    | LegC3          | +               |
| 0038    | LegA10/AnkQ    | ++              | 1797    | –              | +               |
| 0086    | –              | ++              | 1950    | RafF           | ++              |
| 0096    | –              | ++              | 1961    | –              | ++              |
| 0195    | –              | +               | 1978    | SetA           | ++              |
| 0210    | –              | +               | 2144    | LegAU13/Ceg27/AnkB | +         |
| 0227    | Ceg7           | +               | 2147    | –              | +               |
| 0246    | Ceg9           | +               | 2147    | –              | +               |
| 0275    | SdbA           | ++              | 2155    | SidJ           | ++              |
| 0376    | SdhA           | ++              | 2157    | SdeA           | NR              |
| 0390    | VipA           | ++              | 2176    | LegS2          | +               |
| 0401    | Ceg11          | ++              | 2199    | –              | +               |
| 0402    | LegA9/Ceg12/AnkY | ++         | 2257    | –              | +               |
| 0422    | LegY           | +               | 2271    | –              | +               |
| 0483    | LegA12/AnkC    | ++              | 2298    | YllA/LegC7     | ++              |
| 0621    | SidA           | +               | 2322    | LegA5/AnkK     | ++              |
| 0634    | –              | +               | 2327    | –              | +               |
| 0642    | WipB           | ++              | 2391    | SdbC           | +               |
| 0695    | LegA8/AnkN/AnkX | ++              | 2410    | VpdA           | ++              |
| 0696    | –              | +               | 2425    | –              | ++              |
| 0898    | Ceg18          | ++              | 2444    | –              | +               |
| 0940    | LidA           | ++              | 2452    | LegA14/Ceg31/AnkF | +         |
| 0944    | –              | ++              | 2464    | SidM/DrrA      | ++              |
| 0969    | –              | ++              | 2465    | SidD           | +               |
| 1109    | –              | +               | 2482    | SdbB           | ++              |
| 1111    | –              | +               | 2490    | LepB           | ++              |
| 1121    | Ceg19          | +               | 2504    | –              | NR              |
| 1137    | Ceg20          | ++              | 2510    | SdcA           | +               |
| 1152    | –              | +               | 2526    | –              | +               |
| 1154    | –              | ++              | 2529    | –              | +               |
| 1166    | –              | +               | 2588    | LegS1          | +               |
| 1183    | –              | ++              | 2603    | –              | ++              |
| 1290    | –              | ++              | 2718    | WipA           | +               |
| 1328    | LegT           | +               | 2793    | LepA           | +               |
| 1355    | SidG           | ++              | 2815    | DimB           | +               |
| 1368    | Lgt1           | NR              | 2829    | SidH           | ++              |
| 1426    | VpdC           | ++              | 2831    | Vpd            | +               |
| 1483    | LegK1          | ++              | 2862    | LegC8/Lgt2     | NR              |
| 1488    | LegC5/Lgt3     | NR              | 2879    | –              | ++              |
| 1489    | –              | NR              | 2999    | LegP           | +               |
| 1642    | SidB           | +               |         |                |                 |

a. A (+) symbol indicates production of the L. pneumophila protein resulted in a slow growth/intermediate phenotype in yeast, while a (++) symbol indicates the L. pneumophila protein was extremely toxic and caused a severe growth defect. NR (no recombinants) indicates that correctly spliced recombinants could not be obtained during the cloning process, suggesting that the respective L. pneumophila ORF was toxic even at low levels of expression.
sorting of secretory proteins to the yeast vacuole, the equivalent of the mammalian lysosome (Bowers and Stevens, 2005). Proteins that cause this phenotype are likely to interact with host cell vesicle trafficking factors and therefore may be involved in LCV biogenesis during infection (Derre and Isberg, 2005; Shohdy et al., 2005; de Felipe et al., 2008). To this end, we analysed protein sorting by assaying the trafficking of the well-characterized secretory proteins carboxypeptidase Y (CPY) and alkaline phosphatase (ALP). These proteins are subjected to organelle-specific post-translational modifications as they are trafficked from the ER to the yeast vacuole via transport vesicles. These modifications alter their electrophoretic mobility as assessed by SDS-PAGE and immunoblotting, and therefore they provide sensitive and stage-specific readouts of secretory processes (Fig. 3A). CPY is resident in the ER as the p1 precursor form of 67 kDa and then modified in the Golgi to produce the p2 form of 69 kDa. Finally, CPY is proteolytically processed in the vacuole to yield the mature form of 61 kDa (Stevens et al., 1982). Similarly, the ER form of ALP is a p1 precursor of 66 kDa, and is transported through the Golgi and cleaved in the vacuole to generate the 60 kDa mature form (Klionsky and Emr, 1989).

In control strains containing empty vector, CPY and ALP were detected primarily as the mature forms, indicating efficient transport to the vacuole (Fig. 3B). Interestingly, immunoblot analysis revealed that production of 21 different L. pneumophila proteins out of the 127 tested caused the accumulation of precursor forms of CPY and/or ALP to varying degrees (see Fig. 3B for a representative immunoblot). This group included the substrates LidA, VipD, SidM/DrrA, RalF and LegA8/AnkN/AnkX, each of which has previously been shown to manipulate host cell secretory processes (Nagai et al., 2002; Derre and Isberg, 2005; Machner and Isberg, 2006; Pan et al., 2008). Of the remaining 16 candidates, 7 caused strong or intermediate accumulation of both CPY and ALP precursors when produced in yeast (Fig. 3B and Table 2). This group included the proteins LegS2, LegK1, VpdA, LepA, Ceg9, Ceg19 and Lpg1978. Four candidates caused weaker defects in both CPY and ALP processing (Lpg0518, Lpg0634, Lpg2603 and LegA14/Ceg31/AnkF), while the remaining five appeared to cause selective defects either in CPY transport (LegA7/AnkG/AnkZ and LegA12/AnkC) or ALP transport (Lpg1108, Lpg1961 and LegU2/LubX) (Table 2). A full compilation of yeast expression data, growth defects and secretory defects is provided in Table S1.

Given the secretory defects associated with the production of these proteins in yeast, we hypothesized that they represent strong candidates for Dot/Icm substrates that modulate host cell vesicle trafficking during infection. As Lpg1978, Ceg19 and Ceg9 caused significant secretory phenotypes and were relatively uncharacterized, we investigated these candidates further. Based on the ability of Lpg1978 to impinge upon yeast protein trafficking, we have termed this protein SetA for subversion of eukaryotic vesicle trafficking A.
We next analysed the secretory defects caused by production of SetA, Ceg19 and Ceg9 in greater detail. Specifically, we examined the transport kinetics of CPY in yeast strains producing SetA, Ceg19 and Ceg9 compared with a strain containing empty vector. Strains were pulse labelled for 7 min with [35S] methionine and cysteine and then chased with excess unlabelled amino acids for 20 min. Maturation of CPY was monitored by immunoprecipitation with anti-CPY antibodies. Most of the CPY was converted to the mature form after 20 min chase in the control strain (Fig. 4A). In contrast, strains producing SetA accumulated both the p1 and p2 forms of CPY, indicating a delay in trafficking between the ER and the Golgi as well as between the Golgi and the vacuole. As shown in Fig. 4B, densitometry analysis revealed that SetA-producing cells converted \(~40\%\) less CPY to the mature form relative to the empty vector strain over the chase period. Production of Ceg19 caused the specific accumulation of the p2 form of CPY, indicating a block in trafficking between the Golgi and the vacuole. Ceg19-producing cells converted \(~60\%\) less CPY to the mature form relative to the control strain during the chase period (Fig. 4B). Lastly, production of Ceg9 caused the specific accumulation of p1 CPY, indicating a delay in trafficking between the ER and the Golgi complex. These cells converted \(~45\%\) less CPY to the mature vacuolar form relative to the empty vector strain over the chase period. Taken together, these data indicate that SetA, Ceg19 and Ceg9 cause distinct trafficking defects in yeast. Therefore, these proteins likely modulate host cell vesicle trafficking by different molecular mechanisms.
SetA, Ceg19 and Ceg9 have tropism for eukaryotic secretory organelles

The subcellular localization of ectopically produced bacterial effector proteins in eukaryotic cells can provide important clues as to their function (Sisko et al., 2006). As production of SetA, Ceg19 and Ceg9 caused secretory defects in yeast, we hypothesized that these proteins may localize to organelles of the eukaryotic secretory pathway. EGFP fusions to the N-terminus of SetA, Ceg19 and Ceg9 were produced in transiently transfected HeLa cells and analysed by fluorescence microscopy.

We observed that EGFP-SetA localized to pleiomorphic vesicular structures that were found in a perinuclear locale as well as throughout the cell (Fig. 5A). A significant portion of these structures co-stained with antibodies directed against the late endosomal/lysosomal proteins LAMP-1 and Rab7. The EGFP-SetA positive structures did not co-stain with antibodies directed against ER proteins, Golgi markers, or the autophagy marker Atg12 (Fig. S1 and data not shown), indicating that the SetA localization pattern was specific and that SetA was not targeted to an autophagic compartment. These results indicate that SetA localizes to late endosomal/lysosomal compartments when ectopically produced in mammalian cells. EGFP-Ceg9 localized to relatively smaller vesicular structures that could be found in a perinuclear position as well as throughout the cell periphery. In some cells, a portion of EGFP-Ceg9 also exhibited a cytoplasmic distribution. A significant number of these EGFP-Ceg9 positive compartments co-stained with anti-LAMP1 antibodies, indicating that they were endosomal in nature (Fig. 5B). Interestingly, these structures rarely stained positively for Rab7, suggesting they may be a different class of late endosomal compartments as compared with those targeted by SetA.

In contrast to the subcellular localization patterns displayed by SetA and Ceg19, EGFP-Ceg9 localized to the nuclear rim, juxtanuclear compartments and membrane tubules that extended towards the cell periphery (Fig. 5C). This localization pattern was heterogeneous, as some transfected cells contained EGFP-Ceg9 primarily in tubular structures, while other cells contained EGFP-Ceg9 mainly at the nuclear rim and in juxtanuclear structures. Regardless, these structures co-stained with antibodies directed against the ER proteins calnexin and PDI, indicating that ectopically produced EGFP-Ceg9 has tropism for ER membranes. We also observed that EGFP fusions to SetA, Ceg19 and Ceg9 co-fractionated with membranes to varying extents when ectopically produced in mammalian cells (Fig. 5D). Taken together, these data indicate that SetA, Ceg19 and Ceg9 localize to secretory organelles

![Image](https://via.placeholder.com/150)

**Fig. 4.** Production of SetA, Ceg19 and Ceg9 cause distinct trafficking defects in yeast.

A. Pulse-chase analysis of yeast expression strains. Yeast strains grown in the presence of 2% galactose were pulsed for 7 min with 35S-labelled cysteine/methionine to label nascent proteins and then chased for 20 min with excess unlabelled amino acids. Pulse-labelled CPY was immunoprecipitated, resolved on an 8% acrylamide gel and visualized by autoradiography. The positions of mature and precursor forms of CPY are indicated on the left side of the autoradiogram.

B. Quantification of CPY trafficking defects in yeast strains producing SetA, Ceg19 and Ceg9. Mature CPY (mCPY) levels for each strain were visualized as described in (A) and quantified using densitometry. Levels were normalized to the mature CPY signal from the empty vector control strain and plotted as a bar graph. Data are representative of two separate experiments.

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**Table 2.** Summary of *L. pneumophila* proteins found to cause secretory defects in *S. cerevisiae* in this study.

| Lpg No. | Alias | CPY defect<sup>a</sup> | ALP defect<sup>b</sup> |
|---------|-------|------------------------|------------------------|
| 0246    | Ceg9  | ++                     | ++                     |
| 0403    | LegA7/AnkG/AnkZ | + | – |
| 0483    | LegA12/AnkC | + | – |
| 0518    | –     | +                      | +                      |
| 0634    | –     | +                      | +                      |
| 0695    | LegA8/AnkN/AnkX | +++ | +++ |
| 0940    | LidA  | +++                    | +++                    |
| 1108    | –     | –                      | ++                     |
| 1121    | Ceg19 | ++                     | ++                     |
| 1483    | LegK1 | +++                    | +++                    |
| 1950    | RalF  | +++                    | +++                    |
| 1961<sup>b</sup> | – | – | ++ |
| 1978    | SetA  | ++                     | ++                     |
| 2176    | LegS2 | +++                    | +++                    |
| 2410    | VpdA  | +++                    | +++                    |
| 2452    | LegA14/Ceg31/AnkF | + | + |
| 2464    | SldM/DrrA | +++ | +++ |
| 2603    | –     | +                      | +                      |
| 2793    | LegA8/AnkG/AnkZ | + | + |
| 2830    | LegU2/LubX | – | + |
| 2831    | VipD  | ++                     | ++                     |

<sup>a</sup> *L. pneumophila* proteins were assayed by immunoblot for their ability to cause defects in the trafficking of CPY and/or ALP to the yeast vacuole. A (−) symbol indicates production of the protein did not impinge upon trafficking. A (+) symbol indicates a mild defect in trafficking, a (+++) symbol indicates an intermediate defect while a (+++) symbol indicates the *L. pneumophila* protein caused a strong delay in the trafficking process.

<sup>b</sup> Parologue of SetA/Lpg1978.
when produced in mammalian cells, consistent with the ability of these proteins to cause yeast secretory defects.

SetA, Ceg19 and Ceg9 have Dot/Icm translocation signals

As production of SetA, Ceg19 and Ceg9 caused specific phenotypes in eukaryotic cells, we hypothesized that these proteins are substrates of the Dot/Icm system. To test this possibility, we employed the CyaA fusion assay. This assay has been successfully used to study the translocation of many bacterial effector proteins into eukaryotic cells (Chen et al., 2004; de Felipe et al., 2005; Shohdy et al., 2005; Cambronne and Roy, 2007). CyaA activity is dependent on host cell calmodulin for activity, so increased cellular cAMP levels after infection with strains producing Cya fusion proteins indicates Cya translocation from the bacteria to the host cytosol (Sory and Cornelis, 1994). L. pneumophila strains harbouring the catalytic domain of CyaA fused to the N-terminus of SetA, Ceg19 or Ceg9 were used to challenge U937 cells at multiplicity of infection (moi) = 1 for 30 min. We observed a ~30-fold increase in cAMP levels relative to the uninfected control following incubation of cells with each of the wild-type L. pneumophila strains producing Cya fusion proteins (Fig. 6A). This increase in cAMP was comparable to the amount generated by a strain producing Cya-RalF, a known Dot/Icm substrate (Nagai et al., 2002). Furthermore, the increase in cAMP was dependent on a functional Dot/Icm system, as no increase in cAMP levels was observed for dotA- strains producing Cya fusions. This was not due to differential production of the Cya fusion proteins in the wild-type and dotA- strain backgrounds, as assessed by immunoblot with anti-Cya antibodies (Fig. S2). These data indicate that SetA, Ceg19 and Ceg9 are delivered into the cytosol of host cells in a Dot/Icm-dependent manner.

We next assessed the subcellular distribution of SetA during infection. The mammalian cell localization data
suggest that a pool of SetA may associate with host cell membranes during infection. To test this possibility, we performed fractionation experiments on infected cells. U937 cells were incubated at moi = 5 for 30 min with wild-type or dotA L. pneumophila strains over-producing SetA. Lysates were collected, subjected to low-speed spins to remove bacteria and unbroken host cells, and the low-speed supernatant was subjected to further fractionation by centrifugation at 100 000 g. The low-speed, bacteria-free supernatant obtained from host cells infected with wild-type L. pneumophila contained a significant amount of SetA, indicating that the protein was translocated (Fig. 6B, ‘Supe.’ lane). In contrast, the non-translocated protein EnhC was not detected in this fraction (Liu et al., 2008). Importantly, the presence of SetA in the supernatant was dependent on a functional Dot/Icm system, as SetA was not detected in low-speed supernatants obtained from a dotA incubation. After high-speed centrifugation, SetA fractionated with both host cell cytosol and membrane fractions in a Dot/Icm-dependent manner (Fig. 6B, ‘Cytosol’ and ‘Mem.’ lanes). These results indicate that a portion of SetA associates with host cell membranes after translocation by the Dot/Icm system, consistent with our observations that SetA has tropism for late endocytic compartments (Fig. 5A).

SetA is induced in the post-exponential phase of growth

A large body of evidence indicates that the expression of factors important for L. pneumophila virulence, including several Dot/Icm substrates, is increased during the post-exponential phase of growth (Byrne and Swanson, 1998; Hammer and Swanson, 1999; Nagai et al., 2002; Luo and Isberg, 2004; Bardill et al., 2005; Bruggemann et al., 2006; VanRheenen et al., 2006; Banga et al., 2007; Zusman et al., 2007). Affinity purified anti-SetA antibodies were used to immunoprobe bacterial lysates harvested from L. pneumophila strains at different stages of growth. SetA protein levels were increased approximately threefold in the post-exponential phase of growth. This protein was absent in lysates derived from a ΔsetA strain, indicating the antibodies were specific (Fig. 7A).

Intracellular growth characteristics of a ΔsetA mutant strain

To determine whether SetA is important for efficient intracellular growth of L. pneumophila, the ΔsetA mutant strain was analysed for growth in both murine bone marrow-derived macrophages and the soil amoeba Dictyostelium discoideum (Fig. 7B). The ΔsetA mutant strain grew similarly to wild-type L. pneumophila in both host cell types, indicating that SetA is not required for efficient intracellular replication of L. pneumophila under laboratory growth conditions, typical of knockouts that affect most of the Dot/Icm substrate genes.

A functional glycosyltransferase domain is required for SetA to interfere with yeast cell function

Inspection of the amino acid sequence of SetA revealed structural features that may provide clues as to
function. First, analysis of the SetA sequence with the MARCOIL algorithm predicted the presence of coiled-coil structure in the C-terminal region of the protein (Fig. 8A). Several confirmed Dot/Icm substrates are predicted to possess coiled-coil domains (Conover et al., 2003; Campodonico et al., 2005; de Felipe et al., 2005). Second, BLAST searches revealed significant homology of the N-terminal region of SetA with both prokaryotic and eukaryotic glycosyltransferases (Fig. 8A).
Glycosyltransferases catalyze the transfer of a sugar moiety from an activated sugar donor onto carbohydrate and protein acceptors (Breton et al., 2006). Some secreted bacterial toxins are known to act as glycosyltransferases towards host cell targets, such as the clostridial glucosylating toxins which catalyze the transfer of a glucose moiety onto host cell Rho GTPases (Aktories and Just, 2005). Recently, three paralogous L. pneumophila proteins (Lgt1, Lgt2/LegC8 and Lgt3/LegC5) were shown to possess cytotoxin-like glucosylating activity towards the host cell translation elongation factor eEF1A (Belyi et al., 2003; 2006; 2008). At least two of these proteins (Lgt2/LegC8 and Lgt3/LegC5) have also been shown to be Dot/Icm substrates (de Felip et al., 2005).

Alignment of the catalytic regions of known and putative glucosylating toxins with the glycosyltransferase domain of SetA revealed a conserved DxD motif, shown to be essential for enzymatic activity of these proteins (Fig. 8B) (Busch et al., 1998). Given these findings, we tested if the DSD motif in the glycosyltransferase domain of SetA was important for the generation of yeast growth and secretory phenotypes. Mutation of the DSD motif to ASA alleviated the toxicity of SetA to yeast growth (Fig. 8C). This was not due to instability of the ASA mutant protein as assessed by immunoblot (Fig. 8D). Furthermore, the ASA mutant protein was no longer able to cause the accumulation of precursor forms of CPY and ALP (Fig. 8D). These results demonstrate that a functional glycosyltransferase domain is required for the ability of SetA to interfere with yeast cell function. These data are consistent with a model where SetA may act as a glycosyltransferase towards host cell targets involved in vesicle trafficking.

**SetA is ubiquitinated in eukaryotic cells**

Immunoblot analysis of SetA produced in yeast consistently revealed the presence of higher molecular weight forms of SetA (Fig. 8D). These higher forms were present for the DSD to ASA mutant protein, although they were less intense. We also observed that EGFP-SetA localized to endocytic organelles in mammalian cells (Fig. 5A), organelles known to be associated with the ubiquitin-dependent sorting of membrane proteins (Babst, 2005; Piper and Katzmann, 2007; Piper and Luzio, 2007). Therefore, we hypothesized that the larger forms of SetA may represent ubiquitinated species of the protein. To test this idea, we prepared detergent-solubilized lysates from yeast strains harbouring an empty vector or a vector encoding epitope-tagged SetA and performed immunoprecipitations with affinity-purified antibodies directed against SetA. SetA and its higher order forms could be efficiently immunoprecipitated in this manner, as SetA was depleted from the input mate-
random *L. pneumophila* genomic libraries in yeast has uncovered distinct phenotypes for individual substrates. This has allowed the identification of several substrates, and has also demonstrated that *L. pneumophila* proteins can perturb sorting of a model secretory protein to the yeast vacuole at distinct steps in the trafficking pathway (Campodonico *et al.*, 2005; Shohdy *et al.*, 2005; de Felipe *et al.*, 2008).

In this report, we screened a defined set of 127 confirmed or candidate Dot/Icm substrates for their ability to generate yeast growth and secretory phenotypes. We found that 79 *L. pneumophila* proteins significantly impacted yeast cell growth. Furthermore, we suspect that six other genes caused such severe growth defects that they were unclonable by the yeast homologous recombination cloning strategy we employed (Table 1). Specifically, we utilized a multicopy expression vector, and this likely allowed for low-level production of *L. pneumophila* proteins due to ‘leaky’ expression from the GAL1 promoter even under repressive conditions. Recent evidence indicates that the generation of yeast growth phenotypes by the ectopic expression of bacterial proteins is a unique property of translocated substrates, as expression of non-translocated proteins rarely causes growth defects (Slagowski *et al.*, 2008). Therefore, the growth phenotypes we observed are likely due to disruption of specific host cell targets. Importantly, these growth defects are genetically tractable phenotypes and provide means to investigate the action of these substrates towards host cell factors.

We also identified a group of *L. pneumophila* proteins that inhibited the trafficking of secretory proteins to the yeast vacuole. Nearly all of these proteins also caused growth phenotypes (Tables 1 and 2). Of the 21 proteins in our expression library that disrupted trafficking of the secretory proteins CPY and/or ALP to the yeast vacuole, five (LidA, SidM/DrrA, RaIF, VipD and LegA8/AnkN/AnkX) had already been implicated in modulating secretory processes, and thus served as an important validation of our approach (Nagai *et al.*, 2002; Derre and Isberg, 2005; Shohdy *et al.*, 2005; Machner and Isberg, 2006; Pan *et al.*, 2008). We focused on three uncharacterized proteins (SetA/Lpg1978, Ceg19/Lpg1121 and Ceg9/Lpg0246) identified from our secretion screen for further study. Lpg1121 and Lpg0246 have been previously annotated as Ceg proteins (correlated with effector-encoding genes) (Zusman *et al.*, 2007). The ceg genes are predicted to be under transcriptional control of the response-
regulator PmrA, an important regulator of virulence factor expression in both *L. pneumophila* and *C. burnetii* (Zusman *et al.*, 2007). We demonstrated that SetA, Ceg19 and Ceg9 were translocated into host cells by the Dot/Icm system, as assessed by the Cya translocation assay. Furthermore, SetA co-fractionation with host cell cytosol and membranes in a Dot/Icm-dependent manner during infection, providing a second line of evidence for translocation. Interestingly, Ceg9 is predicted to possess a hydrophobic putative membrane-spanning region. Other Dot/Icm substrates are also predicted to contain hydrophobic domains (Campodonico *et al.*, 2005; de Felipe *et al.*, 2005; Cambronne and Roy, 2007; Kubori *et al.*, 2008). These observations indicate that the Dot/Icm system is capable of translocating proteins with significant hydrophobic character, and raises mechanistic questions as to the folding state of such proteins as they are engaged by and pass through the Dot/Icm complex. As Ceg9 has a robust translocation signal, this substrate may serve as a useful model to investigate the mechanisms by which hydrophobic substrates interface with the Dot/Icm machinery.

In vivo labelling experiments revealed that SetA, Ceg19 and Ceg9 modulated yeast protein trafficking in distinct ways. The precise molecular mechanisms by which these proteins impinge upon vesicular trafficking are unknown at present. However, some information can be gained from inspection of their protein sequences and subcellular localization patterns. Ceg19 is predicted to possess an N-terminal coiled-coil region and displays tropism for late endocytic organelles. We also observed that Ceg19 localized to the vacuolar membrane when ectopically produced in yeast cells (data not shown), the equivalent of the mammalian lysosomal membrane. Further, Ceg19 overproduction in yeast specifically delayed CPY transport between the Golgi complex and the vacuole, consistent with its localization to late endocytic compartments. Given these data, we speculate that Ceg19 may interact through its coiled-coil domain with host cell proteins involved in vesicular transport between the Golgi complex and late endocytic organelles. Similarly, Ceg9 has no primary sequence homology to any known protein. However, as this protein delays CPY transport between the ER and the Golgi complex and exhibits tropism for ER membranes, we hypothesize that Ceg9 interacts with host cell factors involved in ER-to-Golgi membrane trafficking, perhaps to recruit or redirect ER-derived vesicles to the LCV surface.

We have focused much of our attention on the SetA protein. SetA is a coiled-coil domain containing protein with homology to large clostridial glucosylating toxins. These toxins target host cell Rho GTPases by catalysing the transfer of a glucose moiety to a conserved threonine residue within their catalytic region, resulting in inactiva-

\[ \text{GDP} + \text{Glucose} \rightarrow \text{GTP} + \text{GluCoRhoGTP} \]

tion of the GTPase and disruption of downstream signaling events (Aktories and Just, 2005). This homology raises the possibility that SetA may act as a toxin-like factor that glucosylates a host cell protein involved in vesicle trafficking. Consistent with this model, we found that mutation of a conserved DxD motif within the glycosyltransferase domain of SetA blocked the ability of the protein to cause yeast growth and secretory defects. One possibility is that SetA glucosylates a component of the endosomal ubiquitin-dependent protein sorting machinery. Through the glucosylation and inactivation of such a factor, SetA might inhibit ubiquitin-dependent endocytic trafficking and therefore prevent entry of the LCV into the endocytic network. Three lines of evidence are consistent with this hypothesis: (i) overproduction of SetA in yeast inhibits CPY and ALP transport to the vacuole/lysosome; (ii) ectopically produced EGFP-SetA localizes to late endosomal/lysosomal organelles, compartments known to harbour ubiquitin-dependent sorting complexes; and (iii) a pool of SetA is ubiquitinated during ectopic expression in host cells, suggesting that a subset of SetA is in close proximity to the endosomally localized ubiquitination machinery.

Based on these properties, SetA may be a member of an emerging class of Dot/Icm substrates that prevent entry of the LCV into the endocytic network. Recent work indicates that the substrate LegA8/AnkN/AnkX may also be in this functional class of effectors, as this protein appears to inhibit microtubule-dependent fusion of the LCV with LAMP1 positive compartments (Pan *et al.*, 2008). Alternatively, glucosylation of a host cell trafficking factor by SetA may act to promote the recruitment of ER-derived vesicles to the LCV. Glucosylation of a host trafficking factor might alter its function such that the downstream effect is a rerouting of ER-derived material to the LCV surface.

It should be noted that there are other explanations for the function of SetA ubiquitination. The most obvious model is that the function of ubiquitination is to simply direct SetA to proteasomes for degradation once SetA function is no longer required. Previous work, however, raises another possibility that this modification could play a role in the dislocation of the protein from the LCV. Ubiquitinated proteins have been observed surrounding the LCV membrane during infection of host cells (Dorer *et al.*, 2006). The identities of these ubiquitinated proteins are unknown, although their dislocation from the LCV surface is promoted by cytosolic components of the host cell ER-associated degradation pathway (ERAD). It has been proposed that *L. pneumophila* may hijack the ERAD pathway to promote removal of ubiquitinated Dot/Icm substrates from the LCV membrane. Therefore, ubiquitination could be a strategy to facilitate removal of SetA from the vacuolar membrane and movement to other sites in the
cell. At present, we have been unable to detect ubiquitination of SetA during \textit{L. pneumophila} infection of host cells. This may be due to technical issues or may be due to inhibition of SetA ubiquitination by the activity of other Dot/Icm substrates. For example, \textit{L. pneumophila} produces several Dot/Icm substrates that modulate or are predicted to impact host cell ubiquitination pathways, including a deubiquitinase (Cazalet \textit{et al}., 2004; Catic \textit{et al}., 2007; Kubori \textit{et al}., 2008).

In summary, we have utilized a defined yeast expression library to identify a large group of confirmed and candidate \textit{L. pneumophila} Dot/Icm substrates that cause yeast growth and secretary defects. These growth phenotypes will provide new opportunities for functional analysis of these proteins towards host cell targets using yeast genetic approaches. Furthermore, we demonstrated that 21 \textit{L. pneumophila} proteins subvert yeast protein trafficking, 16 of which had not been previously implicated in this process. For three of these proteins (SetA, Ceg19 and Ceg9), our initial characterization studies are consistent with a model where they function during infection to facilitate biogenesis of the LCV by manipulating host cell vesicle trafficking factors. Future studies on the precise modes of action of SetA, Ceg19 and Ceg9 towards host cell targets will provide insight towards the molecular mechanisms by which \textit{L. pneumophila} manipulates host cell membrane trafficking to establish a replication vacuole.

**Experimental procedures**

**Strains and media**

Bacterial strains, yeast strains and plasmids used in this study are listed in Table S2. The \textit{L. pneumophila} strains used are derivatives of the \textit{L. pneumophila} strain Philadelphia-1 strain Lp02 (\textit{thyA \Delta(hsdRI-lv) rpsL}) (Berger and Isberg, 1993), and were grown on casamino acids-yeast extract (CYE) solid medium or in ACES-yeast extract (AYE) broth. For \textit{L. pneumophila}, antibiotics were used at the following concentrations: chloramphenicol 5 \(\mu\)g ml\(^{-1}\) and kanamycin 40 \(\mu\)g ml\(^{-1}\). The growth media were supplemented with thymidine at 100 \(\mu\)g ml\(^{-1}\) or 5\% sucrose when appropriate. The \textit{L. pneumophila} strains used in all assays were grown to post-exponential phase (A\textsubscript{600} 3.2–3.5) unless stated otherwise. Lp02 \textunderscore{\Delta}setA harbouring an in-frame deletion of \textit{setA} was constructed as described previously (Dumenil and Isberg, 2001; Luo and Isberg, 2004).

For \textit{E. coli} strains, ampicillin was added to 100 \(\mu\)g ml\(^{-1}\) when appropriate. Yeast strains were grown in complete synthetic medium (CSM) dropout mixes (US Biological) supplemented with glucose or galactose as indicated in the text. All yeast methods were based on protocols as outlined (Sherman, 1991).

**Construction of an \textit{L. pneumophila} yeast expression library**

\textit{Legionella pneumophila} ORFs were cloned into the commercially available yeast expression plasmid pYES/NT (Invitrogen) using yeast homologous recombination (Sisko \textit{et al}., 2006). Oligonucleotides were designed such that the 3’ ends were specific to the gene of interest and the 5’ ends were complementary to the sequences flanking the EcoRI–Xhol sites within the multicloning site of pYES/NT (a complete list of oligonucleotides used is available in Table S3). Pfu Ultra II DNA polymerase (Stratagene) was used for the amplification of \textit{L. pneumophila} ORFs from Lp02 genomic DNA. PCR-amplified \textit{L. pneumophila} ORFs were cotransformed with EcoRI–Xhol digested pYES/NT into BY4742 yeast cells using the lithium acetate/PEG method (Sherman, 1991). Transformation reactions were plated to CSM media lacking uracil to select for recombinants. Plasmids were recovered from recombinant yeast strains using the ‘smash and grab’ method (Sherman, 1991), sequenced to confirm correct insertion of the amplified \textit{L. pneumophila} ORF and retransformed into the \textit{S. cerevisiae} strain BY4742. For immunoblot analysis of \textit{L. pneumophila} proteins produced in yeast, strains were grown to saturation at 30°C (A\textsubscript{600} 5) in CSM-ura media containing 2\% glucose. Strains were then back-diluted to an A\textsubscript{600} of 1.5 in CSM-ura media containing 2\% galactose to induce protein expression and grown for 5 h at 30°C. Cells were harvested (6 A600 units) and lysed by vigorous agitation with glass beads in M2 buffer (20 mM Hapes pH 7.0, 150 mM NaCl) containing a protease inhibitor cocktail (Roche). Cleared lysates were mixed with sample buffer, boiled for 5 min, resolved on 8\% or 10\% polyacrylamide gels, transferred to PVDF membranes and probed with anti-Xpress monoclonal antibodies (Invitrogen). For analysis of yeast secretory defects, expression strains were grown as described above and lysates were immunoblotted with antibodies against CPY, ALP and PGK (Molecular Probes).

**Pulse-chase experiments**

Labelling of growing yeast strains was performed as previously described (Belden and Barlowe, 1996) with some modifications. Yeast expression strains and an empty vector control strain were grown to stationary phase (A\textsubscript{600} 5) in CSM-ura containing 2\% glucose. Cells were then back-diluted to an A\textsubscript{600} of 0.2 in 30 ml low-sulfate CSM-ura medium (US Biological) containing 2\% glucose and grown for 4 h at 30°C (approximately two cell doublings). Cells were then pelleted, re-suspended in low-sulfate CSM-ura media containing 2\% galactose and grown for 5 h to induce expression of \textit{L. pneumophila} proteins. Next, 15 A\textsubscript{600} units of each culture were pelleted, re-suspended in 5 ml medium, pre-cultured for 10 min and pulse-labelled with 12 \(\mu\)l ProMx\(^{[\text{35S}]}\) methionine and cysteine (Amersham) for 7 min. Cultures were then chased by the addition of excess unlabelled methionine and cysteine (10 \(\mu\)l of a 20 mg ml\(^{-1}\) solution). Cell lysates were taken at the end of the pulse period and after 20 min of chase. Cell lysates were prepared by glass bead lysis and labelled species were precipitated from a common extract with specific antibodies for CPY (a kind gift of Dr C. Barlowe, Darmouth Medical School, Hanover, NH). Immunoprecipitations were performed as described below.

**Analysis of yeast growth defects**

To determine the effect of \textit{L. pneumophila} protein production on yeast cell growth, strains were grown to saturation in CSM-ura media containing 2\% glucose. Strains were then back-diluted to
an A600 of 1.0, diluted 10-fold serially, and 5 µl of each dilution was spotted onto CSM-ura plates containing either 2% glucose or 2% galactose. Plates were incubated at 30°C for 72 h. For all experiments, growth of yeast strains was compared with a control strain harbouring empty vector. Strains were scored visually for growth defects and placed in three categories: (i) no defect; (ii) intermediate growth defect; and (iii) severe growth defect. For more quantitative analysis of growth defects, strains were grown to saturation in CSM-ura containing 2% glucose. Cultures were adjusted to an A600 of 1.0, diluted 1:1000 with sterile water, and 100 µl was plated to CSM-ura plates containing either 2% glucose or 2% galactose. Plates were incubated at 30°C for 45 h. For each strain, ~25 colonies were imaged using SPOT microscope digital imaging software. Images were exported to IP Laboratory Spectrum for measurement of colony diameter in pixels. The average colony diameter and standard deviations for each strain was normalized to the empty vector control strain for each experiment.

Mammalian cell culture, transfections and immunofluorescence

HeLa cells and HEK-293T cells were maintained in DMEM containing 10% heat-inactivated FBS (Gibco). Macrophage-like U937 cells were cultured as described (Berger and Isberg, 1993). For transfection of EGFP-SetA, EGFP-Ceg19 and EGFP-Ceg9 expression plasmids, HeLa cells were seeded onto glass coverslips in 24-well dishes and were transfected the next day with 0.5 µg plasmid DNA using Lipofectamine 2000 (Invitrogen). Transfections were allowed to proceed for 8–16 h, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with PBS/0.1% Triton X-100 for 10 min at room temperature, and then blocked for ~16 h in PBS/4% goat serum at 4°C. Cells were then stained with the appropriate antibodies and coverslips were mounted using FluoroGuard Anti-fade Reagent (Bio-Rad). For immunofluorescence, the following antibodies and dilutions were employed: anti-calnexin (1:100, BD Biosciences), anti-PDI (1:50, BD Biosciences), anti-LAMP1 (1:100, Abcam), anti-Rab7 (1:100, Santa Cruz Biotechnology) and anti-Atg12 (1:100, Cell Signalling). Texas red-conjugated goat anti-rabbit or anti-mouse IgG (Molecular Probes) were utilized as secondary antibodies.

For subcellular fractionation experiments, 1 × 10⁶ HEK-293T cells were transiently transfected with the indicated plasmids for 16–20 h. Cells were pelleted and re-suspended in 250 µl lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25 M sucrose, 2 mM MnCl₂, 1 mM MgCl₂) containing protease inhibitors and lysed on ice by 30 passes through a 26-gauge needle. Lysates were centrifuged at 500 g for 2 min at 4°C to remove unbroken cells. Cleared lysates were then centrifuged at 100 000 g for 12 min at 4°C to generate cytosol and membrane fractions. Samples were volume adjusted with buffer, mixed with sample buffer, boiled for 5 min and then analysed by immunoblot.

Translocation assay

Translocation of SetA, Ceg19 and Ceg9 into host cells was assessed using the Cya fusion system (Sory and Cornelis, 1994). Specifically, the L. pneumophila ORFs setA (lp1987), ceg19 (lp1121) and ceg9 (lp0246) were amplified by PCR from Lp02 genomic DNA, digested with BamHI and ligated into the CyaA fusion vector pJB2581 (a kind gift of Dr J. Vogel, Washington University, St Louis, MO). Sequenced plasmids were transformed into WT and dotA L. pneumophila strains by electroporation. Production of Cya fusion proteins was assessed by immunoblotting using monoclonal antibodies directed against CyaA (Santa Cruz Biotechnology). For the translocation assay, differentiatied U937 cells were plated at a density of 2.5 × 10⁶ cells per well of a 24-well tissue culture dish. The next day, the monolayers were infected with post-exponential phase (A600 ~3.5) L. pneumophila strains expressing the Cya fusion proteins of interest at moi = 1. Incubations were allowed to proceed for 30 min, cells were washed three times with warm media, and the monolayer was lysed by the addition of a solution containing 50 mM HCl and 0.1% Triton X-100. Lysates were then boiled for 5 min, the pH was neutralized with NaOH, 0.2 ml cold ethanol was added to the lysate, and samples were incubated on ice for 5 min. Samples were spun at 13 000 g for 5 min, the supernatant was transferred to a new tube, and dried under a vacuum. cAMP levels were measured using the cAMP Biotrak Enzymeimmunoassay System (GE healthcare/Amersham). Values were plotted graphically and are the result of three independent infections ± standard deviation.

Intracellular growth assays

Bone marrow-derived macrophages from A/J mice were prepared as previously described (Conover et al., 2003). D. discoideum AX4 was plated in MB medium prior to incubation with L. pneumophila (Li et al., 2005). For assays of L. pneumophila growth within bone marrow-derived macrophages or D. discoideum, host cells were cultured in 24-well plates and infected with L. pneumophila at moi = 0.05 for 2 h. At each time point, monolayers were lysed with saponin, dilutions of the lysate were plated onto bacteriological media, and colony-forming units were determined from triplicate wells at each time point. Each time point represents the mean number of bacteria recovered from three independent wells ± standard deviation.

Fractionation of infected U937 cells

To detect the fractionation of SetA with host cell membranes after contact with L. pneumophila, differentiated U937 cells were plated at a density of 4 × 10⁷ cells in 10 cm tissue culture dishes. The next day, monolayers were incubated at moi = 5 for 30 min with post-exponential phase cultures of L. pneumophila wild-type or dotA strains over-producing SetA from the plasmid pJB908. Monolayers were washed three times with PBS to remove extracellular bacteria, cells were lifted with 1 ml of trypsin/EDTA, washed once with cold PBS and pelleted. Cells were then re-suspended on ice in 1.5 ml of cold lysis buffer (PBS w/250 mM sucrose) containing 1 mM EDTA, protease inhibitor cocktail (Roche) and 5 µl of Halt phosphatase inhibitor (Thermo Scientific). Cells were mechanically lysed on ice by 12 passes within a chilled Dounce homogenizer. Lysates were spun at 4000 g for 5 min at 4°C to pellet unbroken cells and reduce the viscosity of the sample. The sample was then spun again at 10 000 g for 5 min at 4°C to pellet any uninternalized bacteria and intact bacterial phagosomes. The resulting bacteria-free, vesicle-rich supernatant was then spun at 100 000 g to separate host cell
cytosol from host cell membranes. Samples were collected from each fraction, volume adjusted with lysis buffer, mixed with sample buffer and boiled for 5 min. Samples were analysed by immunoblot with antibodies directed against SetA, EnhC (Liu et al., 2008), calnexin and tubulin (Sigma).

For analysis of growth stage-specific induction of SetA, *L. pneumophila* strains were grown at 37°C in AYE broth supplemented with thymidine. The equivalent of 0.5 *A* 600 units of each strain was harvested at various phases of growth, pelleted, re-suspended in 100 μl sample buffer and boiled for 5 min. Samples were resolved on SDS-PAGE gels, transferred to PVDF membranes and immunoprobed with antibodies directed against SetA and DotG (VanRheenen et al., 2004).

**Immunoprecipitations**

For immunoprecipitations from yeast cells, 25 *A* 600 units of cells expressing Xpress-SetA or an empty vector control were pelleted, re-suspended in 0.3 ml lysis buffer (PBS, 250 mM sorbitol and protease inhibitor cocktail) and disrupted by glass bead lysis. Lysates were cleared of unbroken cells by centrifugation at 500 *g* for 2 min at 4°C. Lysates were solubilized with 1% Triton X-100 and clarified by centrifugation at 16 000 *g* for 10 min at 4°C. Solubilized material (150 μl) was transferred to a new tube on ice, diluted fivefold with ice-cold PBS, and SetA was immunoprecipitated by the addition of 0.2 μg of affinity-purified anti-SetA antibodies and 50 μl 20% protein A-sepharose beads (Amersham). After binding for 3 h at 4°C, beads with bound protein were washed four times with lysis buffer/0.1% Triton X-100. Finally, the bound protein was released from the beads by the addition of 40 μl sample buffer and boiled for 5 min. Proteins from the immunoprecipitates were resolved on polyacrylamide gels and analysed by immunoblot.

For immunoprecipitations from tissue culture cells, 1 × 10⁶ HEK-293T cells were transfected for 16 h with pEGFP-SetA, pEGFP-Ceg19 or empty vector. Cells were harvested, pelleted and solubilized in 0.3 ml buffer (PBS, 250 mM sucrose and protease inhibitors) containing 1% Triton X-100. Samples were centrifuged at 16 000 *g* for 10 min at 4°C to remove debris. Solubilized material (0.2 ml) was transferred to a new tube on ice, diluted fivefold with buffer, and proteins were immunoprecipitated by the addition of affinity-purified anti-SetA antibodies (0.2 μg) or anti-GFP antibodies (2 μg) and 50 μl 20% protein A-sepharose beads. Monoclonal antibodies directed against ubiquitin were obtained from BD Biosciences, monoclonal antibodies specific for polyubiquitin were acquired from Biomol, antibodies specific for polyubiquitin were obtained from BD Biosciences, monoclonal anti-GFP antibodies were obtained from Invitrogen.

**Generation of anti-SetA antibodies**

Full-length *setA* was amplified by PCR and cloned into the bacterial expression vector pQE80 (Qiagen) to generate a 6×His-SetA fusion construct (Table S2). 6×His-SetA was expressed and purified from BL21-DE3 *E. coli* by incubating bacterial cell lysates with Ni-NTA agarose, extensive washing of the resin, and elution of 6×His-SetA with PBS/250 mM imidazole. The fusion protein was used to immunize rabbits by standard procedures (Pocono Rabbit Farm and Laboratory, Canadensis, PA).

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Supporting information

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

**Fig. S1.** EGFP-SetA does not colocalize with the autophagy protein Atg12. HeLa cells were transfected for 8 h with pEGFP-SetA, fixed, permeabilized and stained with antibodies directed against Atg12. Left panel, EGFP-SetA; centre panel, Atg12; right panel, merge (DAPI-stained DNA in blue, EGFP-SetA in green, and Atg12 in red). Bar, 10 μm.

**Fig. S2.** Cya fusions to SetA, Ceg19 and Ceg9 are produced in both wild-type and dotA. pneumophila strains. Immunoblot of whole-cell bacterial extracts from wild-type (WT) and dotA strains. Antibodies used for immunoprecipitation with Atg12. Left panel, Atg12 variant; centre panel, CyaA to assess fusion protein levels. Immunoblot of ICDH is a control to show equivalent loading. White dots indicate bands of the predicted molecular weight for each fusion protein, while positions of molecular weight markers are indicated on the left side of the blot.

**Fig. S3.** A subset of EGFP-SetA is polyubiquitinated when ectopically produced in eukaryotic cells. HEK-293T cells were transfected with pEGFP-SetA for 16 h. Lysates were subjected to immunoprecipitation with anti-SetA antibodies (Experimental procedures). Input lane (I) represents 0.2% of the total starting material. Four per cent of the total immunoprecipitated material was loaded into the lane marked IP. Antibodies used for immu-
noblotting are indicated on the right side of each panel. Arrow indicates position of EGFP-SetA. Large asterisks indicate higher molecular weight forms of EGFP-SetA. Small asterisk indicates the position of a band that likely represents a truncated polyubiquitinated form of EGFP-SetA.

Table S1. Compilation of yeast expression phenotypes (production of *L. pneumophila* proteins, growth defects and secretory defects).

Table S2. Strains, primers and plasmids utilized in this study.

Table S3. Primers used for amplification and cloning of *L. pneumophila* ORFs by homologous recombination in yeast.

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