Life Stage-specific Proteomes of *Legionella pneumophila* Reveal a Highly Differential Abundance of Virulence-associated Dot/Icm effectors*\(^\text{§}\)

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Major differences in the transcriptional program underlying the phenotypic switch between exponential and post-exponential growth of *Legionella pneumophila* were formerly described characterizing important alterations in infection capacity. Additionally, a third state is known where the bacteria transform in a viable but nonculturable state under stress, such as starvation. We here describe phase-related proteomic changes in exponential phase (E), postexponential phase (PE) bacteria, and unculturable microcosms (UNC) containing viable but nonculturable state cells, and identify phase-specific proteins. We present data on different bacterial subproteomes of E and PE, such as soluble whole cell proteins, outer membrane-associated proteins, and extracellular proteins. In total, 1368 different proteins were identified, 922 were quantified and 397 showed differential abundance in E/PE. The quantified subproteomes of soluble whole cell proteins, outer membrane-associated proteins, and extracellular proteins; 841, 55, and 77 proteins, respectively, were visualized in Voronoi treemaps. 95 proteins were quantified exclusively in E, such as cell division proteins MreC, FtsN, FtsA, and ZipA; 33 exclusively in PE, such as motility-related proteins of flagellum biogenesis FlgE, FlgK, and FliA; and 9 exclusively in unculturable microcosms soluble whole cell proteins, such as hypothetical, as well as transport/binding-, and metabolism-related proteins. A high frequency of differentially abundant or phase-exclusive proteins was observed among the 91 quantified effectors of the major virulence-associated protein secretion system Dot/Icm (> 60%). 24 were E-exclusive, such as LepA/B, YlfA, MavG, Lpg2271, and 13 were PE-exclusive, such as RalF, VipD, Lem10. The growth phase-related specific abundance of a subset of Dot/Icm virulence effectors was confirmed by means of Western blotting. We therefore conclude that many effectors are predominantly abundant at either E or PE which suggests their phase specific function. The distinct temporal or spatial presence of such proteins might have important implications for functional assignments in the future or for use as life-stage specific markers for pathogen analysis.

*Legionella pneumophila* is a Gram-negative bacterium ubiquitously found in water and soil. In their natural environment, the bacteria infect, counteract host defense mechanisms, and intracellularly replicate in protozoa, especially amoebae. When inhaled by humans, *L. pneumophila* uses similar strategies to propagate in alveolar macrophages leading to Legionnaires disease, a severe pneumonia (1–4). Upon infection of amoebae and human cells, the intracellular life cycle of *L. pneumophila* includes at least two distinct stages, a replicative phase, where the bacteria efficiently proliferate, and a transmissive phase, where bacterial replication halts as nutrients become scarce. During transmissive phase, the expression of transmission traits, such as motility, cytotoxicity, accumulation of the storage lipid polyhydroxybutyrate, increased osmotic robustness, production of virulence traits, is triggered and the bacteria are highly infectious (5–7). The phenotypic switch is regulated in a complex fashion and involves the action of various regulators, such as CsrA, RpoS, LetA/S, FilA, RelA, and the small ncRNAs RsmY/Z (8–10). Accordingly, it has been shown that phase transition goes along with significant transcriptomic changes (11–14) which must impact the life stage-specific proteomes. Remarkably, broth-grown *L. pneumophila* show a comparable phenotypic switch by expressing a phenotype similar to the replicative phase during
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exponential phase (E)\(^1\) and to transmissive phase in post-exponential phase (PE) (8, 12, 14).

Currently, several proteomic studies are available for *L. pneumophila*. One of the first proteomic maps characterizing *L. pneumophila* grown on agar media was published by Lebeau et al. One hundred ten different proteins of bacterial cell lysates were identified by means of 2D gel electrophoresis (2DE), tryptic digest, and mass spectrometry (MS) (15). Hayashi et al. conducted a similar approach and analyzed the growth phase-dependent *L. pneumophila* cell extract proteome after growth in broth. They determined four proteins overabundant in E and 64 proteins accumulating in PE. About 60% of the differentially abundant proteins were defined as enzymes and were categorized to: carbohydrate metabolism, amino acid metabolism, and lipid metabolism (16).

In the context of bacterial pathogenicity, exported proteins designed to directly interact with host cells are of particular interest. Several protein secretion systems of *L. pneumophila* are associated with bacterial virulence, such as the Defect in organelle trafficking/Intracellular multiplication (Dot/Icm) type IVB secretion system (T4BSS), the *Legionella* type II secretion pathway (Lsp) (T2SS), the type I (Lss), and the Twin-arginine translocation (Tat) systems (15, 17–24). More than 300 and 25 proteins are translocated into the host cell by the T4BSS or are exported by the T2SS, respectively (18, 25). Previous proteomics studies gave an insight into the variety of *L. pneumophila* proteins present in the culture supernatant and on their dependence on type II secretion, Tat secretion, or export via outer membrane vesicles. In those studies, 2DE/MS analysis was used and identified at least 20 type II-secreted proteins, 20 proteins with differential abundance in wild type and a Tat mutant as well as 181 culture supernatant proteins of which 33 specifically were associated with outer membrane vesicles (20, 25–28). Furthermore, in a recent study by Hoffmann et al., the proteome of intracellular *L. pneumophila* 1h post infection was analyzed by means of 1D gel electrophoresis and subsequent liquid chromatography-MS/MS (29).

Four hundred thirty-four *Legionella* proteins were detected in *Legionella*-containing vacuoles (LCV) purified from RAW 264.7 macrophages, or 944 obtained from *D. discoideum* including 29 or 60 Dot/Icm effectors, respectively (29).

In addition to E and PE, various studies reported on *L. pneumophila* in a viable but nonculturable (VBNC) state which would normally grow, but retain insignia of life, such as intact membranes, metabolic activity, transcription, respiration, and some attributes of virulence (35). It has been shown that unculturable *L. pneumophila* may resuscitate to a fully virulent form by passage through an amoeba host (30, 33, 34, 36) and therefore provide a largely undetected source of infections. Unculturable *Legionellae* have been observed for example after prolonged incubation in laboratory tap water microcosms at different temperatures, including elevated temperature at 42 °C (36), heat shock (50–70 °C) (37), following disinfection (31–33), and after treatment with heavy metals (38).

Major differences in the transcriptional program underlying the phenotypic switch between E and PE in *L. pneumophila*, which are important in understanding bacterial virulence, were formerly described (11–14). This and the study by Hayashi et al. indicated that cell-associated proteomes of E and PE *L. pneumophila* indeed reveal differences (16). However, the big variety and differential abundance of proteins present at the three *L. pneumophila* life stages and distinct subproteomes (soluble whole cell, outer membrane-associated, and extracellular) have so far not been comprehensively described. Here, we present a global proteomic analysis of E, PE, and UNC of *L. pneumophila* and visualize the protein composition and occurring changes in Voronoi treemaps. Our results reveal (1) different life stage- and accordingly virulence-related protein patterns, (2) phase-dependent differential protein abundance including many Dot/Icm effectors, (3) life stage-exclusive proteins, and (4) protein localization of a wide array of proteins.

**EXPERIMENTAL PROCEDURES**

**Experimental Design and Statistical Rationale—** To delineate growth phase-related proteomic changes of *L. pneumophila*, E/PE bacteria and UNC were generated and analyzed by mass spectrometry. E and PE bacteria were assessed for swcp, extr, and omap. Each sample was analyzed in biological triplicates to allow for statistical tests and to improve consistency. Protein identification and quality criteria were very strict throughout the study (see section Proteome Analysis). Only proteins identified in each of three biological replicates were quantified by means of normalized spectral abundance factors (NSAFs) (see section Data Analysis). In order to reduce mainly cytosolic contaminants, quantified extr and omap were assessed for quantitative enrichment of individual proteins compared with swcp, and only enriched proteins were further analyzed (see section Data Analysis of omap and extr). To test for phase related differential abundance of proteins, a t test as implemented in Scaffold (v.4.4.5) was used (p < 0.05). Ratios of quantity of significantly different proteins were log2 transformed and only those were approved who exceeded 1 or fell below −1 (49). Within proteins with an apparent phase specific abundance, subfractions termed “on/off proteins” and “phase exclusive proteins” were defined (see section Data Analysis).

Generation of E, PE, and UNC of *L. pneumophila*—*L. pneumophila* was routinely grown on buffered charcoal yeast extract (BCYE) agar for 2–3 days at 37 °C (39). For growth in liquid laboratory medium, *L. pneumophila* was inoculated at an OD\(_{660}\) = 0.2–0.3 and was cultured in buffered yeast extract (BYE) broth at 37 °C with continuous shaking at 250 rpm. Bacterial growth was checked by determining the optical

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\(^1\) The abbreviations used are: E, exponential phase; 2DE, 2D gel electrophoresis; BCYE, buffered charcoal yeast extract; BYE, buffered yeast extract; extr, extracellular proteins; HA, haemagglutinin; LCV, *Legionella*-containing vacuole; NSAF, normalized spectral abundance factor; omap, outer membrane-associated proteins; PE, post-exponential phase; SPI, signal peptidase I signal peptide; swcp, soluble whole cell protein; T2SS, type II secretion system; T4BSS, type IVB secretion system; Tat, Twin-arginine translocation system; UNC, unculturatable microcosms; VBNC, viable but nonculturable.
density of the culture at wavelength 660 nm (OD \textsubscript{660}) using a Beckman spectrophotometer DU520 (Beckman Coulter, Brea, CA). E and PE L. pneumophila for proteomics analysis were obtained from liquid cultures. E starter cultures were diluted in BYE broth to an OD \textsubscript{660} = 0.2. E samples were removed after ~4.5 h shaking at 37 °C. PE samples were obtained at 13.5 h when the bacteria entered stationary growth phase. To confirm that the bacteria reached PE, samples were subjected to Western blotting with antibodies directed against flagellin (kindly provided by Klaus Heuner, Robert Koch-Institut Berlin (40)), a growth phase-related marker of PE L. pneumophila (5). For the generation of L. pneumophila UNC, PE cells were washed two times in sterilized tap water and subsequently inoculated in sterilized tap water to an OD\textsubscript{660} of 0.3, corresponding to sterilized tap water.

Proteome Analysis—For proteome analysis, protein concentration of the whole cell and the extracellular protein samples was determined (Roti-Nanoquant, Carl ROTH, Karlsruhe, Germany). Subsequently, the samples were analyzed by liquid chromatography-MS according to the workflow described by Bonn et al. (45). Briefly, 20 µg protein (swcp and extr) or the complete sample (omap) were separated by 1D electrophoresis and stained with colloidal Coomassie Brilliant Blue G250 overnight. Subsequently, protein containing gel lanes were cut into 10 equisized pieces and destained by washing at least three times for 15 min with 700 µl of gel wash buffer (0.2 M ammonium bicarbonate in 30% (v/v) acetonitrile) at 37 °C under vigorous shaking. The destained gel pieces were desiccated in a vacuum centrifuge at 30 °C and rehydrated with trypsin solution (2 µg of modified trypsin (Promega, Madison, WI) in 1 ml of water) for 15 min. Remaining trypsin solution was removed, and the digest was performed overnight at 37 °C. The gel pieces were covered with water, and the peptides were eluted from the gel matrix by immersion of the reaction tube in an ultrasonic bath for 15 min. The supernatant containing the peptides was removed, transferred to a glass vial, and concentrated to a final volume of 10 µl in a vacuum centrifuge.

For LC-MS/MS measurements, the tryptic digest was subjected to a reversed-phase column chromatography run on an EASYnLC (Proxeon, Odense, Denmark). Emitter tips for the self-packed columns were prepared by pulling out tips using 100 µm i.d. fused silica capillaries with an o.d. of 360 µm with a laser puller (P2000, Sutter Instruments, Novato, CA). These emitter tips were packed at >200 bar using Aeris C18 material (3.6 µm). Self-packed columns with a length of 20 cm were used in an open vented one-column setup with a loading volume of 10 µl at a flow of 500 nl/min at a maximum of 220 bar and a subsequent flow rate of 300 nl/min. Separation of the peptides was achieved by the application of a binary nonlinear 75-min gradient from 5% to 75% acetonitrile in 0.1% acetic acid. The self-packed columns were mounted in a modified nanoelectrospray ion source with liquid junction of the voltage (2400 V) applied between orifice and emitter tip.

MS and MS/MS data were acquired with the LTQ-Orbitrap (Thermo Fisher). After a survey scan at a resolution of 30,000 in the Orbitrap with activated lock mass correction, the five most abundant precursor ions were selected for fragmentation. Singly charged ions as well as ions without detected charge states were not selected for MS/MS analysis. Collision-induced dissociation (CID) fragmentation was performed for 30 ms with normalized collision energy of 35, and the fragment ions were recorded in the linear ion trap.

Sorcerer-Sequence (v 27, rev 11) was used to create peaklists and to search a L. pneumophila forward-reverse protein sequence database. This Database (5970 entries) included the complete proteome set of L. pneumophila Philadelphia-1 extracted from UniprotKB (2013.04.25) and a set of common laboratory contaminants finally compiled with Scaffold (version Scaffold_4.0.3, Proteome Software Inc., Portland, OR). The search was performed assuming the digestion enzyme trypsin. Data were searched with a fragment ion mass tolerance of 1.00 Da and a search tolerance of 5 ppm for the overview scans. Oxidation of methionine was specified in Sequest as a variable modification and two missed cleavages were allowed. Scaffold (version Scaffold_4.4.5, Proteome Software Inc.) was used to validate MS/MS-based peptide and protein identifications. Proteins were only considered as identified if at least two unique peptides matched solid quality criteria (deltaCn > 0.1 and XCorr > 2.2; 3.3; 3.5 for doubly, triply, or higher charged peptides). Peptide quality criteria were very strict to result in a false discovery rate of 0% on protein level in all experiments. False discovery rate was calculated in Scaffold accord-
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**Table I**

Overview of identified subproteomes of *L. pneumophila* exponential phase (E), postexponential phase (PE), and unculturable microcosms (UNC). E/PE-up include only quantified proteins with significantly different abundance between E/PE (t-test, \( p < 0.05 \)) and log2 NSAF E/PE ratios above +1 or below -1, and include on/off proteins (E-on or PE-on). E/PE-on proteins constitute a subfraction of E/PE-up proteins, and were quantified in the respective cell fraction only in the respective growth phase. UNC-on proteins were only detected in swcp of UNC microcosms. Phase-exclusive proteins constitute a subfraction of E/PE/UNC-on proteins, and include those that were, independent of the cell fraction, solely identified in samples of the respective growth phase.

| Fraction | E and PE | UNC | E+PE+UNC |
|----------|----------|-----|----------|
|          | \( \%\text{Ident} \) | \( \%\text{Quant} \) | \% Quant PE | \% Diff (\( p < 0.05 \)) | \% E-up | \% PE-up | \% E-on | \% PE-on | \% Ident UNC | \% Quant UNC | \% UNC-on | \% Total quant |
| Total    | 2743     | 973  | 908      | 859       | 416  | 179     | 157     | 113     | 64          | -              | -          | -        |
| Total different | 1368  | 922  | 863      | 814       | 397  | 176     | 147     | 113     | 62          | 348           | 223        | 15       | 960       |
| Percentage* | 46     | 31   | 29       | 28        | 13   | 6       | 5       | 4       | 2           | 12            | 8          | 1        | 33        |

*Swcp-each phase* | 1273     | 841  | 783      | 741       | 336  | 152     | 120     | 99      | 57          | 348           | 223        | 15       | 890       |

*Omap-each phase* | 837     | 55   | 55       | 43        | 33   | 21      | 8       | 12      | 0           | -             | -          | -        |

*Extr-each phase* | 633     | 77   | 70       | 75        | 47   | 6       | 29      | 7       | -           | -             | -          | -        |

Phase exclusive | -       | -    | -        | -         | -    | -       | -       | -       | -           | 95            | 33         | 9        |

*Percentage of total different proteins found compared to 2943 ORFs corresponding to the *L. pneumophila* Philadelphia-1 genome NC_002932.5.*

**Table I**

| Fraction | E and PE | UNC | E+PE+UNC |
|----------|----------|-----|----------|
|          | \( \%\text{Ident} \) | \( \%\text{Quant} \) | \% Quant PE | \% Diff (\( p < 0.05 \)) | \% E-up | \% PE-up | \% E-on | \% PE-on | \% Ident UNC | \% Quant UNC | \% UNC-on | \% Total quant |
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|----------|----------|-----|----------|
|          | \( \%\text{Ident} \) | \( \%\text{Quant} \) | \% Quant PE | \% Diff (\( p < 0.05 \)) | \% E-up | \% PE-up | \% E-on | \% PE-on | \% Ident UNC | \% Quant UNC | \% UNC-on | \% Total quant |
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Phase exclusive | -       | -    | -        | -         | -    | -       | -       | -       | -           | 95            | 33         | 9        |

*Percentage of total different proteins found compared to 2943 ORFs corresponding to the *L. pneumophila* Philadelphia-1 genome NC_002932.5.*
All constructs were sequenced to confirm identity and the lack of errors. Allelic exchange was performed as described elsewhere (62). Allelic exchange was confirmed by PCR with primer HA_f which binds the HA-tag gene sequence, and effector-specific reverse primers localized outside of the homology region used for recombination. Resulting clones were grown comparably to the strains for proteomic analysis (see above) and equal amounts of material from E and PE bacteria were subjected to SDS-PAGE and Western blotting with protein detection via anti HA.11 mAb (Covance).

RESULTS

Overview of L. pneumophila E and PE Proteomes—Because of their distinctive phenotypes and infection capacity, the aim of our study was to acquire a comprehensive proteomic view on the L. pneumophila life stages and to identify stage-exclusive proteins. For E/PE sample preparation, L. pneumophila was grown in broth until E or PE and enrichment of flagellin FliC (also designated FlaA in some studies) was used to confirm entry into PE (12, 63, 64) (Fig. 1A and 1B). Our data set of E and PE bacteria comprised proteins from three different bacterial subfractions: (1) soluble whole cell proteins (swcp), (2) outer membrane-associated proteins (omap), and (3) extracellular proteins (extr). A total of 2743 L. pneumophila proteins was identified in the different cell fractions of E and PE corresponding to 1368 different proteins (Table I) or 46%

Fig. 1. Generation of E and PE L. pneumophila upon growth in broth and corresponding soluble whole cell proteins (swcp). A, L. pneumophila was inoculated in BYE broth and grown at 37 °C. E and PE samples were harvested in triplicates for proteomic analysis at the time points indicated by an asterisk. B, Western blot (top) and corresponding Coomassie-stained SDS-PAGE gel showed expression of flagellin in the PE samples. Left lane: protein ladder [kDa]. C and D, The Voronoi treemaps are based on quantitative NSAF values and visualize abundance of proteins during (C) exponential phase (E) and (D) post-exponential phase (PE). Individual NSAF values are encoded by area of mosaic tiles. Large tiles indicate abundant proteins; small tiles show less abundant proteins. Clusters of tiles without black frames represent respective gene operons. Clusters of identically colored tiles represent functional relatedness (see supplemental Fig. S1); i.e. blue = genetic information processing, dark gray = no function assigned, orange/yellowish = metabolism, red = cellular processes, purple = pathogenesis, pink = cell structure, green = signal transduction.
of the annotated theoretical *L. pneumophila* Philadelphia-1 proteome covering 2943 open reading frames (53). From the E/PE data set, 67% corresponding to 922 different proteins were quantified by using normalized spectral abundance factors (NSAFs) (48) (see experimental procedures). Eight hundred sixty-three proteins were quantified in E and 814 in PE bacteria (Table I). The abundance of 397 proteins showed significant differences between E and PE (*t* test, *p* < 0.05); and included 176 proteins more abundant in E (E-up) and 147 proteins more abundant in PE (PE-up) which were above or below the threshold of +1 and −1 (log2NSAF(E/PE)), respectively (see experimental procedures). Both groups contained a high number of on/off proteins, which were, corresponding to a specific cellular subfraction, solely identified either in E or PE. Specifically, a total of 113 proteins was E-on (synonymously PE-off), and a total of 62 was PE-on (synonymously E-off) in swp, omap and extr. Ninety-five E-on proteins were E-exclusively and 33 PE-on proteins were PE-exclusively identified and quantified (Table I), i.e. were solely detected at either E or PE (and also not in UNC) considering all cellular subfractions. Complete lists of proteins quantified or identified in swp, and quantified in omap, or extr are given as supplementary data (supplemental Table S3 to S13).

Overview of *L. pneumophila* E and PE Soluble Whole Cell Proteomes—To comprehensively visualize proteomic data of E and PE, and transition between the phases, we generated Voronoi treemaps (65). Voronoi treemaps have previously been used to combine quantitative proteomic data and functional relatedness of proteins for *S. aureus* and *B. subtilis* (48, 66). Functional relatedness is represented in hierarchical clusters of irregular tiles and (1) quantitative information is given by the defined size of the respective tile or (2) changing ratios between two conditions are expressed in a color-encoded manner.

Fig. 1 C, 1D (functional categories see supplemental Fig. S1) and supplemental Table S3 give an overview about the 841 proteins quantified in E and PE swp. Proteins identified but not quantified are provided in supplemental Table S4. Very abundant proteins in E and PE included chaperones such as GroEL, GroES, and DnaK; proteins involved in translation, such as elongation factor EF-Tu, Tsf/EF-Ts, FusA/EF-G; RNA polymerase subunit alpha RpoA; DNA-binding protein HupB; Lpg0689; single strand-binding protein SSB; moreover, more than 20 ribosomal proteins (Rps and Rpl proteins) and others (Fig. 1C, 1D, supplemental Table S3). The three most abundant proteins in both phases were GroEL, RplL, and EF-Tu, in total contributing to around 10% of the entire protein quantity (67–69). Interestingly, the general pattern of the major (most abundant) swp proteins did not change substantially with entry into PE, showing that most of the proteome remains stable upon E/PE transition (Fig. 1C, 1D).

Next, we analyzed proteins with significantly different abundance between E and PE, i.e. E-up and PE-up proteins (Table I, supplemental Table S3): arranged according to locus tag and supplemental Table S5: arranged according to log2NSAF(E/PE). The “up” proteins included those exclusively found in a specific bacterial subfraction (swcp, omap, or extr) of E or PE, which were designated “E-/PE-on” proteins and their log2(E/PE) transform was set to 8 for E-on and ~8 for PE-on proteins (Table I, supplemental Table S3, S5). Three hundred thirty-six swp proteins were found differentially abundant by the above mentioned criteria correlating to about 11% of the annotated *L. pneumophila* Philadelphia-1 proteins (53) or to about 40% of the 841 different swp proteins quantified in E/PE (Table 1). Proteins with most obvious changes in quantity were related to the flagellum apparatus (FliA, FlgD, FlgE, FlgK, FliD, FliC (70)); to stress (KatG2, IbpA, HspC2/Lpg2493); virulence, such as 23 Dot/Icm effectors and IcmB, IcmW, EnhA, EnhB, EnhC (Lpg2539–2541); carbon and energy metabolism (20 proteins), as well as lipid and fatty acid metabolism (six proteins) (Fig. 1C, 1D, supplemental Table S3, S5). 152 of those were E-up (including 99 E-on proteins) and 120 were PE-up (including 57 PE-on proteins) (Table I, Fig 2A). Ratios of protein abundance in E/PE swp are highlighted in a Voronoi map (Fig. 3, supplemental Fig. S2). Most functional categories, as visible in the Voronoi treemaps, contained protein members of both, the E-up and the PE-up group. Categories “Cell division,” “LspTII secretion,” “Transcription” contained members of both, the E-up and the PE-up group. Categories “Carbon and energy metabolism,” “Cellular processes,” “Chemotaxis and motility,” “Fatty acid, lipid, and phospholipid metabolism,” “Pathogenesis,” “Oppositely,” “Cell division,” “Cell envelope,” “DNA metabolism,” “Dot/Icm effector,” “LspTII secretion,” “Protein synthesis,” “Purines, pyrimidines, nucleosides, and nucleotides” contained substantially more E-up proteins (Fig. 2B, Fig. 3 and supplemental Fig. S2).

Overview of *L. pneumophila* E and PE Outer Membrane-associated Proteomes—Outer membrane-associated proteins (omap) are important players in pathogen adhesion, communication, environmental sensing, signal transduction, and virulence. To delineate *L. pneumophila* omap, biotinylation of intact *L. pneumophila* with Sulfo-NHS-SS biotin, protein pull down, MS-based protein identification, quantification, and protein enrichment analysis was used (see experimental procedures for details). Fifty-five omap were quantified in E/PE samples (Table I, Fig. 4 and supplemental Fig. S3, supplemental Table S6: arranged according to locus tag, supplemental Table S7: arranged according to log2NSAF(E/PE)). The ten most abundant proteins in omap contributed in terms of quantity to ~70%. Five of those, Mip, Com1, Lpg0844, YjeA, TolB, were identified in comparable quantity at E/PE and five were E-up, such as OmpH (Lpg0507), DsbA, SurA, HtrA, and LolA. This group includes a number of known and/or predicted outer membrane proteins.
(by means of LocateP) and all of which contain a predicted signal peptidase I (SPI) signal peptide, such as OmpH (Lpg0507), TolB, and Lpg0844/Ttg2D. The most abundant omop detected was the prefoldin-like chaperon OmpH which is also known as Skp (Lpg0507) (Fig. 4 and supplemental Fig. S3, supplemental Table S6). Although it forms trimeric periplasmic complexes, its ability to directly interact with membrane lipids and LPS has been described (71–73). TolB is a periplasmic constituent of TolB-Pal cell envelope complex linked to the outer membrane with function in colicin import in E. coli (74, 75). Furthermore, several known membrane proteins or some that are involved in the biogenesis of outer membrane proteins are among this group. These include the virulence factor and peptidyl-prolyl cis/trans isomerase Mip (76, 77); Com1, another protein promoting bacterial pathogenicity and formation of stable disulfide-bond complexes, e.g. in Dot/Icm effectors (78, 79); the periplasmic chaperone SurA, required for biogenesis of outer membrane proteins (80); DsbA, a disulfide oxidoreductase forming disulfide bonds when proteins emerge in the periplasm (81, 82); LolA, an outer membrane lipoprotein carrier protein and part of the LolAB complex (83) which aids export of lipoproteins to the outer membrane in E. coli; and YjeA, a peptidoglycan deacetylase (84) (Fig. 4, supplemental Table S6).
Regarding relative protein abundance, among the 55 quantified omap, 29 showed significantly changed abundance at E/PE. 21 were E-up or -on and 8 were PE-up (Fig. 4, supplemental Fig. S2, S4, supplemental Table S7). Moreover, the omap fraction comprised 12 E-on (but no PE-on) proteins 9 of which contained a SPI signal peptide. The 12 included one Dot/Icm effector (PPlase LilB/Lpg1662) and one Lsp substrate (aminopeptidase LapA). Furthermore, another thiol-disulfide oxidoreductase (ResA) involved in cytochrome C synthesis (85–88), several predicted metabolic or substrate-binding proteins, such as amino acid-binding protein Lpg0498, polysaccharide deacetylase Lpg0633, amine oxidase Lpg1153, hydroxymethylglutaryl CoA-reductase Lpg2052, G3P ABC transporter substrate-binding protein UgpB, were detected as omap E-on. Among the remaining 16 E- or PE-up proteins, five belonged to the functional categories “protein fate” (E-up: DsbA, HtrA, LolA, PE-up: metallopeptidase PepO, carboxyl terminal protease Lpg0499) (79, 83, 89, 90), two to “pathogenesis” (E-up: Lpg1585, PE-up: EnhA/Lpg2641) (91), two to “transport and binding” (PE-up: arginine ABC transporter substrate-binding protein ArtJ (92), E-up: sugar ABC transporter substrate-binding protein Lpg0184), and one to “cellular processes” (PE-up: carbonic anhydrase Lpg2500) (Fig. 4, supplemental Fig. S2, S4, supplemental Table S7).

Overview of L. pneumophila E and PE Extracellular Proteomes—To characterize the extracellular proteome of L. pneumophila during E and PE, cell-free culture supernatants were precipitated, subjected to MS analysis, and subsequently assessed for enrichment compared with swcp (see Experimental Procedures). We quantified 77 extr of L. pneumophila and 35 of which were E- or PE-up (Fig. 5, supplemental Fig. S5, supplemental Table S8: arranged according to locus tag, supplemental Table S9: arranged according to: log2NSAF(E/PE)). The eleven most abundant proteins contributed to 53% of the total extracellular protein quantity. Remarkably, eight of those were substrates of the Lsp T2SS (zinc metalloproteinase ProA, aminopeptidase LapA, chitinase ChiA, endoglucanase CelA, tail-fiber-like protein ScIB, hypothetical proteins Lpg1832, Lpg0873, Lpg0956), and only two of which (ChiA, Lpg0956) were differentially abundant, specifically PE-up (20, 25). In addition, we quantified 10 more T2SS substrates and therefore quantified the almost entire known T2SS secretome (except Nta/Lpg1385 and lcmX/...
Lpg2889) of *L. pneumophila* (20, 25) (Fig. 5, supplemental Fig. S5, supplemental Table S8, S9). Remarkably, two proteins clearly formed the most abundant extr during E and PE, these were ProA (mean NSAF E/H11005 1.2, PE/H11005 1.7) and LapA (mean NSAF E/H11005 1.0, PE/H11005 1.5), followed by hypothetical protein Lpg0956 (mean NSAF E/H11005 0.2, PE/H11005 1.1, PE-up). Similarly, Lpg0957, encoded adjacent to Lpg0956, belonged to the majorly abundant extr and was also PE-up (mean NSAF E/H11005 0.1, PE/H11005 0.5; log2NSAF(E/PE)H11002 2.1). An overview of the type II-secreted proteins quantified in the study is given in supplemental Table S10.

The 35 differentially abundant extr contained six E-up proteins (major outer membrane protein Lpg2961, cell division protein FtsI4, Dot/Icm apparatus core complex protein IcmG/DotF, invasion protein Lpg1585/EnhB) including 2 E-on proteins (hemagglutinin/zinc metalloprotease Lpg0019, hypothetical protein Lpg0198). The remainder was PE-up and included seven extracellular PE-on proteins (ABC transporter periplasmic-binding protein ArtJ, hypothetical protein Lpg0264, flagellar hook protein FlgK, phospholipase C PlcB, protease/elastase LasB, polysaccharide deacetylase Lpg1993, Lpg2443) (70, 92–94). The further PE-up extr majorly included 8 proteins without assigned function (Lpg2220, Lpg1647, Lpg1645, Lpg0957, Lpg0301, Lpg1318, Lpg2206, Lpg2246), 5 T2SS substrates (chitinase ChiA, phospholipase A/acyltransferase PlaC, hypothetical protein Lpg0956, Lpg0189, Lpg0264), three Dot/Icm effectors (LegP, Lpg1667, Lpg2443) and Dot/Icm apparatus core complex protein DotC, as well as further (in addition to FlgK) motility-related proteins (FliC, FliD) (26, 70, 95–97). The phase-stable extracellular proteome (42 proteins) included 13 T2SS substrates (phospholipase C PlcA, NttC, endoglucanase CelA, lysophospholipase A PlaA, T2 ribonuclease SrnA, NttB, Lpg1832, ScbB, aminopeptidases LapB, LapA, zinc metalloprotease ProA, major acid phosphatase Map, Lpg0873), five Dot/Icm effectors (LegY/GamA which was also detected as T2SS substrate (25), LirB, MavL, LegS, Lpg0041), three proteins involved in carbon and energy metabolism (enolase Eno, thioredoxin reductase TrxB1, xylanase-like protein YjeA), as well as nine functionally unasigned proteins (Fig. 5, supplemental Fig. S6, supplemental Table S8, S9) (25, 26, 97–102).

**Growth Phase-dependent Abundance of Dot/Icm Effectors**—A high frequency of differentially abundant proteins in E/PE was observed among the Dot/Icm effectors. Of the 86 quantified effectors in the swcp fraction, 55 were E- or PE-up, and of those 37 were on/off proteins. Remarkably, as much as 24 effectors were E-on, whereas 13 were PE-on. The remaining 18 split into 8 E-up and 10 PE-up proteins (Fig. 1, Fig. 3, Fig. 6A, supplemental Table S11). Among the E-up effectors were the eukaryotic-like effector AnkJ which is required for full virulence of *L. pneumophila* in mice (103), and SidI which has...
lethal effects to yeast and mammalian cells because of perturbation of translational machinery (104). E-on effectors included LegS2, a bacterial homologue of the eukaryotic enzyme sphingosine-1-phosphate lyase (SPL) (105), YlfA which is observed on the endoplasmic reticulum-derived replicative vacuole at later stage of a host cell infection (106, 107), as well as LepA and LepB promoting nonlytic release of \textit{L. pneumophila} from protozoa, the latter is a GTPase-activating protein that regulates removal of Rab proteins from membranes (108–110) (Fig. 6A, supplemental Table S11). The PE-up proteome included SidC, SdcA, SidM/DrrA, and LpnE. SidC has been shown before to accumulate in PE bacteria and, after anchored to phosphatidylinositol-4 phosphate on LCVs, supports efficient intracellular growth and recruitment of endoplasmic reticulum-derived vesicles to the LCV. SdcA is a paralog of SidC with similar functions whose gene is localized directly upstream of \textit{sidC} (111, 112). SidM/DrrA is a Rab1 guanine nucleotide exchange factor (GEF), which regulates the transport of endoplasmic reticulum-derived vesicles and binds to phosphatidylinositol-4 phosphate (109, 113–116). LpnE binds phosphatidylinositol 3-phosphate, is required for invasion and the establishment of an infection in macrophages, amoebae as well as A/J mice (117, 118) (supplemental Table S11). PE-on Dot/Icm effectors included RalF, a guanine nucleotide exchange factor activating ARF on LCVs (119); VipD, a phospholipase A which blocks endosome fusion with LCVs (120, 121); AnkX interfering with fusion of the LCVs with late endosomes (122, 123); RavZ inhibiting autophagy during infection (124); and SidH which is negatively regulated by another effector protein, the E3 ubiquitin ligase LubX (112, 125) (supplemental Table S11).

To further address the finding of growth phase-specific abundance of Dot/Icm effectors, we chose seven apparently inversely abundant effectors, specifically RavX, AnkF, MavG, Lpg2271 (E-on); VipD, Lem10, RalF (PE-on) and verified their phase specificity in E or PE. To this purpose, the effectors were chromosomally tagged with the HA-tag sequence (27 bp corresponding to about 1.1 kDa). The HA strains were subsequently broth grown to E or PE, and relative effector abundance was determined by Western blotting with anti HA antibodies. Indeed, HA-VipD (70.4 kDa), HA-Lem10, RalF (PE-on) and RalF (43.3 kDa) solely were detected in PE samples whereas HA-Lpg2271 (25.8 kDa), HA-MavG (52.6 kDa), HA-AnkF (105 kDa), and HA-RavX (40 kDa) almost solely were detected in E samples. Therefore the data obtained by means of Western blotting completely confirmed the proteome data (Fig. 6B). It should be noted that HA-RavX migrated in two independently generated strains as an ~50 kDa protein. This is in contrast to its predicted apparent molecular weight of ~40 kDa and may suggest post-translational modification.

**Generation of UNC for Proteome Analysis**—We further aimed to describe the major proteins present in bacteria when
they are kept prolonged periods in tap water, a situation that is very common for Legionella bacteria. Under those conditions, the bacteria have only limited access to nutrients, switch into a VBNC state, and may persist until they get in contact with a host or nutrient-rich condition (30, 34, 36). To generate VBNC L. pneumophila, we inoculated the bacteria into sterilized tap water and compared the decrease of CFU at the following temperatures: 4 °C, 21 °C, and 42 °C. Incubation at 42 °C efficiently yielded in VBNC bacteria in a time frame of ~110 days; a finding that relates well to the results of others (34, 36). Microcosms at 4 °C or 21 °C showed only moderate CFU decrease and therefore were not considered for further analysis (Fig. 7A). Samples with complete loss of culturability at 42 °C, in the following termed UNC, were assessed for total bacterial counts, the presence of intact viable cells by means of cFDA hydrolysis as a measure of esterase activity and live/dead staining (126–129). We found that an almost unchanged number of total bacteria contained a fraction of more than 50% intact viable cells as indicated by cFDA hydrolysis (Fig. 7B). Live/dead stain analysis yielded in about 25% clearly membrane-intact cells and comprised a similar fraction of cells with intermediate red/green stains which we here did not rate as membrane intact but still might represent viable cells (Fig. 7B and data not shown).

Proteomic Analysis of Unculturable Cells—A total of 348 L. pneumophila proteins were identified and 223 were quantified in UNC swcp (Fig. 7C, Table I, supplemental Table S12). The 12 most abundant proteins in UNC were in decreasing quan-

![Figure 6](image_url)

**Fig. 6.** Differential abundance of 55 Dot/Icm effectors in E and PE. A, 32 effectors were quantified E-up/on and 23 were PE-up/on in swcp. B, Verification of proteomic results by Western blot analysis of HA-tagged effector proteins. Dot/Icm effector genes vipD, lem10, ralF, lpg2271, mavG, ankF, and ravX were chromosomally tagged with the HA-gene sequence in L. pneumophila. The strains were grown to E and PE and comparable amounts of cell lysates were applied and their phase-specific abundance as indicated by the proteome study was analyzed by means of detection via anti-HA antibodies. The left lane shows the molecular weight standard in kilodaltons. AnkF (105kDa) and RavX were run in a 7.5% SDS-PAGE gel whereas other samples were separated in 10% SDS-PAGE gel.
Proteomic Analysis of *L. pneumophila* Life Stages

Fig. 7. Generation of unculturable microcosms, determination of CFU, total counts, live/dead status, and map of UNC proteome. A. Generation of long-term starved and unculturable *L. pneumophila* microcosms (UNC) in tap water at 42°, 21°, or 4 °C. At various time points, culturability was determined by means of CFU analysis, and the presence of VBNC bacteria was confirmed by live/dead (L/S) staining. Bars indicate the amount of VBNC bacteria per ml at the indicated time points. B) Quantification of VBNC cells within the UNC obtained at 42 °C taken for proteome analysis. Esterase positive bacteria (cFDA “live”) and propidium iodide impermeable, Syto 9 positive bacteria (L/D “live”) were quantified by microscopy. C) The treemap visualizes functionally organized quantitative information on abundance of proteins in UNC expressed as area of mosaic tiles. Large tiles indicate abundant proteins; small tiles show less abundant proteins. Clusters of tiles without black frames represent operons. Clusters of identically colored tiles represent functional relatedness (see also supplemental Fig. S7); i.e. blue = genetic information processing, dark gray = no function assigned, orange/yellowish = metabolism, red = cellular processes, purple = pathogenesis, pink = cell structure, green = signal transduction.

Further, four Dot/Icm secretion machinery proteins were quantified in UNC samples, such as IcmS, an adaptor to facilitate effector translocation, IcmE/DotG, a protein of the secretion system central channel spanning inner and outer membranes (137), as well as DotA (85); and 18 Dot/Icm effectors, including LpnE, Ceg28, SidC, RalF, and LidA (all except Ceg28 PE-on or PE-up) (supplemental Table S12) (95, 111, 112, 117, 119, 134, 138–144). Overall, it is interesting that the UNC proteome is not generally dominated by stress proteins, and rather shows signatures of active life. Indeed, we quantified 55 proteins linked to “genetic information processing” (including protein synthesis) and 85 to “metabolism.” The category “cellular processes,” which includes stress-related proteins comprises 17 proteins and none of them belongs to the top abundant proteins in these cells. Stress-related proteins in UNC samples included superoxide dismutase, diverse
heat shock proteins (small heat shock protein HspC2 Lpg2493, small HspC2 heat shock protein Lpg2192, heat shock protein GrpE, heat shock protein 90/HtpG), catalase/ (hydro)peroxidase KatG, general stress protein GspA, AhpC/ Tsa family peroxyxinitrite reductase, multidrug resistance protein Lpg0720, as well as Dps-like DNA-binding stress protein Lpg0689. Within the quantified UNC proteome, all but 15 UNC-on proteins overlapped with the identified swcp E/PE proteome.

Definition of Life Stage-exclusive Proteins—Our goal was to define specific proteomic markers of the three distinct *L. pneumophila* life stages. After removal of all not strictly phase-exclusive proteins, 95 of the 113 different E-on proteins, 33 of the 62 PE-on proteins, and nine of the 15 UNC-on proteins were defined as E-, PE-, or UNC-exclusive proteins (Table I, Tables II, III, IV).

E-exclusive Proteins—Regarding protein function, the E-exclusive proteome is dominated by five (out of 23) functional groups; “Dot/Icm effectors,” “not assigned,” “Protein synthesis,” “Protein fate,” and “Cell envelope,” which contained 62 of the 95 E-exclusives (65%) (Table II, supplemental Fig. S8). The 23 Dot/Icm effectors in this group contained five Ceg proteins (coregulated with the effector encoding genes; Ceg18, Ceg19, Lpg1171/Ceg21, Ceg29, AnlK/Ceg31 (145); LepAB (146), MavGM (11), YlfA/Lpg2298 (9), and others (Table II). The 19 E-exclusive proteins from “Protein synthesis/Protein fate” mainly included various ribosomal tRNA and rRNA base modification proteins (MiaB, RlmH, RlmN, QueA, TruB, TrmE), protein and peptide secretion and trafficking proteins (ToIC, Lpg0934, BamB), and protein modification and repair proteins (Pcm, Def) (Table II). Proteins linked to “Cell envelope” include three enzymes involved in biosynthesis and degradation of murein sacculus and peptidoglycan (Lpg0066 (phosphoribosylglycinamidet synthetase ATP-grasp domain-containing protein), MreC (Lpg0812, rod shape-determining protein), Lpg2522 (metal-activated pyridoxal enzyme), moreover Lpg0756 (RmlC, dTDP-6-deoxy-β-glucose-3,5-epimerase), RfbB (Lpg0758/RmlB, dTDP-glucose 4,6-dehydratase), Lpg2838 (rhodanese domain-containing protein), each related to biosynthesis and degradation of surface polysaccharides and lipopolysaccharides, as well as rare lipoprotein A (Lpg1508). Furthermore, some cell division-associated proteins were only detected in E, such as FtsA, FtsN, and Lpg2619/ZipA (Table II).

PE-exclusive Proteins—The PE-exclusive proteome is dominated by four functional groups; “Dot/Icm effectors,” “not assigned,” “Carbon and energy metabolism,” and “Chemotaxis and motility,” which contained 18 of the 33 PE-exclusives (55%) (Table III, supplemental Fig. S8). Remarkably, as seen in the E-exclusive proteome, Dot/Icm effectors constitute the major fraction among the PE-exclusive proteins (VipA, AnKX, Lem7, Lem9, Lem10, RavZ, LirC, Lpg2443, LegC8) (Table III, supplemental Fig. S8). In addition to proteins classified in “without assigned function” (Lpg0902, Lpg1603/PlaB, Lpg2803) and “Carbon and energy metabolism” (PhaB, Lpg2681, PteB), “chemotaxis/motility-associated proteins” were, as a hallmark of transmissive traits, identified in PE bacteria only (FlgE, FlgK, FlIA). Lpg1603/PlaB, although classified in the category “without assigned function”, has been previously characterized as a phospholipase A and virulence factor of *L. pneumophila* (147–149).

UNC-exclusive Proteins—Interestingly, only three of the nine UNC-exclusives are functionally annotated proteins: glyoxylase Lpg0619 ("Central intermediary metabolism"), major facilitator transporter Lpg0652 ("Transport and binding"), and PiiA/PiiE-like Tfp pilus assembly protein ("Cell envelope" (Table IV). Two hypothetical proteins, Lpg0741 and Lpg2958, were functionally predicted to the category “energy metabolism” (TIGR00393) and “Protein fate” (TIGR00706), respectively. The further four hypothetical proteins in that fraction do not match any functional prediction. Lpg0619 is annotated as glyoxylase and possesses limited protein homology to the SgaA_N-like (e-value 4.8e-43) and Glycoxalase_2 domains (e-value 4.8e-21), however there is no overall homology to *E. coli* glyoxalase I and II. Lpg0619 is the *L. pneumophila* enzyme most closely related to *Streptomyces griseus* SgaA, a protein involved in *Streptomyces* growth regulation (150). Lpg0652 bears a major facilitator protein domain (MFS) which comprises a large and diverse group of secondary transporters facilitating transport of various metabolites across membranes (151). Hypothetical protein Lpg0741 carries a CBS domain (Pfam00571), a conserved domain known from cystathionine beta synthase which is frequently found in different bacterial and eukaryotic proteins. The PiiA/PiiE-like protein Lpg1915, although annotated as PiiE, is only distantly related to the previously described PiiE protein involved in twitching motility (BlastP: 26% coverage, e-value 6e-07), adherence to cells, and natural competence in *L. pneumophila* (152–154). However, its exclusiveness to UNC contradicts the transcriptional findings of others (155), showing that expression of PiiA is affected by sensor kinase LqsS and its homolog LqsT in the stationary phase and that the protein was identified in purified *Legionella*-containing vacuoles (29). Lpg2958 carries a conserved Clp_protease_NfE_D1 domain (2.31e-94), a domain family which includes membrane-bound Clp class serine proteases acting in protein quality control (156).

To summarize, we analyzed and portrayed the major protein composition of E, PE and UNC of *L. pneumophila* as well as swcp, omp, and extr of E/PE. We here identified a series of proteins with growth phase-specific abundancies, some of which with even exclusive presence in E, PE, or UNC. Interestingly many Dot/Icm effectors showed differential abundance especially in E and PE which suggests their phase-specific function. The distinct temporal or spatial presence of such proteins might have important implications for functional assignments in the future or for use as life-stage specific markers for pathogen analysis.
| Locus tag | Gi number | Identified proteins (95) | Name | Loc | mean NSAF | S.D. | Pathway | Subcellular localization pred. | T4SSTS [24] |
|-----------|-----------|--------------------------|------|-----|-----------|------|---------|--------------------------------|-------------|
| lpg0011   | 52840267  | thiol-disulfide oxidoreductase | ResA | omap | 0.033 | 0.013 | Sec(SPI) | Periplasmic protein |
| lpg0019   | 52840275  | hemagglutinin/protease, zinc metalloprotease | Lpg0019 | extr | 0.018 | 0.003 | Sec(SPI) | Secreted/Released |
| lpg0066   | 52840322  | GAR synthetase ATP-grasp domain-containing protein | Lpg0066 | swcp | 0.005 | 0.002 | - | Intracellular Protein |
| lpg0075   | 52840330  | hypothetical protein lpg0075 | Lpg0075 | swcp | 0.016 | 0.001 | - | Intracellular Protein |
| lpg0112   | 52840367  | hypothetical protein lpg0112 | Lpg0112 | swcp | 0.005 | 0.002 | - | Intracellular Protein |
| lpg0125   | 52840380  | ribosome biogenesis GTP-binding protein YsxC | EngB | swcp | 0.008 | 0.001 | - | Intracellular Protein |
| lpg0133   | 52840388  | activator of ProP osmoprotectant transporter | ProQm | swcp | 0.010 | 0.002 | - | Intracellular Protein |
| lpg0134   | 52840389  | hypothetical protein lpg0134 | Lpg0134 | omap | 0.031 | 0.004 | Sec(SPI) | Periplasmic protein |
| lpg0198   | 52840453  | hypothetical protein lpg0198 | Lpg0198 | extr | 0.007 | 0.001 | Sec(SPI) | Periplasmic protein |
| lpg0362   | 52840607  | 3-oxoacyl-ACP synthase | FabF | swcp | 0.014 | 0.001 | Sec(SPI) | Periplasmic protein |
| lpg0379   | 52840624  | ribosomal protein S6 modification protein | Lpg0379 | swcp | 0.007 | 0.001 | - | Intracellular Protein |
| lpg0410   | 52840652  | hypothetical protein lpg0410 | Lpg0410 | swcp | 0.016 | 0.004 | Sec(SPI) | Periplasmic protein |
| lpg0482   | 52840672  | endo-1,4 beta-glucanase | Lpg0482 | omap | 0.022 | 0.007 | Sec(SPI) | Periplasmic protein |
| lpg0490   | 52840682  | 3-oxoacyl-ACP synthase FabF | Lpg0490 | omap | 0.021 | 0.004 | Sec(SPI) | Periplasmic protein |
| lpg0676   | 52840727  | hypothetical protein lpg0676 | Lpg0676 | swcp | 0.004 | 0.001 | - | Intracellular Protein |
| lpg0775   | 52840740  | hypothetical protein lpg0775 | Lpg0775 | swcp | 0.009 | 0.002 | - | Intracellular Protein |
| lpg0780   | 52840746  | hypothetical protein lpg0780 | Lpg0780 | swcp | 0.008 | 0.002 | - | Intracellular Protein |
| lpg0812   | 52840754  | hypothetical protein lpg0812 | Lpg0812 | swcp | 0.005 | 0.001 | - | Intracellular Protein |
| lpg0827   | 52840762  | hypothetical protein lpg0827 | Lpg0827 | omap | 0.016 | 0.003 | Sec(SPI) | Periplasmic protein |
| lpg0840   | 52840767  | hypothetical protein lpg0840 | Lpg0840 | omap | 0.005 | 0.001 | - | Intracellular Protein |
| lpg0898   | 52840777  | hypothetical protein lpg0898 | Lpg0898 | omap | 0.008 | 0.001 | - | Intracellular Protein |
| lpg0934   | 52840783  | hypothetical protein lpg0934 | Lpg0934 | omap | 0.008 | 0.001 | - | Intracellular Protein |
| lpg0966   | 52840792  | hypothetical protein lpg0966 | Lpg0966 | omap | 0.008 | 0.001 | - | Intracellular Protein |
| lpg1064   | 52840801  | hypothetical protein lpg1064 | Lpg1064 | omap | 0.011 | 0.002 | - | Intracellular Protein |
| lpg1085   | 52840810  | hypothetical protein lpg1085 | Lpg1085 | omap | 0.008 | 0.001 | - | Intracellular Protein |
| lpg1171   | 52840820  | hypothetical protein lpg1171 | Lpg1171 | omap | 0.029 | 0.004 | - | Intracellular Protein |
| lpg1181   | 52840830  | hypothetical protein lpg1181 | Lpg1181 | omap | 0.006 | 0.001 | - | Intracellular Protein |
| lpg1200   | 52840840  | hypothetical protein lpg1200 | Lpg1200 | omap | 0.008 | 0.003 | - | Intracellular Protein |
| lpg1215   | 52840850  | hypothetical protein lpg1215 | Lpg1215 | omap | 0.009 | 0.002 | - | Intracellular Protein |
| lpg1334   | 52840860  | hypothetical protein lpg1334 | Lpg1334 | omap | 0.024 | 0.002 | - | Intracellular Protein |
| lpg1370   | 52840870  | hypothetical protein lpg1370 | Lpg1370 | omap | 0.005 | 0.011 | - | Intracellular Protein |

**TABLE II**

List of quantified exponential phase (E)-exclusive proteins and their abundance, Loc = detected in subcellular fraction swcp, omap, or extr, S.D. = standard deviation, Pathway = prediction of sec signal, T4SSTS = Dot/Icm type IVB secretion system translocated substrate.
| Locus tag | Gi number | Identified proteins (95) | Name | Loc | mean NSAF | S.D. | Pathway | Subcellular localization | T4SSTS [24] |
|-----------|-----------|--------------------------|------|-----|-----------|------|----------|--------------------------|------------|
| lpg1372   | 52841602  | oxidoreductase           | Lpg1372 | swa | 0.017     | 0.004 | -        | Intracellular Protein    |            |
| lpg1376   | 52841606  | rRNA large subunit methyltransferase | RlmH | swa | 0.016     | 0.001 | -        | Intracellular Protein    |            |
| lpg1391   | 52841621  | 50S ribosomal protein L32 | RmlF | swa | 0.056     | 0.013 | -        | Intracellular Protein    |            |
| lpg1392   | 52841622  | glycerol-3-phosphate acyltransferase PtaX | PtaX | swa | 0.006     | 0.001 | -        | Intracellular Protein    |            |
| lpg1489   | 52841719  | hypothetical protein lpg1489 | RavX | swa | 0.017     | 0.002 | -        | Intracellular Protein    |            |
| lpg1508   | 52841738  | rare lipoprotein A        | Lpg1508 | swa | 0.012     | 0.001 | Sec(SPI) | Periplasmic protein       | T4SSTS     |
| lpg1542   | 52841772  | PQQ WD-40-like repeat-containing protein | BamB | swa | 0.012     | 0.003 | Sec(SPI) | Periplasmic protein       |            |
| lpg1547   | 52841777  | radical SAM protein       | RimN | swa | 0.008     | 0.002 | -        | Intracellular Protein    |            |
| lpg1550   | 52841780  | RNA-(ms(2)j0(6a)-hydroxylase(5RNA) hydroxylase) | Lpg1550 | swa | 0.014     | 0.006 | -        | Intracellular Protein    |            |
| lpg1565   | 52841795  | thiamine biosynthesis protein NMT-1 | Thl3 | swa | 0.006     | 0.002 | -        | Intracellular Protein    |            |
| lpg1576   | 52841806  | Holliday junction DNA helicase RuvB | RuvB | swa | 0.010     | 0.002 | -        | Intracellular Protein    |            |
| lpg1564   | 52841912  | hypothetical protein lpg1684 | Lpg1684 | swa | 0.006     | 0.002 | -        | Intracellular Protein    |            |
| lpg1576   | 52841944  | hypothetical protein lpg1716 | Lpg1716 | swa | 0.018     | 0.003 | -        | Intracellular Protein    |            |
| lpg1591   | 52842119  | hypothetical protein lpg1901 | Lpg1901 | swa | 0.009     | 0.002 | -        | Intracellular Protein    |            |
| lpg1912   | 52842130  | sensory box histidine kinase/response regulator | Lpg1912 | swa | 0.003     | 0.002 | Sec(SPI) | Intracellular Protein     |            |
| lpg1924   | 52842142  | hypothetical protein lpg1924 | Lpg1924 | swa | 0.003     | 0.001 | -        | Intracellular Protein    | T4SSTS     |
| lpg1953   | 52842170  | hypothetical protein lpg1953 | Lpg1953 | swa | 0.016     | 0.003 | -        | Intracellular Protein    | T4SSTS     |
| lpg1964   | 52842181  | hypothetical protein lpg1964 | Lpg1964 | swa | 0.004     | 0.000 | -        | Intracellular Protein    | T4SSTS     |
| lpg1972   | 52842189  | hypothetical protein lpg1972 | Lpg1972 | swa | 0.003     | 0.015 | -        | Intracellular Protein    | T4SSTS     |
| lpg2004   | 52842221  | S-adenosylmethionine-5'-ribosyltransferase-isomerase | QueA | swa | 0.008     | 0.003 | -        | Intracellular Protein    |            |
| lpg2030   | 52842247  | hypothetical protein lpg2030 | FtsN | swa | 0.007     | 0.000 | -        | Intracellular Protein    |            |
| lpg2052   | 52842269  | hydroxymethylglutaryl-CoA reductase | Lpg2052 | OMAP | 0.005     | 0.001 | -        | Intracellular Protein    |            |
| lpg2176   | 52842389  | sphingosine-1-phosphate lyase I | LegS2 | swa | 0.011     | 0.000 | -        | Intracellular Protein    | T4SSTS     |
| lpg2271   | 52842484  | hypothetical protein lpg2271 | Lpg2271 | OMAP | 0.033     | 0.008 | Sec(SPI) | Integral Inner Membrane Protein | T4SSTS |
| lpg2293   | 52842486  | glycerol-3-phosphate ABC transporter substrate binding protein | UgpB | OMAP | 0.006     | 0.001 | Sec(SPI) | Periplasmic protein       |            |
| lpg2298   | 52842508  | inclusion membrane protein A | YIA | swa | 0.004     | 0.001 | -        | Intracellular Protein    | T4SSTS     |
| lpg2328   | 52842538  | hypothetical protein lpg2328 | Lnm22 | swa | 0.013     | 0.001 | -        | Intracellular Protein    | T4SSTS     |
| lpg2345   | 52842555  | ATP-dependent RNA helicase | DeaD | swa | 0.015     | 0.005 | -        | Intracellular Protein    |            |
| lpg2409   | 52842618  | hypothetical protein lpg2409 | Ceg9 | swa | 0.008     | 0.002 | -        | Intracellular Protein    | T4SSTS     |
| lpg2414   | 52842623  | hypothetical protein lpg2414 | Lpg2414 | swa | 0.039     | 0.005 | -        | Intracellular Protein    | T4SSTS     |
| lpg2424   | 52842633  | hypothetical protein lpg2424 | MavG | swa | 0.011     | 0.001 | -        | Intracellular Protein    | T4SSTS     |
| lpg2452   | 52842660  | ankyrin repeat-containing protein | AnKf | swa | 0.007     | 0.002 | -        | Intracellular Protein    | T4SSTS     |
| lpg2463   | 52842671  | peptide aspartate b-dioxygenase | Lpg2463 | swa | 0.007     | 0.000 | -        | Intracellular Protein    | T4SSTS     |
| lpg2490   | 52842698  | hypothetical protein lpg2490 | LeyB | swa | 0.007     | 0.002 | -        | Intracellular Protein    | T4SSTS     |
| lpg2507   | 52842715  | hypothetical protein lpg2507 | Lpg2507 | swa | 0.020     | 0.001 | -        | Intracellular Protein    | T4SSTS     |
| lpg2519   | 52842727  | hypothetical protein lpg2519 | Lpg2519 | swa | 0.052     | 0.000 | -        | Intracellular Protein    |            |
| lpg2522   | 52842730  | metal-activated pyridoxal enzyme | Lpg2522 | swa | 0.008     | 0.003 | -        | Intracellular Protein    |            |
Here, we provide comprehensive maps of a total of 960 different proteins quantified in three life stages, E, PE, and UNC, of *L. pneumophila* and in different bacterial subfractions (Table I). Such data may be important for selection of significantly abundant proteins for future analysis and may facilitate investigations of protein function and localization.

Three hundred seventy-nine *L. pneumophila* proteins showed differential abundance in E/PE. 176 of those additionally exceeded the threshold of $\log_{2}(\text{NSAF}(\text{E/PE})/H11005) > 1$ and therefore were considered E-up, whereas 147 fell below the threshold of $-1$ and therefore were considered PE-up (Table I). Our data therefore indicate an almost comparable number of proteins differentially abundant in E or PE. This is in contrast to the results of Hayashi et al., who identified in a 2DE-based proteomic study 68 differentially abundant *L. pneumophila* proteins in E/PE with 64 of which ($\approx 94\%$) being more abundant in PE than in E (16). Although, several highly abundant proteins associated with flagellum biogenesis were found overabundant in PE samples in our study, especially FliC (mean NSAF PE swcp $= 0.31$) which was also used as PE marker, the protein strikingly was not among the differentially abundant proteins of Hayashi et al. (Fig. 1, Fig. 5).

FliC gene up-regulation is among the highest in amoebae infections with *L. pneumophila* or during switch from E to PE upon growth of the bacteria in broth (12, 14). The transcriptional study by Brüggemann et al. revealed that 84% of the up-regulated genes in replicative phase (upon amoebae infection) are also up-regulated during E (upon growth in broth) and 77% of transmissive phase up-regulated genes are also found up-regulated in PE (12). Conformingly, we found that there is a high overlap of the PE-exclusive proteins defined in our study and the transcriptional up-regulation of those genes during transmissive phase in *A. castellanii* ($> 80\%$) as well as for the E-exclusive proteins with respect to replicative phase ($> 60\%$). The more limited representation of the E-exclusive proteins by the replicative phase up-regulated genes might be because of post-transcriptional processes that contribute to reduction of protein abundance not reflected in the transcriptomic data. Brüggemann et al. further found that some genes are only transcriptionally up-regulated under *in vivo* infection conditions, including ralF, lidA, and sidC (12). However, in our study, using broth-grown bacteria, the respective proteins were differentially abundant in E/PE swcp. RalF was PE-exclusive, and both LidA and SidC were PE-on proteins ($\log_{2}(\text{NSAF}(\text{E/PE})/H11005) = -1.4$, and $-2.4$, respectively) (supplemental Table S11). Interestingly, RalF, LidA, and SidC were shown before to accumulate to higher protein levels in PE suggesting for some proteins major differences between transcript and protein abundance (112, 119).

Our proteome data included a large number of Dot/Icm effectors and some of which showed substantial effects on *L.*
### Table III

List of quantified postexponential phase (PE)-exclusive proteins and their abundance, Loc = detected in subcellular fraction swcp,ormap,出境extr,S.D. = standard deviation, Pathway = prediction of sec signal, T4SSTS = Dot/Icm type IVB secretion system translocated substrate

| Locus tag | gi-Nr | Identified proteins (33; 2 in two subcellular fractions) | Name | Loc | mean NSAF | S.D. | Pathway | Subcellular localization pred. | T4SSTS [9] |
|-----------|-------|----------------------------------------------------------|------|-----|-----------|------|---------|-------------------------------|------------|
| lpg0242   | 52840497 | c-3-phosphoglycerate dehydrogenase                       | SerA | swcp | 0.017    | 0.002 | -       | Intra cellular Protein       |            |
| lpg0277   | 52840532 | sensory box protein, GGDEF family protein, LasE         | Lpg0277 | swcp | 0.010    | 0.004 | -       | Intra cellular Protein       |            |
| lpg0390   | 52840635 | VipA                                                     | VipA | swcp | 0.027    | 0.016 | -       | Intracellular Protein         |            |
| lpg0415   | 52840660 | hypothetical protein lpg0415                            | Lpg0415 | swcp | 0.040    | 0.009 | -       | Intracellular Protein         |            |
| lpg0695   | 52840932 | ankyrin repeat-containing protein                        | AnkX | swcp | 0.006    | 0.002 | -       | Intracellular Protein         |            |
| lpg0891   | 52841126 | sensory box protein                                      | Lpg0891 | swcp | 0.032    | 0.003 | -       | Intracellular Protein         |            |
| lpg0892   | 52841127 | kynurenicine 3-monooxygenase                             | Kyno | swcp | 0.012    | 0.002 | -       | Intracellular Protein         |            |
| lpg0902   | 52841137 | hypothetical protein lpg0902                            | Lpg0902 | swcp | 0.031    | 0.013 | Sec(Sp) | Periplasmic Protein           |            |
| lpg1059   | 52841293 | acebacetyl CoA reductase                                 | PhaB | swcp | 0.033    | 0.001 | -       | Intracellular Protein         |            |
| lpg1145   | 52841379 | hypothetical protein lpg1145                            | Lmp7 | swcp | 0.015    | 0.005 | -       | Intracellular Protein         |            |
| lpg1155   | 52841389 | pyruvate dehydrogenase                                   | Lpg1155 | swcp | 0.010    | 0.001 | -       | Intracellular Protein         |            |
| lpg1193   | 52841426 | phosphoribosyl-AMP cyclohydrolase/ phosphoribosyl-ATP pyrophosphatase | Hsl | swcp | 0.026    | 0.006 | -       | Intracellular Protein         |            |
| lpg1219   | 52841451 | flagellar hook protein FlgE                              | FlgE | swcp | 0.021    | 0.003 | Sec(Sp) | Cell Surface Appendage        |            |
| lpg1225   | 52841457 | flagellar hook-associated protein FlgK                   | FlgK | swcp | 0.022    | 0.007 | -       | Intracellular Protein         |            |
| lpg1225   | 52841457 | flagellar hook-associated protein FlgK                   | FlgK | extr | 0.232    | 0.029 | -       | Intracellular Protein         |            |
| lpg1455   | 52841685 | phospholipase C                                          | PcbB | extr | 0.022    | 0.009 | Sec(Sp) | Periplasmic Protein           |            |
| lpg1491   | 52841721 | hypothetical protein lpg1491                            | Lmp9 | swcp | 0.024    | 0.005 | -       | Intracellular Protein         |            |
| lpg1496   | 52841726 | hypothetical protein lpg1496                            | Lmp10 | swcp | 0.017    | 0.005 | -       | Intracellular Protein         |            |
| lpg1532   | 52841762 | peptidase                                                | MmcC | swcp | 0.004    | 0.001 | -       | Intracellular Protein         |            |
| lpg1603   | 52841831 | phospholipase                                            | Lpg1603 | swcp | 0.008    | 0.004 | -       | Intracellular Protein         |            |
| lpg1626   | 52841854 | copper efflux ATPase                                     | Lpg1626 | swcp | 0.006    | 0.002 | Sec(Sp) | Integral Inner Membrane Protein |            |
| lpg1655   | 52841883 | class 4 metalloprotease                                   | LasB | extr | 0.051    | 0.011 | Sec(Sp) | Secreted/Released             |            |
| lpg1683   | 52841911 | hypothetical protein lpg1683                            | RasV | swcp | 0.018    | 0.004 | -       | Intracellular Protein         |            |
| lpg1782   | 16173249 | flagellar biosynthesis sigma factor                       | FlaA | swcp | 0.041    | 0.010 | -       | Intracellular Protein         |            |
| lpg1963   | 52842180 | hypothetical protein lpg1963                            | Lrc | swcp | 0.006    | 0.003 | -       | Intracellular Protein         |            |
| lpg1993   | 52842210 | polysaccharide deacetylases                              | Lpg1993 | swcp | 0.056    | 0.018 | Sec(Sp) | Periplasmic protein           |            |
| lpg1993   | 52842210 | polysaccharide deacetylases                              | Lpg1993 | extr | 0.138    | 0.032 | Sec(Sp) | Periplasmic protein           |            |
| lpg2212   | 52842425 | acetyl/propionamine aminohydrolase                       | Bcp | swcp | 0.015    | 0.007 | -       | Intracellular Protein         |            |
| lpg2323   | 52842533 | type II secretion system protein (switching motility protein) | UptC | swcp | 0.021    | 0.005 | -       | Intracellular Protein         |            |
| lpg2443   | 52842652 | hypothetical protein lpg2443                            | Lpg2443 | extr | 0.370    | 0.027 | Sec(Sp) | Outer Membrane Associated/ Outer Membrane B-barrel Protein | T4SSTS |
| lpg2681   | 52842867 | 4-hydroxy-2-oxovalerate aldolase                         | Lpg2681 | swcp | 0.010    | 0.003 | -       | Intracellular Protein         |            |
| lpg2688   | 52842894 | intracellular multiplication protein IcmW                 | IcmW | swcp | 0.026    | 0.011 | -       | Intracellular Protein         |            |
| lpg2904   | 52842910 | ubiquinol-cytochrome c reductase, cytochrome b           | PetB | swcp | 0.007    | 0.002 | Sec(Sp) | Integral Inner Membrane Protein |            |
| lpg2803   | 52842999 | hypothetical protein lpg2803                            | Lpg2803 | swcp | 0.024    | 0.004 | -       | Intracellular Protein         |            |
| lpg2862   | 52843057 | coiled-coil containing protein                           | LegC8 | swcp | 0.032    | 0.007 | -       | Intracellular Protein         |            |
Proteomic Analysis of *L. pneumophila* Life Stages

**Table IV**

| Locus tag | gi-Nr   | Identified Proteins (9) | Name          | mean NSAF | S.D.  | Pathway                  | Subcellular localization | T4SSTS [0] |
|-----------|---------|-------------------------|---------------|-----------|-------|--------------------------|--------------------------|------------|
| lpg0132   | 52840387| hypothetical protein    | Lpg0132       | 0.061     | 0.013 | Sec(SPI) Integral Inner Membrane Protein |                         |            |
| lpg0619   | 52840856| glyoxylase              | Lpg0619       | 0.064     | 0.024 | -                        | Intracellular Protein    |            |
| lpg0623   | 52840860| hypothetical protein    | Lpg0623       | 0.537     | 0.189 | Sec(SPI) Integral Inner Membrane Protein |                         |            |
| lpg0652   | 52840889| major facilitator family transporter | Lpg0652 | 0.009     | 0.001 | Sec(SPI) Integral Inner Membrane Protein |                         |            |
| lpg0741   | 52840978| hypothetical protein    | Lpg0741       | 0.057     | 0.009 | -                        | Intracellular Protein    |            |
| lpg1915   | 52842133| Tfp pilus assembly protein, major type IV pilin class A | PiIE | 0.871     | 0.245 | Sec(SPI) Secreted/Released |                         |            |
| lpg1979   | 52842196| hypothetical protein    | Lpg1979       | 0.011     | 0.004 | Sec(SPI) Periplasmic protein |                         |            |
| lpg2633   | 52842839| hypothetical protein    | Lpg2633       | 0.041     | 0.009 | -                        | Intracellular Protein    |            |
| lpg2958   | 52843152| hypothetical protein    | Lpg2958       | 0.014     | 0.005 | Sec(SPI) Integral Inner Membrane Protein |                         |            |

*pneumophila* virulence especially upon intracellular growth or host-cell trafficking. To our surprise, a big variety of Dot/Icm effectors belonged to the differentially abundant proteins. Of the 86 quantified effectors in the swcp fraction, 32 were E-up and 23 were PE-up (Fig. 3, Fig. 6, 6A, 6B, supplemental Table S11). This implies that Dot/Icm effectors play a substantial role in both E and PE and most of the detected effectors may reveal functions associated with a specific life stage. Interestingly, several effectors known to be important at one specific stage in intracellular lifestyle of the bacteria in our study were most abundant in the other phase. Such as, LepA and LepB, supporting nonlytic release of the bacteria from amoebae, here were more abundant in E (108). Accordingly, Yfa was observed on the endoplasmic reticulum-derived replicative vacuole and on punctate structures at later stages of a host cell infection (106) although its abundance in our study was increased in E. It may therefore be an important strategy of *L. pneumophila* to accumulate to higher protein levels in PE (112, 119). Icm effectors, such as SidC, RalF, and LidA were shown to be away applicable to the host cell. Correspondingly, some Dot/Icm effectors, such as SidC, RalF, and LidA were shown before to accumulate to higher protein levels in PE (112, 119), and are important at early stages of host infection (157–160). Nevertheless, the here presented extracellular proteome contained a subset of proteins which, based on predictions, were unlikely candidates for classic secretion. However, *L. pneumophila* is known to shed outer membrane vesicles from intact membranes during intra- and extracellular growth (28). The study by Galka et al. analyzed proteins found in outer membrane vesicles expelled by *L. pneumophila* and culture supernatants devoid of them (28). Seventy percent of our quantified extracellular proteome (54 of 77 proteins) is covered by the secretome data of Galka et al., which is a reasonable overlap and therefore, presence of nonclassically secreted proteins may at least in part be explained by budded outer membrane vesicles. Indeed, eight of the extr in our study which were predicted as periplasmic or membrane-bound were previously identified in outer membrane vesicles (28). Moreover, six of the predicted 14 cytoplasmic, seven of the 11 predicted membrane, and 14 of the 21 predicted periplasmic proteins in our study were previously identified as *L. pneumophila* extracellular proteins (28). Further, export of canonical cytosolic proteins has been described as moonlighting phenomenon. It is however unclear how moonlighting proteins are translocated to the exterior, but their occurrence has been described and is often associated with a shift in function (i.e. in case of GAPDH,
enolase) and may result in an increase of bacterial adhesive-
ess to cells (169).

We here used biotinylation of intact \textit{L. pneumophila} to pull
down and characterize omp. This approach has been em-
ployed successfully both in Gram-positive and Gram-negative
bacteria, and this is the first report in \textit{L. pneumophila} (170–
174). Biotinylation of proteins was performed by incubation of
intact cells with Sulfo-NHS-SS biotin, which is advertised as
membrane impermeable and therefore is expected to react
specifically with proteins residing in the membrane or being
exposed to the exterior (175, 176). By means of that method

coupled to subsequent enrichment analysis, we detected a
variety of known and potentially surface-exposed proteins as
well as outer membrane-interacting proteins, such as Mip,
EnhA, TolB, OmpH/Lpg0507, and Ttg2D/Lpg0844 \textit{(supplemen-
tal Table S6)} (75, 91, 177). However, some studies also
report on the labeling of intracellular proteins when using
Sulfo-NHS-SS biotin (178). Therefore, we further verified the
experimentally achieved proteomes by means of enrichment
analysis of omp \textit{versus} swcp data sets. This led to a signif-
ificant reduction of likely cytosolic contaminants within the
omp fraction (70.6% prior enrichment compared with 16.4% after
enrichment; according PsortB prediction) and to a sig-
ificant increase of spatially unpredicted (22.1% prior compa-
ned with 40.0% after enrichment) and periplasmic (2.6% to
25.5%) proteins in omp. These results imply that the method
not absolutely seems restricted to the detection of proteins
located on or in the outer membrane and especially is prone
to enrich periplasmic contaminations, likely by biotin leakage
through the outer membrane. Nevertheless, the identification/
quantification of a variety of known outer membrane-linked
proteins suggests that also several new outer membrane-
linked proteins were identified in our study. However, their
verification needs further dedicated analysis.

Alleron \textit{et al.} showed by means of an \textit{35S}-labeling approach
coupled to subsequent 2D gel electrophoresis-based pro-

oteomics, that unculturable \textit{L. pneumophila} obtained after dis-

infection with monochloramine actively synthetized proteins
and they identified nine proteins which were accumulating in
higher quantities in the unculturable bacteria compared with
the culturable control (32). Three of those proteins, Mip,
DsbA2/Com1, and L1/RplA were quantified in UNC of our
study although we used long-term starvation in tap water for
generation of UNC.

It was surprising to find IcmX, an essential component with
yet unknown specific function in Dot/Icm transport, as the
second most abundant UNC protein (mean NSAF 1.26). IcmX
has been shown to be essential for bacterial replication in host
cells, to be primarily localized in the bacterial periplasm, and
a protein fragment was also detected outside of the bacteria
(131). However, it is currently not clear which function IcmX
may fulfill in the VBNC state.

It is further remarkable that the Com1 protein was found in
our study in very prominent amounts in UNC (mean NSAF
0.74) suggesting that this protein plays an important role in
this state. It has been described to promote bacterial patho-
genicity as well as formation of stable disulfide-bond com-
plexes with substrate proteins including those of the Dot/Icm
T4BSS (79).

Finally, our study shows that the phase-exclusive pro-

teomes of E and PE \textit{L. pneumophila} are in terms of protein
numbers clearly dominated by Dot/Icm effectors in conjunc-
tion with functionally unassigned and hypothetical proteins.
The latter moreover represent several UNC-exclusives. This
observation suggests that in large part the mediators attrib-
uting for the life stage-related phenotypes of \textit{L. pneumophila}
are unexplored and will require further investigation.

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