

Proteomic regulation during *Legionella pneumophila* biofilm development: decrease of virulence factors and enhancement of response to oxidative stress

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ABSTRACT

Legionella pneumophila (*L. pneumophila*) is a Gram-negative bacterium, which can be found worldwide in aquatic environments. It tends to persist because it is often protected within biofilms or amoebae. *L. pneumophila* biofilms have a major impact on water systems, making the understanding of the bacterial physiological adaptation in biofilms a fundamental step towards their eradication. In this study, we report for the first time the influence of the biofilm mode of growth on the proteome of *L. pneumophila*. We compared the protein patterns of microorganisms grown as suspensions, cultured as colonies on agar plates or recovered with biofilms formed on stainless steel coupons. Statistical analyses of the protein expression data set confirmed the biofilm phenotype specificity which had been previously observed. It also identified dozens of proteins whose abundance was modified in biofilms. Proteins corresponding to virulence factors (macrophage infectivity potentiator protein, secreted proteases) were largely repressed in adherent cells. In contrast, a peptidoglycan-associated lipoprotein (Lpg2043) and a peroxynitrite reductase (Lpg2965) were accumulated by biofilm cells. Remarkably, hypothetical proteins, that appear to be unique to the *Legionella* genus (Lpg0563, Lpg1111 and Lpg1809), were over-expressed by sessile bacteria.

Key words | biofilms, bioreactor, *Legionella pneumophila*, proteomics, stainless steel coupon

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INTRODUCTION

Legionellosis is a severe and sometimes life-threatening form of pneumonia (Jarraud *et al.* 2007). This disease is caused by a bacterium of the genus *Legionella*. Among the members of this group, *Legionella pneumophila* (*L. pneumophila*) was first identified in 1977, after a severe pneumonia outbreak in Philadelphia, USA (Brenner *et al.* 1979). Although unknown, it is assumed that the infectious dose of *L. pneumophila* is low. Indeed, some patients had been infected at up to 10 km from the source of contamination. In these situations, the source of the infection is generally difficult to establish and its spread is highly dependent on the level of bacterial water contamination, the bacterial capacity to disseminate and the strain virulence

(Pastoris *et al.* 1992; Garcia *et al.* 2004). Recently, we have shown that *L. pneumophila* in the viable but non-culturable state (which may be associated with Pontiac fever; Steinert *et al.* 1997) were still able to synthesise proteins, some of which are involved in virulence (Alleron *et al.* 2013). In general, outbreaks are related to poorly maintained artificial water systems, particularly cooling towers or evaporative condensers associated with air conditioning and industrial cooling (Stout *et al.* 1985; Dennis & Lee 1988). This is where *L. pneumophila* persists the most in a biofilm-associated state (Beveridge *et al.* 1997). In these artificial environments, where the water temperature is kept between 28 and 50 °C, *L. pneumophila* growth is favoured as

compared to natural ecosystems (Yamamoto 1992; Bentham 2000; Turetgen *et al.* 2005). Within these biofilms, *L. pneumophila* replicates intracellularly in a wide range of protozoa (Fields 1996; Harb *et al.* 2000; Steinert *et al.* 2002). Unsurprisingly, amoebae have been observed in water systems associated with Legionnaires' disease (Barbaree *et al.* 1986; Breiman *et al.* 1990).

Prokaryotic biofilms that predominate in a diverse range of ecosystems, in natural as well as in artificial environments, are composed of highly structured multi-species communities (Allison 2003). Indeed, biofilms represent 95% of the overall biomass in water distribution systems, while only 5% occurs in the bulk water phase (Wingender & Flemming 2004). Biofilm are now being recognised as the preferred status for bacterial development, thus it is necessary to study the maintenance of water systems, which seem to be of fundamental interest in attempts to reduce biofilm formation. The present techniques of prevention include ultraviolet irradiation (Triassi *et al.* 2006), ultrasonic treatment (Dadjour *et al.* 2006), instantaneous heating systems or chemical treatment (Muraca *et al.* 1987). Unfortunately, biofilms are very resistant to conventional disinfectants (Stewart & Costerton 2001; Donlan & Costerton 2002). It is now believed that the adaptations and interrelationships within biofilms are mediated by the expression of sets of genes resulting in phenotypes that differ profoundly from those of planktonically grown cells (Davies *et al.* 1985; Stewart & Costerton 2001; Whiteley *et al.* 2001; Sauer *et al.* 2002). To investigate the proteins specifically translated within biofilms, studies using the proteomic approach have emerged recently and rapidly (Oosthuizen *et al.* 2002; Tremoulet *et al.* 2002; Helloin *et al.* 2003; Vilain *et al.* 2004b; Collet *et al.* 2007; Lo *et al.* 2007), but have also raised new questions, for example, about the uniqueness of the biofilm phenotype.

We have chosen here to investigate *L. pneumophila* biofilms at the protein level by using two-dimensional gel electrophoresis, which allows direct semi-quantitative comparison of protein patterns across multiple experimental conditions. Thereafter, we used principal component analysis (PCA) and analysis of variance (ANOVA) to interpret spot quantity variations on electropherograms. Moreover, some of the proteins displaying biofilm-specific regulation were highlighted and identified using mass spectrometry.

MATERIALS AND METHODS

Strains and media

L. pneumophila (strain Philadelphia CIP no. 103854) was provided by the Pasteur Institute of Paris. Bacteria were maintained as glycerol stocks and stored at -80°C . Pre-cultures were performed in 5 mL buffered charcoal yeast extract (BCYE) medium with a selective supplement (Sigma), composed of colistin sulphate (7.500 U), vancomycin (2.50 mg), trimethoprim (1.25 mg) and amphotericin B (1.25 mg). Flasks were incubated for 72 h at 37°C under agitation (115 rpm). The medium also contained α -ketoglutarate and was supplemented with L-cysteine and ferric pyrophosphate (Edelstein 1981).

Free-cell cultures

One hundred microlitres of pre-culture was suspended in 200 mL of BCYE- α medium, without selective supplement and incubated at 37°C . The bacterial growth was controlled by monitoring the optical density at 600 nm (OD_{600}), until the stationary phase was reached ($\text{OD}_{600} = 2$, generally between 48 and 72 h of growth).

From an initial pre-culture, 100 μL of BCYE medium was added to 50 mL of culture medium to obtain an initial OD_{600} of 0.1. This OD was measured over the entire culture period and allowed an estimation of generation time of about 90 min.

Colony growth

Bacteria were streaked onto solid BCYE- α medium with selective supplement GVPC (glycine, vancomycin, polymyxin and cycloheximide; AES laboratory). After 48 h of growth at 37°C , white colonies were scraped off and suspended in 5 mL deionized water.

Biofilm growth

L. pneumophila biofilms were grown using a CDC Biofilm Reactor (CBR, Biosurface Technologies, Bozeman, MT, USA) (Donlan & Costerton 2002). The CBR consists of a

1-L glass vessel with an effluent spout positioned to provide approximately 400 mL operational fluid capacity. In this reactor, 24 removable stainless steel coupons (discs, 1.27 cm diameter and 0.3 cm thick) were incubated. The glass vessel was placed on a digitally controlled stirring plate to provide constant homogeneous agitation.

Before experiments, stainless steel coupons were cleaned and rinsed and then the assembled reactor was sterilised by autoclaving. The sterile reactor was filled with 400 mL of BCYE sterile medium. One millilitre of the *L. pneumophila* pre-culture in selective BCYE medium was then added to the reactor. After inoculation, the reactor was placed at room temperature in a sterile cabinet and biofilms were grown in batch conditions for 8 days. The culture medium was changed once after 72 h of incubation. Thereafter, all 24 coupons of the reactor were picked up and plunged in cold water to collect sufficient biomass for subsequent analysis. Biofilm bacteria were recovered by sonication of the coupons (one step of 4 min) in 10 mL of sterile distilled water (Deltasonic bath, Meaux, France).

Scanning electron microscopy

Free-cell culture bacteria were collected after 2 days' incubation and rinsed twice with sterile phosphate buffer. A film of poly-lysine was coated onto a glass slide and 20 μ L of the bacterial suspension was deposited. For biofilm imaging, coupons obtained from the CBR reactor were desiccated and directly used for the succeeding step.

For both samples, the biological material was fixed in 2% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.4) for 30 min and rinsed (3×10 min) in 0.2 M cacodylate buffer (pH 7.4). Samples were then dehydrated by passing them through the following ethanol series: 30, 50 and 80%, each for 10 min; 100% ethanol, 2×10 min. Samples were then dried at 37 °C for 24 h. Once coated with gold-palladium (sputtering device), samples were examined under a scanning electron microscope (Cambridge S200).

Protein extraction and solubilisation

Culture preparations were centrifuged at $2,000 \times g$ for 10 min, and the bacterial pellet was freeze-dried for 2 h

and resuspended in iso-electric focalisation (IEF) buffer (7 M urea, 2 M thiourea, 0.1% amidosulfobetaine (ASB14), 2 mM tributyl phosphine, 0.5% (v/v) carrier ampholytes (pH 3–10; Sigma)). The mixture was incubated twice for 10 min at 37 °C and 10 min at –80 °C successively and subsequently submitted to four cycles of 30 s sonication. Protein extracts were then harvested in the supernatant after elimination of cellular debris by centrifugation ($10,000 \times g$ for 15 min). Protein concentrations were evaluated using the Bio-Rad protein assay and protein extracts were stored at –20 °C until further analysis.

Two-dimensional gel electrophoresis

For IEF, an additional volume of IEF buffer was added to the protein solution to obtain 100 μ g of proteins in a final volume of 400 μ L (with 0.4% of Coomassie blue). IEF was carried out with immobilised pH gradients (Immobiline Dry Strips, pH 3–10, 18 cm NL; Amersham Pharmacia Biotech) using an IEF-Cell (Bio-Rad) as follows: active rehydration for 12 h at 50 V, 250 V for 15 min, gradient from 250 to 10,000 V for 3 h and final focusing for 12 h at 10,000 V. After IEF, the strip was equilibrated in a buffer containing 1% dithiothreitol for 10 min. A second equilibration step was performed for 10 min in equilibration buffer containing 4% iodoacetamide. Finally, the second separation dimension was ensured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% polyacrylamide resolving gel (width 16 cm, length 20 cm and thickness 0.75 cm) using the Protean II xi vertical system (Bio-Rad). The proteins were stained using the silver nitrate method modified to be compatible with mass spectrometry (Shevchenko *et al.* 1996). Images of gels were acquired using a fluoroi-mager (ProXpress, PerkinElmer) and gels were stored in water at 4 °C before spot excision.

For each condition, that is planktonic (P), colonies (C) and biofilm (B), three independent cultures were obtained and the corresponding gels prepared in duplicate, except for biofilms for which the protein extract titration did not reveal a sufficient protein amount to carry out many gels from the same culture. The experiment was repeated in the same conditions in order to reach a significant number of experimental gels.

Image analysis

The gel images were analysed using the SameSpots software V2 (Nonlinear Dynamics). Images were primarily tested for similar dynamic range and absence of saturation and entirely pre-aligned before detection, which resulted in an optimal matching between all gels. The statistical tool integrated into the software combines calculation of *p*-values (ANOVA), *q*-values, power, correlation analysis and PCA. Spot quantity variation among the different experimental conditions tested was interpreted by using PCA. This analysis is based upon the generation of a covariance matrix describing how each spot varies with respect to every other spot in the data set. For this analysis, about 800 protein spots per condition were obtained and matched. Thereafter, ANOVA was calculated using a variance stabilisation technique based on the logarithm of normalised volume. The spots with a *p*-value under 0.01 and a power over 0.90 were selected. Then, in order to remove false positives, spots with *q*-values over 0.15 were rejected (Storey & Tibshirani 2003).

Protein identification

Protein spots were excised from two-dimensional gels using an automatic spot exciser (ProXCISION, PerkinElmer). The excised fragments were washed several times and dried in a SpeedVac centrifuge for a few minutes. Trypsin digestion (with 10 μ L of a 10 ng/ μ L trypsin solution) was performed overnight with a dedicated automate (Multi-PROBE II, PerkinElmer). Thereafter, gel fragments were subsequently incubated twice for 15 min in a H₂O/CH₃CN (1:1) solution to allow peptides extraction from the gel. The peptide extracts were then dried and solubilised in starting buffer made of CH₃CN 3%/HCOOH 0.1% in water for chromatographic elution. The peptides were enriched and separated by reversed-phase liquid chromatography with a precolumn/analytical column nano-flow setup (HPLC-Chip cube; Agilent Technologies). The peptides were further fragmented after a full survey scan (*m/z* 300–200) using an on-line ion trap mass spectrometer (model 6330, Agilent). Tandem mass spectrometry (MS/MS) readings for the five most abundant precursor ions were acquired and the fragmentation data

were interpreted using the DataAnalysis Software (version 3.4, BrukerDaltonic).

In order to identify proteins, extracted MS/MS peak lists were compared to the *L. pneumophila* open reading frame protein database using the MASCOT Daemon (version 2.1.3) search engine. All searches were performed with no fixed modification and with variable modifications for carbamidomethylation of cysteines and for oxidation of methionines and with a maximum of one missed cleavage. MS/MS spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.6 Da for fragment ions, respectively. The protein identification was validated if two peptides were observed with a fragmentation profile score higher than the average default value for significance using MASCOT. When this criterion was not met, peptides were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion.

Bioinformatic tools

For identified proteins, and in particular for unknown proteins, a first prediction of their location within the bacterial cell was obtained from the genome annotation of *L. pneumophila* (<http://www.cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=ntlp04>) or by using the SignalP bioinformatics tool (<http://www.cbs.dtu.dk/services/SignalP/>). For extensive screening of identified proteins, the 'String' search engine (accessible at <http://www.xi.embl.de>) was routinely used (von Mering *et al.* 2007).

RESULTS AND DISCUSSION

The deciphering of several *L. pneumophila* genomic sequences allowed a new era of investigations for this ubiquitous pathogen (Cazalet *et al.* 2004; Chien *et al.* 2004). As a clear illustration, bioinformatics studies revealed that *L. pneumophila* expresses an arsenal of eukaryote-like proteins, which clearly plays a major role in intracellular replication and host-cell lysis (Bruggemann *et al.* 2006; Khemiri *et al.* 2008a). In addition, post-genomic investigations, such as transcriptomics and proteomics, are nowadays sufficiently powerful to depict the physiological status of this microorganism under different conditions and thus to

access the molecular reprocessing associated with a biological event (Hindre *et al.* 2008). Here, we compare the proteome of *L. pneumophila* biofilms to colony-grown and to planktonic bacteria in order to highlight specific regulation associated with the immobilised status.

Polymorphism of *L. pneumophila*

Planktonic cells

The observation of *L. pneumophila* cells using scanning electron microscopy revealed a heterogeneous bacterial morphology. During the exponential phase, the bacterial cells are homogeneous, displaying a rod shape of approximately 3 µm length, and are undergoing division. At the end of the exponential phase, the bacteria become filamentous, and later in the stationary phase, *L. pneumophila* forms longer filaments of up to 30–40 µm (Figure 1(a)). This polymorphic behaviour has already been reported for *L. pneumophila* grown either *in vitro* or *in vivo* (Berg *et al.* 1985; Mauchline *et al.* 1992). Previous investigations also described the morphology of *L. pneumophila* from different serogroups (1–6) in different conditions of growth (Katz *et al.* 1984). Morphological differences were also detected according to the growth medium's iron level. Specifically, iron excess conditions promoted the transition from the short rod to the filament, these shape alterations being accompanied by a modification of the virulence of the bacterium (James *et al.* 1995).

Biofilm cells

Bacterial shape evolved during biofilm growth. In the colonisation period, a long rod shape is found. As the biofilm matures, the bacterial size is reduced and tends towards a rod-coccioid shape with an increased presence of polymer matrix (Figure 1(b)). As already described for a wealth of microorganisms, the planktonic mode of growth is very often a transitory situation. In its natural environment, *L. pneumophila* is either associated with protozoa within which the bacteria replicate (Fields 1996) or as a component of complex natural biofilms (Barbaree *et al.* 1986; Murgia *et al.* 2001). Previous microscopic observations of *L. pneumophila* biofilms revealed that the temperature had

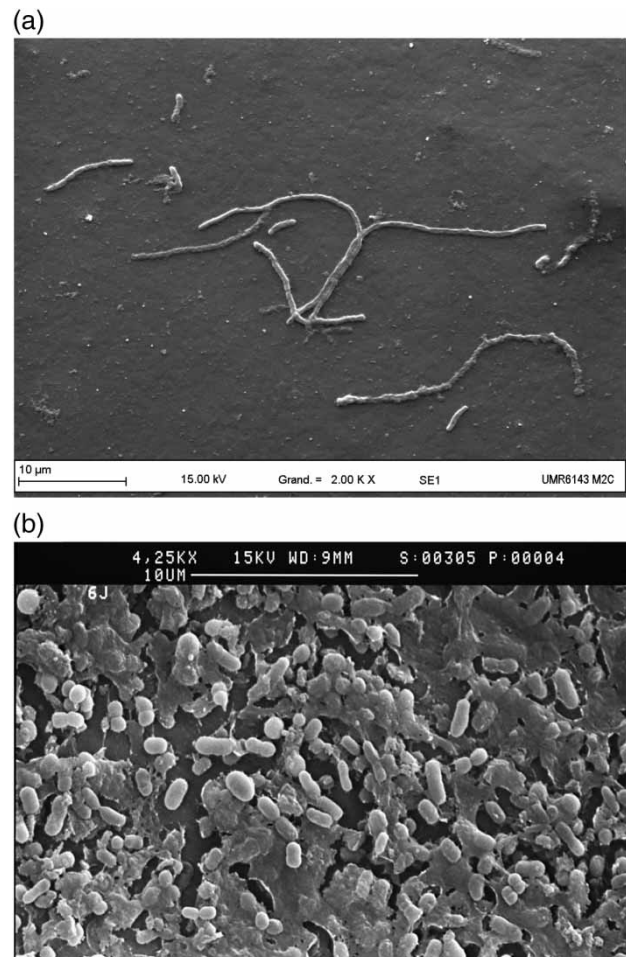


Figure 1 | Scanning electron micrographs of *L. pneumophila*. (a) Free cells in the stationary phase (72 h), most often observed as long filamentous structures, and (b) 7-day-old biofilms formed on stainless steel coupons; the bacteria appear as coccioid rod structures.

an impact on the bacterial shape. Indeed at room temperature, sessile bacteria were observed as rods as in our study, whereas at 37 °C, the bacteria became filamentous (Donlan & Costerton 2002; Piao *et al.* 2006).

Biofilm phenotype

For each growth condition, three two-dimensional gels were selected for statistical analysis. The interpretation was led either at the macroscopic level with PCA or at the spot abundance level in order to identify key proteins involved in biofilm formation.

Principal component analysis

The analysis was performed on normalised data (vertically and horizontally) and demonstrates that the proteome of *L. pneumophila* biofilms is significantly different from the two other proteomes (i.e. colonies and free cells). Indeed, PCA reveals that the first component strongly discriminates the biofilm proteome with respect to the other culture conditions, explaining more than 61% of the observed modifications in the whole data set (Figure 2). Accounting for another 21%, the second component separates colonies and planktonic proteomes, with nearly no weight for biofilm in this component. This observation is reminiscent of a previous study comparing different conditions of growth for *Pseudomonas aeruginosa* (*P. aeruginosa*) including biofilms developed on different supports (Vilain et al. 2004a). The latter data also demonstrated specific protein expression for the immobilised cells as compared with free cells in different stages of growth and, more interestingly, the immobilised bacteria protein profile was dependent on the condition of colonisation (agar-entrapped, glass fibres or clay beads). Again for *P. aeruginosa*, another recent proteomic report also described similar differences between biofilms and other modes of bacterial growth (Mikkelsen et al. 2007). The fact that *L. pneumophila* exhibits some particularities as compared to other bacteria (e.g. the presence of a battery of eukaryotic-like proteins) (Cazalet et al. 2004;

Khemiri et al. 2008a) is already known, but that its proteomic behaviour in biofilms resembles that of other microorganisms must draw one's attention. In general, the number of modified proteins was consistent with other studies which use two-dimensional gel electrophoresis as a separation tool for biofilm proteomic study. However, *Streptococcus pneumoniae* proteome biofilm analysis has shown the upregulation of proteins involved in virulence, adhesion and resistance (Allegrucci et al. 2006). Proteomic analysis of *Escherichia coli* biofilms reveals the overexpression of the outer membrane protein OmpA (Orme et al. 2006). In *Staphylococcus xylosus* amino acid biosynthesis and protein translation, protein determinants related to protein secretion were upexpressed in biofilm (Planchon et al. 2009). *Acinetobacter baumannii* biofilm was characterised by the upregulation of many proteins, including outer membrane proteins involved in iron transport, a sensor histidine kinase/response regulator and diguanylate cyclase (PAS-GGEDF-EAL domain) (Shin et al. 2009). Nonetheless, in the pathogen *Neisseria meningitidis*, proteomic analysis of meningococcal biofilms was conducted and metabolic changes related to oxygen and nutrient limitation and upregulation of proteins involved in reactive oxygen species defence were observed (van Alen et al. 2010).

Spot abundance analysis

The differential analysis of reference gels (containing information for spot average abundance within a particular growth condition) was undertaken on the 827 detected spots (see Figure 2). According to our selected thresholds, the level of 46 protein spots statistically varies between planktonic, colony and immobilised organisms, which represent 5.6% of the detected spots. Cluster analysis allows the separation of two groups of proteins. The first one corresponds to 23 proteins, which are either over expressed in biofilms or down regulated in colonies. Within the first cluster, 16 proteins are accumulated within biofilms and 7 down regulated in colonies. The second cluster is composed of the same number of proteins, but they are down regulated in biofilms (17 proteins) or over expressed in planktonic (3) and colony-grown bacteria (3). Thus, the major modifications observed in proteomic maps are correlated to the

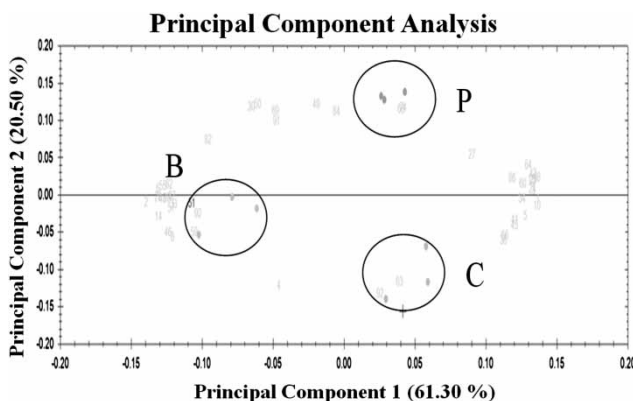


Figure 2 | Principal components analysis. Biplot of PC1 × PC2 after data normalisation (horizontal, spot abundances across the different conditions and vertical, total volume of spots related to protein loading). B refers to biofilm bacteria, P to planktonic cells and C to colony-grown bacteria. The first extracted component accounts for 61% of the variations and the second one for another 21%.

biofilm status, in agreement with the PCA analysis described above.

The degree of modification observed here for *L. pneumophila* switching between different lifestyles is relatively low compared with our previous investigation with *P. aeruginosa* and *Escherichia coli* (Collet et al. 2008). The latter reported 10–25% variation depending on the support used for immobilisation for an equivalent number of spots detected (Vilain et al. 2004b). However, the high adaptability in widely diverse contexts of *P. aeruginosa*, with its potential of more than 5,500 gene products and more than 50 transcriptional factors, could explain such discrepancy. However, a direct parallel is difficult to draw because the filtering used in this study for significant differential expression results from a combined statistical analysis (*p*-value, power and *q*-value). This asserts the validity of the observed regulation but also diminishes in a drastic fashion the number of validated differentially expressed spots.

Protein regulation within biofilms

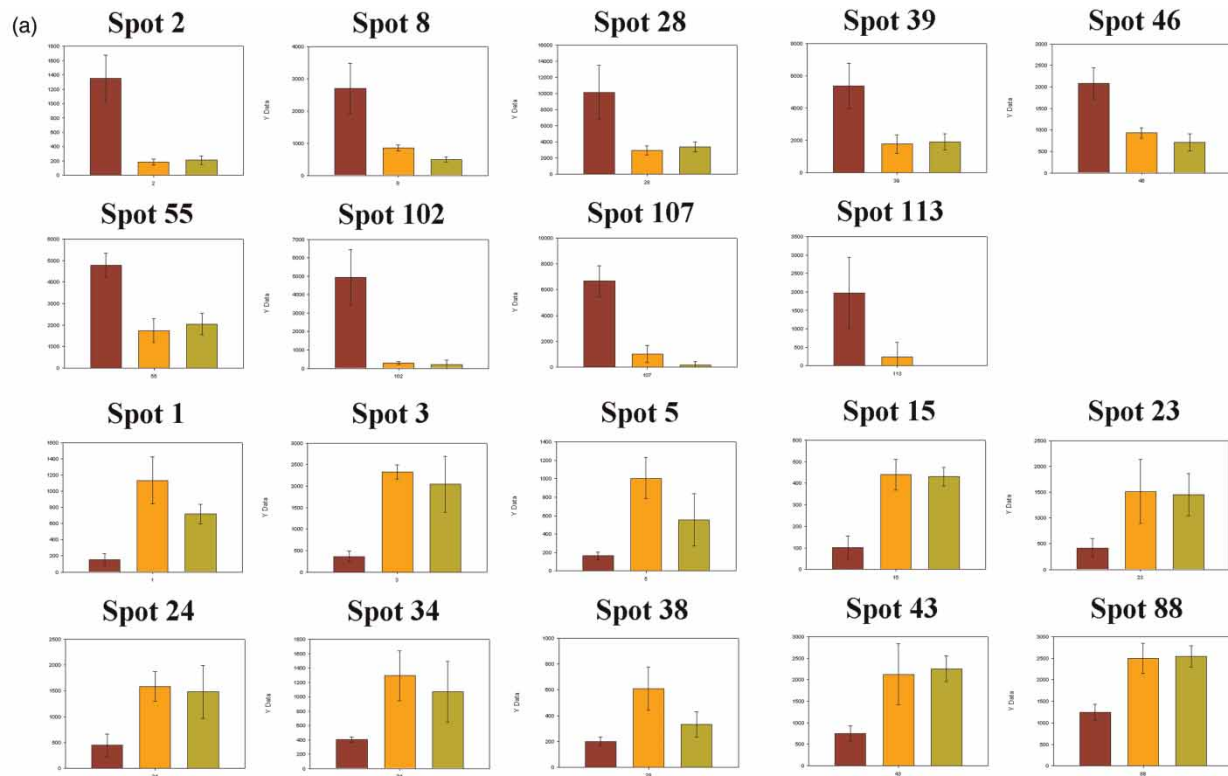
Among the 46 spots whose abundance was modified according to the mode of growth, 26 were identified by MS/MS and the identification of 17 of them, regulated according to the biofilm status, is presented in Figure 3(b) (see also supplementary material for details of identification, available online at <http://www.iwaponline.com/jwh/012/103.pdf>). Accordingly, their differential expression is presented in Figure 3(a). For example, in the biofilm state, the sigma-54 modulation factor (Lpg0476, lower part of the proteomic map) is specifically accumulated. Its highly related functional partner the sigma-54 modulation factor (Lpg0477; RpoN) is a transcriptional factor involved in many regulatory functions, including carbon assimilation, flagella synthesis or virulence (Kustu et al. 1989; Studholme & Buck 2000) that was also shown to control proteins involved in biofilms (Thompson et al. 2003; Wolfe et al. 2004; Saldias et al. 2008). In contrast, proteins involved in one-carbon metabolism and lipid metabolism were expressed less in biofilms (GlyA, AtoB). It is interesting to note that these two proteins were also down regulated in other model biofilms: GlyA in *Pseudomonas putida* (Sauer & Camper 2001) and AtoB in *P. aeruginosa* (Vilain et al. 2004b).

Decrease of virulence factors

For most of the proteins that display a decrease of abundance (Lpg0187, Lpg0467, Lpg1331, Lpg1571 and Lpg0791), a signal peptide was detected, suggesting involvement in secretion or transport. Recently, a fraction of these proteins was identified after outer membrane enrichment (Khemiri et al. 2008b). These classes of proteins are often associated with virulence (Dowling et al. 1992), as for example the macrophage infectivity potentiator (MIP) protein, which constitutes one of the best-known *L. pneumophila* virulence factors (Cianciotto & Fields 1992). Mutations studies and complementation have clearly shown differential lethality when the MIP protein was repressed. Other virulence factors whose abundance is diminished within biofilms include the zinc metalloprotease Lpg0467, which shares significant homology with the elastase of *P. aeruginosa* (Black et al. 1990), another zinc metallopeptidase (Lpg0187) or the protease DO (Lpg1331), which is essential for mammalian intracellular infection (Pedersen et al. 2001). This experimental observation of decrease of virulence factors is in line with recent insights in the field of bacterial biofilms signalling the transition from planktonic virulent bacteria to biofilms by the sensing of c-di-GMP levels (Romling et al. 2005; Cotter & Stibitz 2007). The c-di-GMP signal was shown to be mediated by GGDEF and EAL domain proteins, which participate in the synthesis and degradation of this small molecule to coordinate the phenotypic response. However, the mechanisms underlying this switch between lifestyles is not clearly understood in many model microorganisms and has not been described in *L. pneumophila* though many GGDEF and EAL domain proteins are detected in its genomic sequence.

Enhancement of response to oxidative stress

Thanks to a recent investigation of *L. pneumophila* biofilms carried out at the transcriptomic level (Hindre et al. 2008), it was possible to compare results from the two approaches. In the transcriptomic study, two clusters were detected: the first was of proteins involved in siderophore biosynthesis (no evidence of over-expression of this cluster was observed in our conditions); the other cluster, found in both studies, was due to over expression of alkyl hydroperoxide reductase



(b)

N°	Mr	pI	Score	Protein	Code	Gene	Function
Upregulated in biofilms							
8	24.3	4.98	110 (2)	Hypothetical protein Lpg1111	Lpg1111		ORFs of unknown function (unique)
28	18.9	6.59	413 (8)	Peptidoglycan associated lipoprotein (signal-P)	Lpg2043		Named proteins of general function
39	26.4	6.52	119 (3)	LvhB5 (signal-P)	Lpg1253	<i>virB5</i>	Transport and binding / secretion
46	12.9	4.60	102 (2)	50S ribosomal protein L7/L12	Lpg0321	<i>rplL</i>	Translation
55	22.1	5.89	116 (2)	Peroxytrinitrate reductase, AhpC/Tsa family	Lpg2965	<i>ahpC</i>	Detoxification/adaptation
102	13.7	7.77	149 (3)	Hypothetical protein Lpg1809 (signal-P)	Lpg1809		ORFs of unknown function (unique)
107	13.9	7.74	244 (6)	Hypothetical protein Lpg0563	Lpg0563		ORFs of unknown function (unique)
113	11.4	6.05	189 (4)	Sigma-54 modulation protein	Lpg0476		Transcription factors / DNA binding proteins
Downregulated in biofilms							
1	45.5	6.36	173 (3)	Serine hydroxymethyltransferase	Lpg0725	<i>glyA</i>	Amino acid metabolism, metabolism of cofactors and vitamins, energy metabolism
3	21.1	7.96	218 (5)	Transcription antitermination protein N	Lpg0317	<i>nusG</i>	Transcription
15	45.4	8.67	245 (5)	TolB colicin import protein (signal-P)	Lpg1571	<i>tolB</i>	Transport and binding
23	52.5	8.51	161 (4)	Zinc metalloprotein (signal-P)	Lpg0187		Protein fate/hydrolases /secretion
24	37.1	6.50	99 (2)	Zinc-binding dehydrogenase	Lpg2208		Carbohydrate metabolism, lipid metabolism, amino acid metabolism
34	41.2	6.46	261 (5)	Acyl CoA C-acetyltransferase	Lpg1825	<i>atoB</i>	Lipid metabolism, amino acid metabolism, carbohydrate metabolism
38	60.6	5.33	172 (4)	Zinc metalloprotease (signal-P)	Lpg0467		Toxin production/ hydrolases /secretion
43	49.5	8.96	139 (3)	Protease DO (signal-P)	Lpg1331	<i>htrA</i>	Protein fate/hydrolases/secretion
88	25.1	9.19	280 (5)	Macrophage infectivity potentiator (signal-P)	Lpg0791	<i>mip</i>	Toxin production/other pathogen functions

Figure 3 | (a) Details of protein abundance regulations between experimental conditions: biofilms (first bar), colonies (second bar) and planktonic (third bar). The data are averaged from three different gels and the error bars indicate the standard deviation from the mean. (b) Identification of proteins whose abundance was specifically modified within biofilms (upper part: over-expressed in biofilms as compared to planktonic or colony-grown bacteria and lower part: opposite regulation). Mr, and pI correspond to calculated molecular weights and isoelectric points, respectively. Score refers to MASCOT scoring with number of identified peptides between brackets. The code indicates the reference in the *L. pneumophila* strain Philadelphia annotated genome available online from: <http://www.cmj.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=ntlp04>. (See also supplementary data for additional details of protein identification, available online at <http://www.iwaponline.com/jwh/012/103.pdf>.)

family proteins (Lpg2895 in our study). These proteins are important actors in peroxide regulation (LeBlanc *et al.* 2006) and, as such, might have a role in protecting biofilms from oxidative stress. More interestingly, the PAL protein, which is one of the important surface antigens of *L. pneumophila* (Kim *et al.* 2003), also up regulated in biofilms, displays a significant homology with the OprL protein of *P. aeruginosa*. It was recently shown that from an OxyR deletion background, the superimposition of an OprL mutation promotes bacterial sensitivity to H₂O₂ exposure in planktonic as well as in biofilm conditions. In microscopic assays, the double mutant envelope integrity was compromised (Panmanee *et al.* 2008). This suggests a particular need for bacterial membrane reinforcement within biofilms in order to promote oxidative protection.

New routes of investigations for biofilms

Additionally, many hypothetical proteins were up regulated in the biofilm state, namely Lpg0563, Lpg1111 and Lpg1809. Lpg1111 is unique to *L. pneumophila*, being found only in *L. pneumophila* Philadelphia and Paris strains. According to predictive methods (String; von Mering *et al.* 2007), this protein would interact with its neighbour in the genomic sequence Lpg1112, which could be implied in a complex in contact with the bacterial outer membrane. Lpg0563 is unique to the *Legionella* genus, more than 90% homology being found with proteins from the three other sequenced *L. pneumophila* genomes. Lpg1809 is also specific to *L. pneumophila*. The analysis of its primary sequence allows prediction of the presence of a signal peptide and suggests outer membrane localisation. All of these proteins constitute potential candidates that may play a crucial role for *L. pneumophila* biofilm development.

CONCLUSION

To conclude, this study describes for the first time the proteomic modifications accompanying surface-associated growth for *L. pneumophila*. We showed that the biosynthesis of virulence effectors was decreased and that factors

contributing to oxidative protection were accumulated within biofilms. In addition to those known functional levels, hypothetical proteins unique to the *Legionella* genus were also accumulated (namely Lpg0563, Lpg1111 and Lpg1809). We suggest that these molecular actors, as yet functionally uncharacterised, will prove privileged targets for future molecular biology work and biofilm phenotypic investigations. The immobilised status of *L. pneumophila* involves the biofilm lifestyle, but also may be associated with intracellular survival within amoebae. We plan to investigate in the near future, the effect of the intra-amoebal growth and the synergistic behaviour in multiple-species biofilms.

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REFERENCES

- Allegrucci, M., Hu, F. Z., Shen, K., Hayes, J., Ehrlich, G. D., Post, J. C. & Sauer, K. 2006 Phenotypic characterization of *Streptococcus pneumoniae* biofilm development. *J. Bacteriol.* **188**, 2325–2335.
- Alleron, L., Khemiri, A., Koubar, M., Lacombe, C., Coquet, L., Cosette, P., Jouenne, T. & Frere, J. 2013 VBNC *Legionella pneumophila* cells are still able to produce virulence proteins. *Water Res.* **47**, 6607–6617.
- Allison, D. G. 2003 The biofilm matrix. *Biofouling* **19**, 139–150.
- Barbaree, J. M., Fields, B. S., Feeley, J. C., Gorman, G. W. & Martin, W. T. 1986 Isolation of protozoa from water associated with a Legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. *Appl. Environ. Microbiol.* **51**, 422–424.
- Bentham, R. H. 2000 Routine sampling and the control of *Legionella* spp. in cooling tower water systems. *Curr. Microbiol.* **41**, 271–275.

- Berg, J. D., Hoff, J. C., Roberts, P. V. & Matin, A. 1985 Growth of in continuous culture. *Appl. Environ. Microbiol.* **49**, 1534–1537.
- Beveridge, T. J., Makin, S. A., Kadurugamuwa, J. L. & Li, Z. 1997 Interactions between biofilms and the environment. *FEMS Microbiol. Rev.* **20**, 291–303.
- Black, W. J., Quinn, F. D. & Tompkins, L. S. 1990 *Legionella pneumophila* zinc metalloprotease is structurally and functionally homologous to *Pseudomonas aeruginosa* elastase. *J. Bacteriol.* **172**, 2608–2613.
- Breiman, R. F., Fields, B. S., Sanden, G. N., Volmer, L., Meier, A. & Spika, J. S. 1990 Association of shower use with Legionnaires' disease. Possible role of amoebae. *JAMA* **263**, 2924–2926.
- Brenner, D. J., Steigerwalt, A. G. & McDade, J. E. 1979 Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family Legionellaceae, familia nova. *Ann. Intern. Med.* **90**, 656–658.
- Bruggemann, H., Cazalet, C. & Buchrieser, C. 2006 Adaptation of *Legionella pneumophila* to the host environment: role of protein secretion, effectors and eukaryotic-like proteins. *Curr. Opin. Microbiol.* **9**, 86–94.
- Cazalet, C., Rusniok, C., Bruggemann, H., Zidane, N., Magnier, A., Ma, L., Tichit, M., Jarraud, S., Bouchier, C., Vandenesch, F., Kunst, F., Etienne, J., Glaser, P. & Buchrieser, C. 2004 Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nat. Genet.* **36**, 1165–1173.
- Chien, M., Morozova, I., Shi, S., Sheng, H., Chen, J., Gomez, S. M., Asamani, G., Hill, K., Nuara, J., Feder, M., Rineer, J., Greenberg, J. J., Steshenko, V., Park, S. H., Zhao, B., Teplitskaya, E., Edwards, J. R., Pampou, S., Georghiou, A., Chou, I. C., Iannuccilli, W., Ulz, M. E., Kim, D. H., Geringer-Sameth, A., Goldsberry, C., Morozov, P., Fischer, S. G., Segal, G., Qu, X., Rzhetsky, A., Zhang, P., Cayanis, E., De Jong, P. J., Ju, J., Kalachikov, S., Shuman, H. A. & Russo, J. J. 2004 The genomic sequence of the accidental pathogen *Legionella pneumophila*. *Science* **305**, 1966–1968.
- Cianciotto, N. P. & Fields, B. S. 1992 *Legionella pneumophila* mip gene potentiates intracellular infection of protozoa and human macrophages. *Proc Natl Acad Sci USA* **89**, 5188–5191.
- Collet, A., Vilain, S., Cosette, P., Junter, G. A., Jouenne, T., Phillips, R. S. & Di Martino, P. 2007 Protein expression in *Escherichia coli* S17–1 biofilms: impact of indole. *Antonie Van Leeuwenhoek* **91**, 71–85.
- Collet, A., Cosette, P., Beloin, C., Ghigo, J. M., Rihouey, C., Lerouge, P., Junter, G. A. & Jouenne, T. 2008 Impact of rpoS deletion on the proteome of *Escherichia coli* grown planktonically and as biofilm. *J. Proteome Res.* **7**, 4659–4669.
- Cotter, P. A. & Stibitz, S. 2007 c-di-GMP-mediated regulation of virulence and biofilm formation. *Curr. Opin. Microbiol.* **10**, 17–23.
- Dadjour, M. F., Ogino, C., Matsumura, S., Nakamura, S. & Shimizu, N. 2006 Disinfection of *Legionella pneumophila* by ultrasonic treatment with TiO₂. *Water Res.* **40**, 1137–1142.
- Davies, D. H., Hill, E. C., Howells, C. H. & Ribeiro, C. D. 1985 *Legionella pneumophila* in coal miners. *Br. J. Ind. Med.* **42**, 421–425.
- Dennis, P. J. & Lee, J. V. 1988 Differences in aerosol survival between pathogenic and non-pathogenic strains of *Legionella pneumophila* serogroup 1. *J. Appl. Bacteriol.* **65**, 135–141.
- Donlan, R. M. & Costerton, J. W. 2002 Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15**, 167–193.
- Dowling, J. N., Saha, A. K. & Glew, R. H. 1992 Virulence factors of the family Legionellaceae. *Microbiol. Rev.* **56**, 32–60.
- Edelstein, P. H. 1981 Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J. Clin. Microbiol.* **14**, 298–303.
- Fields, B. S. 1996 The molecular ecology of Legionellae. *Trends Microbiol.* **4**, 286–290.
- Garcia, C., Ugalde, E., Campo, A. B., Minambres, E. & Kovacs, N. 2004 Fatal case of community-acquired pneumonia caused by *Legionella longbeachae* in a patient with systemic lupus erythematosus. *Eur. J. Clin. Microbiol. Infect. Dis.* **23**, 116–118.
- Harb, O. S., Gao, L. Y. & Abu Kwaik, Y. 2000 From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. *Environ. Microbiol.* **2**, 251–265.
- Helloin, E., Jansch, L. & Phan-Thanh, L. 2003 Carbon starvation survival of *Listeria monocytogenes* in planktonic state and in biofilm: a proteomic study. *Proteomics* **3**, 2052–2064.
- Hindre, T., Bruggemann, H., Buchrieser, C. & Hechard, Y. 2008 Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. *Microbiology* **154**, 30–41.
- James, B. W., Mauchline, W. S., Fitzgeorge, R. B., Dennis, P. J. & Keevil, C. W. 1995 Influence of iron-limited continuous culture on physiology and virulence of *Legionella pneumophila*. *Infect. Immun.* **63**, 4224–4230.
- Jarraud, S., Reyrolle, M., Meugnier, H., Forey, F. & Etienne, J. 2007 Legionnaires disease. *Presse Med.* **36**, 279–287.
- Katz, S. M., Hashemi, S., Brown, K. R., Habib, W. A. & Hammel, J. M. 1984 Pleomorphism of *Legionella pneumophila*. *Ultrastruct. Pathol.* **6**, 117–129.
- Khemiri, A., Jouenne, T. & Cosette, P. 2008a Presence in *Legionella pneumophila* of a mammalian-like mitochondrial permeability transition pore? *FEMS Microbiol. Lett.* **278**, 171–176.
- Khemiri, A., Galland, A., Vaudry, D., Chan Tchi Song, P., Vaudry, H., Jouenne, T. & Cosette, P. 2008b Outer-membrane proteomic maps and surface-exposed proteins of *Legionella pneumophila* using cellular fractionation and fluorescent labelling. *Analyt. Bioanalyt. Chem.* **390**, 1861–1871.
- Kim, M. J., Sohn, J. W., Park, D. W., Park, S. C. & Chun, B. C. 2003 Characterization of a lipoprotein common to *Legionella* species as a urinary broad-spectrum antigen for diagnosis of Legionnaires' disease. *J. Clin. Microbiol.* **41**, 2974–2979.
- Kustu, S., Santero, E., Keener, J., Popham, D. & Weiss, D. 1989 Expression of sigma 54 (ntrA)-dependent genes is probably

- united by a common mechanism. *Microbiol. Rev.* **53**, 367–376.
- LeBlanc, J. J., Davidson, R. J. & Hoffman, P. S. 2006 Compensatory functions of two alkyl hydroperoxide reductases in the oxidative defense system of *Legionella pneumophila*. *J. Bacteriol.* **188**, 6235–6244.
- Lo, I., Deneff, V. J., Verberkmoes, N. C., Shah, M. B., Goltsman, D., DiBartolo, G., Tyson, G. W., Allen, E. E., Ram, R. J., Detter, J. C., Richardson, P., Thelen, M. P., Hettich, R. L. & Banfield, J. F. 2007 Strain-resolved community proteomics reveals recombining genomes of acidophilic bacteria. *Nature* **446**, 537–541.
- Mauchline, W. S., Araujo, R., Wait, R., Dowsett, A. B., Dennis, P. J. & Keevil, C. W. 1992 Physiology and morphology of *Legionella pneumophila* in continuous culture at low oxygen concentration. *J. Gen. Microbiol.* **138**, 2371–2380.
- Mikkelsen, H., Duck, Z., Lilley, K. S. & Welch, M. 2007 Interrelationships between colonies, biofilms, and planktonic cells of *Pseudomonas aeruginosa*. *J. Bacteriol.* **189**, 2411–2416.
- Muraca, P., Stout, J. E. & Yu, V. L. 1987 Comparative assessment of chlorine, heat, ozone, and UV light for killing *Legionella pneumophila* within a model plumbing system. *Appl. Environ. Microbiol.* **53**, 447–453.
- Murga, R., Forster, T. S., Brown, E., Pruckler, J. M., Fields, B. S. & Donlan, R. M. 2001 Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. *Microbiology* **147**, 3121–3126.
- Oosthuizen, M. C., Steyn, B., Theron, J., Cosette, P., Lindsay, D., Von Holy, A. & Brozel, V. S. 2002 Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. *Appl. Environ. Microbiol.* **68**, 2770–2780.
- Orme, R., Douglas, C. W., Rimmer, S. & Webb, M. 2006 Proteomic analysis of *Escherichia coli* biofilms reveals the overexpression of the outer membrane protein OmpA. *Proteomics* **6**, 4269–4277.
- Panmanee, W., Gomez, F., Witte, D., Pancholi, V., Britigan, B. E. & Hassett, D. J. 2008 The peptidoglycan-associated lipoprotein OprL helps protect a *Pseudomonas aeruginosa* mutant devoid of the transactivator OxyR from hydrogen peroxide-mediated killing during planktonic and biofilm culture. *J. Bacteriol.* **190**, 3658–3669.
- Pastoris, M. C., Berchicci, C. & Pallonari, G. 1992 Isolation of *Legionella pneumophila* serogroup 14 from a human source. *J. Clin. Pathol.* **45**, 627–628.
- Pedersen, L. L., Radulic, M., Doric, M. & Abu Kwaik, Y. 2001 HtrA homologue of *Legionella pneumophila*: an indispensable element for intracellular infection of mammalian but not protozoan cells. *Infect. Immun.* **69**, 2569–2579.
- Piao, Z., Sze, C. C., Barysheva, O., Iida, K. & Yoshida, S. 2006 Temperature-regulated formation of mycelial mat-like biofilms by *Legionella pneumophila*. *Appl. Environ. Microbiol.* **72**, 1613–1622.
- Planchon, S., Desvaux, M., Chafsey, I., Chambon, C., Leroy, S., Hebraud, M. & Talon, R. 2009 Comparative subproteome analyses of planktonic and sessile *Staphylococcus xylosus* C2a: new insight in cell physiology of a coagulase-negative *Staphylococcus* in biofilm. *J. Proteome Res.* **8**, 1797–1809.
- Romling, U., Gomelsky, M. & Galperin, M. Y. 2005 C-di-GMP: the dawning of a novel bacterial signalling system. *Mol. Microbiol.* **57**, 629–639.
- Saldias, M. S., Lamothe, J., Wu, R. & Valvano, M. A. 2008 *Burkholderia cenocepacia* requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. *Infect. Immun.* **76**, 1059–1067.
- Sauer, K. & Camper, A. K. 2001 Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J. Bacteriol.* **183**, 6579–6589.
- Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W. & Davies, D. G. 2002 *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* **184**, 1140–1154.
- Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. 1996 Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analyt. Chem.* **68**, 850–858.
- Shin, J. H., Lee, H. W., Kim, S. M. & Kim, J. 2009 Proteomic analysis of *Acinetobacter baumannii* in biofilm and planktonic growth mode. *J. Microbiol.* **47**, 728–735.
- Steinert, M., Emody, L., Amann, R. & Hacker, J. 1997 Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. *Appl. Environ. Microbiol.* **63**, 2047–2053.
- Steinert, M., Hentschel, U. & Hacker, J. 2002 *Legionella pneumophila*: an aquatic microbe goes astray. *FEMS Microbiol. Rev.* **26**, 149–162.
- Stewart, P. S. & Costerton, J. W. 2001 Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135–138.
- Storey, J. D. & Tibshirani, R. 2003 Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA* **100**, 9440–9445.
- Stout, J. E., Yu, V. L. & Muraca, P. 1985 Isolation of *Legionella pneumophila* from the cold water of hospital ice machines: implications for origin and transmission of the organism. *Infect. Control* **6**, 141–146.
- Studholme, D. J. & Buck, M. 2000 Novel roles of sigmaN in small genomes. *Microbiology* **146**, 4–5.
- Thompson, L. S., Webb, J. S., Rice, S. A. & Kjelleberg, S. 2003 The alternative sigma factor RpoN regulates the quorum-sensing gene rhII in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **220**, 187–195.
- Tremoulet, F., Duche, O., Namane, A., Martinie, B. & Labadie, J. C. 2002 Comparison of protein patterns of *Listeria monocytogenes* grown in biofilm or in planktonic mode by proteomic analysis. *FEMS Microbiol. Lett.* **210**, 25–31.
- Triassi, M., Di Popolo, A., Ribera D'Alcala, G., Albanese, Z., Cuccurullo, S., Montegrosso, S., Crispino, M., Borella, P. & Zarrilli, R. 2006 Clinical and environmental distribution of *Legionella pneumophila* in a university hospital in Italy: efficacy of ultraviolet disinfection. *J. Hosp. Infect.* **62**, 494–501.
- Turetgen, I., Sungur, E. I. & Cotuk, A. 2005 Enumeration of *Legionella pneumophila* in cooling tower water systems. *Environ. Monit. Assess.* **100**, 53–58.

- van Alen, T., Claus, H., Zahedi, R. P., Groh, J., Blazyca, H., Lappann, M., Sickmann, A. & Vogel, U. 2010 Comparative proteomic analysis of biofilm and planktonic cells of *Neisseria meningitidis*. *Proteomics* **10**, 4512–4521.
- Vilain, S., Cosette, P., Zimmerlin, I., Dupont, J. P., Junter, G. A. & Jouenne, T. 2004a Biofilm proteome: homogeneity or versatility? *J. Proteome Res.* **3**, 132–136.
- Vilain, S., Cosette, P., Hubert, M., Lange, C., Junter, G. A. & Jouenne, T. 2004b Comparative proteomic analysis of planktonic and immobilized *Pseudomonas aeruginosa* cells: a multivariate statistical approach. *Analyt. Biochem.* **329**, 120–130.
- von Mering, C., Jensen, L. J., Kuhn, M., Chaffron, S., Doerks, T., Kruger, B., Snel, B. & Bork, P. 2007 STRING 7 – recent developments in the integration and prediction of protein interactions. *Nucleic Acids Res.* **35**, D358–D362.
- Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S. & Greenberg, E. P. 2001 Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**, 860–864.
- Wingender, J. & Flemming, H. C. 2004 Contamination potential of drinking water distribution network biofilms. *Water Sci. Technol.* **49**, 277–286.
- Wolfe, A. J., Millikan, D. S., Campbell, J. M. & Visick, K. L. 2004 *Vibrio fischeri* sigma54 controls motility, biofilm formation, luminescence, and colonization. *Appl. Environ. Microbiol.* **70**, 2520–2524.
- Yamamoto, H. 1992 Detection and identification of *Legionella* species by PCR. *Nippon Rinsho* **50** Suppl., 394–399.

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