Analysis of the transcriptome of *Legionella pneumophila* *hfq* mutant reveals a new mobile genetic element

Hana Trigui,¹ Paulina Dudyk,¹ Janet Sum,² Howard A. Shuman^{2†} and Sebastien P. Faucher¹

Correspondence

Sebastien P. Faucher
sebastien.faucher2@mcgill.ca

¹Department of Natural Resource Sciences, Faculty of Agricultural and Environmental Sciences, McGill University, Ste-Anne-de-Bellevue, QC, Canada

²Department of Microbiology and Immunology, Columbia University Medical Center, New York, NY, USA

Hfq is a small RNA-binding protein involved in the post-transcriptional regulation of gene expression by affecting the stability of the mRNA and by mediating efficient pairing between small regulatory RNAs and their target mRNAs. In *Legionella pneumophila*, the aetiological agent of Legionnaires' disease, mutation of *hfq* results in increased duration of the lag phase and reduced growth in low-iron medium. In an effort to uncover genes potentially regulated by Hfq, the transcriptome of an *hfq* mutant strain was compared to that of the wild-type. Unexpectedly, many genes located within a 100 kb genomic island, including a section of the previously identified efflux island, were overexpressed in the *hfq* mutant strain. Since this island contains a putative conjugative system and an integrase, it was postulated that it could be a new integrated mobile genetic element. PCR analysis revealed that this region exists both as an integrated and as an episomal form in the cell population and that it undergoes differential excision in the *hfq* mutant background, which was further confirmed by *trans*-complementation of the *hfq* mutation. This new plasmid-like element was named pLP100. Differential excision did not affect the copy number of pLP100 at the population level. This region contains a copper efflux pump encoded by *copA*, and increased resistance to copper was observed for the *hfq* mutant strain that was abrogated in the complemented strain. A strain carrying a mutation of *hfq* and a deletion of the right side recombination site, *attR*, showed that overexpression of pLP100 genes and increased copper resistance in the *hfq* mutant strain were dependent upon excision of pLP100.

Received 2 April 2013

Accepted 2 May 2013

INTRODUCTION

Legionella pneumophila is a water-borne pathogen that can be found in almost any natural or man-made water system where it infects and replicates inside protozoa. Following inhalation of contaminated water droplets by humans, *L. pneumophila* infects and replicates within lung alveolar macrophages. The Icm/Dot type IVb secretion system is essential for intracellular multiplication in host cells, and a number of its substrates were shown to modify host cell properties to the benefit of intracellular *L. pneumophila* (reviewed by Franco *et al.*, 2009).

To date, eight strains of *L. pneumophila* have been sequenced: Philadelphia-1 (Chien *et al.*, 2004), Paris

(Cazalet *et al.*, 2004), Lens (Cazalet *et al.*, 2004), Alcoy (D'Auria *et al.*, 2010), Corby (Glöckner *et al.*, 2008), Lorraine (Gomez-Valero *et al.*, 2011), HL 0604 (Gomez-Valero *et al.*, 2011) and 570-CO-H (Amaro *et al.*, 2012). Detailed analyses of the pangenome of *L. pneumophila*, excluding the genome of strain 570-CO-H, which was only recently published, revealed that the genomes are highly syntenic and contain a core genome consisting of ~2000 genes (D'Auria *et al.*, 2010; Gomez-Valero *et al.*, 2011). In addition, it was reported that most of the genes shared by only a subset of the strains are found on genomic islands, probably acquired by horizontal gene transfer events. Bacteriophages, plasmids and other mobile genetic elements, as well as natural competence are key players in genome plasticity (Dagan *et al.*, 2008). Several plasmid-like elements have been described in *L. pneumophila*: LpPI-1, a 65 kb island encoding a type IV secretion system homologous to the Tra proteins of the F-plasmid of *E. coli* (Brassinga *et al.*, 2003); the 30 kb unstable genetic

[†]Present address: Department of Microbiology, Cummings Life Sciences Center, University of Chicago, 920 E. 58th Street, Chicago, IL 60637, USA.

Abbreviations: ICE, integrative and conjugative elements; sRNA, small regulatory RNA.

element responsible for LPS phase variation (Lüneberg *et al.*, 2001); Trb-1/Trb-2 conjugation systems in Corby (Glöckner *et al.*, 2008) and pLP45 and pP36, encoding the *lvh/lvr* type IV secretion system in the Philadelphia-1 and Paris strains respectively (Doléans-Jordheim *et al.*, 2006; Segal *et al.*, 1999). A 100 kb 'efflux island' region containing several ORFs encoding putative efflux systems was identified in Philadelphia-1 (Chien *et al.*, 2004), and a recent study reported that this region is not involved in intracellular multiplication, but that one of the genes (*copA*) confers resistance to CuCl₂ (Kim *et al.*, 2009). The published borders of the efflux island were not confirmed experimentally and, to our knowledge, its mobility has not been previously investigated. Plasmid-like elements sometimes encode conjugative systems, which allow for their transfer to a recipient cell, after excision from the chromosome (reviewed by Wozniak & Waldor, 2010). Such genomic elements are referred to as integrative and conjugative elements (ICEs).

There are a number of gene products known to be involved in the regulation of growth, virulence and response to stressful conditions in *L. pneumophila*, including proteins, such as PmrA, RpoS, CpxR, LetA/S, CsrA (Altman & Segal, 2008; Gal-Mor & Segal, 2003; Hovel-Miner *et al.*, 2009; Molofsky & Swanson, 2003; Zusman *et al.*, 2007), and small regulatory RNAs (sRNAs), such as RsmYZ and 6S RNA (Faucher *et al.*, 2010; Hovel-Miner *et al.*, 2009; Rasis & Segal, 2009; Sahr *et al.*, 2009). The small RNA-binding protein Hfq is often involved in sRNA-mediated regulation of gene expression, but is not always essential (Faucher & Shuman, 2011). Hfq was first identified in *E. coli* as a required host factor for the replication of the RNA phage Q β (Franze de Fernandez *et al.*, 1968). Published evidence supports a model where Hfq acts as a scaffold to facilitate the interaction between a sRNA and its target mRNA (Aiba, 2007). In *L. pneumophila*, an *hfq* deletion mutant strain shows an increase in the duration of the lag phase after dilution of the culture into fresh broth, as well as a growth defect when cultured in a low-iron medium (McNealy *et al.*, 2005). The latter phenotype seems to be due to the reduced expression of the ferric uptake regulator (Fur) in the *hfq* mutant strain (McNealy *et al.*, 2005). Since Hfq-dependent regulation is mediated, at least in part, through sRNA, we hypothesized that the study of the transcriptome of the *hfq* mutant strain could help identify genes potentially regulated by sRNA.

The initial goal of this study was to decipher the transcriptomic impact of the absence of Hfq. Microarray analysis of the transcriptome of the *hfq* mutant during post-exponential phase revealed that many genes located within a large genomic island, comprising a section of the putative 100 kb efflux island, are overexpressed in the *hfq* mutant strain compared to the wild-type. We hypothesize that this island could be a mobile genetic element undergoing excision in an Hfq-dependent manner. In the present study, we provide evidence that this genomic island can be excised from the chromosome and that Hfq regulates the excision of this new genetic element.

METHODS

Media and antibiotics. Media and antibiotics were used as previously described (Sadosky *et al.*, 1993). *L. pneumophila* was grown on BCYE (buffered charcoal yeast extract) plates and in AYE (ACES buffer yeast extract) broth. Liquid cultures were started with an inoculum corresponding to OD₆₀₀ 0.1 and grown with constant agitation at 37 °C. Exponential phase cultures were harvested at an OD₆₀₀ of approximately 0.7. Post-exponential phase cultures were harvested an OD₆₀₀ between 3.5 and 4.0, 6 h after the cessation of growth. Antibiotics and supplements were used at the following concentrations: kanamycin, 50 µg ml⁻¹; chloramphenicol, 5 µg ml⁻¹; gentamicin, 10 µg ml⁻¹; IPTG, 1 mM. *E. coli* strains were grown on LB plates and in LB broth. Chloramphenicol was added to a final concentration of 25 µg ml⁻¹.

Bacterial strains and construction of mutant strains. All the strains used in this study are derivatives of JR32, a streptomycin-resistant, restriction negative mutant of *L. pneumophila* strain Philadelphia-1 (Sadosky *et al.*, 1993) and are described in Table 1. The *hfq* mutant strain was isolated from a bank of transposon insertion mutants constructed with Tn903dII*lacZ* in JR32 (Sadosky *et al.*, 1993). The *hfq* mutation was moved to the competent strain KS79 as previously described (Charpentier *et al.*, 2008). KS79 is used as the control strain for all experiments and will be hereafter referred to as the wild-type strain. The plasmid used for complementation of the *hfq* mutation, ptacHFQ, was constructed by cloning the *hfq* gene in pMMB207c (Morales *et al.*, 1991). The *hfq* gene was first amplified by PCR using *Taq* polymerase (Life Technologies), and primers hfqF and hfqR, which contain *Xba*I restriction sites (underlined in Table 2). The *hfq* amplicon and pMMB207c were digested with *Xba*I (NEB) and the plasmid was further treated with calf intestinal phosphatase (NEB). The fragments were ligated using T4 DNA ligase (NEB) and transformed into *E. coli* DH5 α . Clones were screened for correct orientation of the insert by PCR using primers pMMB-Prom-F (Table 2) and hfqR. The resulting plasmid, ptacHFQ was transformed into the *hfq* mutant strain by electroporation as previously described (Chen *et al.*, 2006). Mutation of *copA* and *attR* was performed as previously described (Hovel-Miner *et al.*, 2009), using primers described in Table 2. Briefly, primers 1,2 and 5,6 were used to amplify a 1 kb fragment homologous to the upstream and downstream region of the target gene respectively. Primers 3,4 were used to amplify a gentamicin-resistance cassette from plasmid pBBR1MCS-5 (Kovach *et al.*, 1995). The three fragments were joined together by long-flanking homology PCR to create an allelic exchange fragment that was then introduced into KS79 as described above. The *copA* and *attR* mutations were also moved to the *hfq* mutant strains as described above. Allelic exchange was confirmed by PCR.

Transcriptomic study by microarray. The wild-type and the *hfq* mutant strains were grown in AYE broth under constant agitation at 37 °C. Cells were harvested when the post-exponential phase was reached, approximately 6 h after the cessation of growth. One millilitre of culture was pelleted by centrifugation, resuspended in 40 µl Tris-EDTA buffer and lysed with the addition of 1 ml TRIzol reagent. RNA extraction was performed with TRIzol reagent according to the manufacturer's protocol. The RNA was subsequently treated with Turbo DNase (Ambion) and purified by phenol/chloroform extraction. The purity and concentration of RNA were determined by UV spectrophotometry and its integrity was confirmed on a formaldehyde-agarose gel. Fifteen micrograms of RNA was labelled with amino-allyl dUTP (Sigma) during reverse transcription (Superscript II, Life Technologies) using random hexamers (Life Technologies) as previously described (Faucher & Shuman, 2012; Hovel-Miner *et al.*, 2009). Genomic DNA was used as a reference channel and was labelled by random priming using Klenow fragments, amino-allyl dUTP and random primers as described

Table 1. Strains used in this study

Name	Relevant genotype	Reference
<i>Legionella pneumophila</i>		
JR32	Philadelphia-1; Sm ^r ; r ⁻ m ⁺	Sadosky <i>et al.</i> (1993)
KS79 (WT)	JR32 Δ comR	de Felipe <i>et al.</i> (2008)
Hfq-FW	JR32 <i>hfq</i> ::Tn903dIIIacZ, Kn ^r	Sadosky <i>et al.</i> (1993)
SPF185 (<i>hfq</i>)	KS79 <i>hfq</i> ::Tn903dIIIacZ, Kn ^r	This work
SPF186 (<i>copA</i>)	KS79 <i>copA</i> :: <i>aacC1</i> , Gm ^r	This work
SPF187 (<i>attR</i>)	KS79 <i>attR</i> :: <i>aacC1</i> , Gm ^r	This work
SPF188 (<i>attR</i> , <i>copA</i>)	SPF187 <i>copA</i> :: <i>aacC1</i> , Kn ^r Gm ^r	This work
SPF189 (<i>hfq</i> , <i>attR</i>)	SPF185 <i>attR</i> :: <i>aacC1</i> , Kn ^r Gm ^r	This work
SPF190 (<i>hfq</i> + ptacHFQ)	SPF185 + pSF35	This work
Plasmid		
pMMB207c	Δ mobA, Cm ^r	Charpentier <i>et al.</i> (2008)
pSF35 (ptacHFQ)	<i>hfq</i> in <i>XbaI</i> site of pMMB207c	This work

previously (Faucher *et al.*, 2006). DNA was subsequently coupled to the succinimidyl ester fluorescent dye (Life Technologies) AlexaFluor 546 (for cDNA) or AlexaFluor 647 (for gDNA) following the

manufacturer's protocols. The microarray used and the protocol for hybridization, data acquisition and data analysis have been published elsewhere (Faucher *et al.*, 2011; Faucher & Shuman, 2012;

Table 2. Primers used in this study

XbaI restriction sites are underlined.

Name	Sequence
hfqF	GCTCTAGACAGTAGCCTGATTCTAGATGTG
hfqR	GCTCTAGAAAGGCGATAGCTCATGATTGAC
pMMB-Prom-F	CGTATAATGTGTGGAATTGTGAG
copA-1	GTTTAAACCAGCCGGATGAAA
copA-2	ATTCAGGTTTCATCATGCCGTTGGTTTGCCCTACCTGTTAAGT
copA-3	ACTTAACAGGTAGGGCAAACCAACGGCATGATGAACCTGAAT
copA-4	CTCATCTGACCATCCTTTTTTTTAGGTGGCGGTACTTGGGTC
copA-5	GACCCAAGTACCGCCACCTAAAAAAAAGGATGGTCAGATGAG
copA-6	CGTGTGATGGGATGGTTTGT
attR-1	GATTCGCGATCAAAAAGGAG
attR-2	ATTCAGGTTTCATCATGCCGTTTAAATAGCTCATGAAGCTGTTG
attR-3	CAACAGCTTCATGAGCTATTAACGGCATGATGAACCTGAAT
attR-4	CCCAAATACGGGGGCATTTTATTAGGTGGCGGTACTTGGGTC
attR-5	ACCCAAGTACCGCCACCTAATAAAATGCCCCGTATTTGG
attR-6	TCAGACATTTGGCTTTTATCG
copA-qF	CAATACCCTGGTGGTCGATAAAAC
copA-qR	TGCCGCAGCTAATGCTAAAG
int-qF	TCCTGAATGGACTGCCCATGACTT
int-qR	AATCCAATGCCTCTTTGCGTTGGG
sodB-qF	ACAGCTGCTACAACATTCGGTTCC
sodB-qR	ATAAGCGTGCTCCCATACGTCACA
lpg1613-qF	CCATTCTGGTTGATGCATTTC
lpg1613-qR	CTTTCGCTGTTCTCGTAATA
tnpR	CTTCCAACAACAGCTTCATGAG
intF	TGCCCAGGAATCCAATGCCTC
0973R	TATTGATGCTCTAACATGATTACC
0971F	GGACCTTAGGAGTGGTATTGC
site2F	AGTTACACGGATGCCAAAGCC
site2R	CCTTCTATAAGCGCATCCTGC
rpoSF	ACAGAATTCGCTTGTTAAGAAGTAAAAAACTA
rpoSR	CGCTCTAGATCAAAACAAATCCTCTTGTGT

Hovel-Miner *et al.*, 2009). Three biological replicates were used for each strain. Statistical analysis between the *hfq* mutant strain and the wild-type control was performed using an unpaired one-tailed Student's *t*-test. Genes were considered differentially expressed if they demonstrated a ratio-to-control value of ± 2 -fold with a $P < 0.005$. The microarray data are available from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE42905.

Analysis of insertion/excision by PCR. Genomic DNA was extracted with the QIAamp DNA kit (Qiagen) and the integrity was determined by electrophoresis on agarose gel. The concentration and purity were determined by UV spectrophotometry. PCR was performed with *Taq* DNA polymerase (Life Technologies) on 10 ng of genomic DNA in a 50 μ l reaction following the manufacturer's recommendations. Primers are described in Table 2. Amplification of the *rpoS* gene was performed to ensure that a similar quantity of gDNA was used for all samples. PCR amplicons were visualized on 0.7% agarose gels in Tris/acetate EDTA buffer.

DNA sequencing. Sequencing of the *attP* and *attB* sites was performed by Sanger sequencing at the Plate-forme d'Analyses Génomiques of Laval University, Québec City, Québec, Canada.

Quantitative PCR. To quantify the copy number of pLP100, genomic DNA was extracted from strains grown to post-exponential phase as described above. The qPCR was then carried out on 10 ng of gDNA using the iTaq Universal SYBR Green Supermix (Bio-Rad). For analysis of gene expression by qPCR, RNA was extracted from strains grown to post-exponential phase as described above for microarray experiments. One microgram of RNA was then converted to cDNA by using random primers and superscript II (Life Technologies) following the manufacturer's instructions. For each sample, a no reverse-transcriptase reaction was carried out to serve as a negative control. qPCRs were then performed with 1 μ l of cDNA using the iTaq Universal SYBR Green Supermix (Bio-Rad). Primers are described in Table 2. A relative quantification strategy was used to perform analysis of the qPCR data. The calculated threshold cycle was normalized to the value of the internal control 16S rRNA amplified from the corresponding samples and the fold-change was calculated as previously described (Livak & Schmittgen, 2001).

Susceptibility to CuCl₂. Bacterial strains were grown to post-exponential phase in AYE broth under constant agitation. CuCl₂ was then added to a final concentration of 4 mM (Kim *et al.*, 2009). After an exposure time of 8 h, c.f.u. were determined by serial dilution and plating on BCYE plates. The values represent the c.f.u. of the treated culture divided by the c.f.u. of the untreated culture.

RESULTS

Transcriptome of the *hfq* mutant strain

The analysis of the transcriptome of the *hfq* mutant strain grown to post-exponential phase reveals a large region of overexpressed genes compared to the wild-type strain (Fig. 1a). Most of the 98 genes in this region, bordered by *lpg0973* and *lpg1070*, show a high expression ratio (average = 3.26, SD = 1.14). The expression of three genes (*lpg0985*, *lpg0992* and *lpg0995*) is unchanged while *lpg1039*, coding for a small hypothetical protein, shows reduced expression (−2.38). The %GC content of this region (41%) is somewhat higher than the average of the whole genome (38%) (Fig. 1b). A tRNA (*tRNA^{thr}*, *lpg0972*) is encoded upstream of the overexpressed region, while an

integrase (*int*, *lpg1070*) and a putative phage protein (*lpg1069*) are encoded at the far end (Fig. 1c). A transposase is encoded by *lpg1071* (*tnp*), but its expression was not affected by the *hfq* mutation. Moreover, a putative conjugation system could be encoded from *lpg1005* to *lpg0983* in the reverse direction (Fig. 1d). It is important to note that an efflux island was previously delimited arbitrarily by genes *lpg1006* and *lpg1096* and that its mobility was not investigated (Chien *et al.*, 2004; Kim *et al.*, 2009). Hence, we hypothesized that the region delimited by *lpg0973* and *lpg1070* represents a mobile genetic element whose expression is upregulated by its excision from the chromosome. Therefore, the first goal was to investigate the excision and circularization of this presumed genetic element.

Circularization of the overexpressed region

We used PCR amplification to investigate the possibility that the overexpressed region could be excised from the genome of *L. pneumophila* and exist as an episome. We designed primers (*intF*/*tnpR*) encompassing the right side junction region between *int* (*lpg1070*) and *tnp* (*lpg1071*) to investigate the presence of the integrated form (Fig. 1c). The integrated form was amplified with similar efficiency from the wild-type and *hfq* mutant grown to exponential and post-exponential phase (Fig. 2a). Primers 0973R/*intF* were used to amplify the junction between *lpg0973* and *int* that presumably exists in the episome to investigate the presence of the episomal form (Fig. 1c). The episomal form was also amplified from both strains grown to exponential phase with similar efficiency (Fig. 2b, E phase); however, this region was not amplified effectively from the wild-type strain grown to post-exponential phase in contrast to the *hfq* mutant strain (Fig. 2b, PE phase). By increasing the amount of gDNA from 10 ng to 20 ng, the episomal form was amplified from the wild-type grown to post-exponential phase, although the signal was definitively lower than the amplification from the *hfq* mutant strain (Fig. 2b; 20 ng, lanes 1 and 2). Thus, this genomic region seems to be an integrated element that undergoes excision and exists as an episome in the cell. Given its size of 100 kb, this element was named pLP100 (Fig. 1d). A similar PCR amplification of the episomal junction region from the wild-type and *hfq* mutant strains during exponential phase indicates a similar amount or frequency of the episomal form. In contrast, the reduced amplification observed in the wild-type strain compared to the *hfq* mutant strain during post-exponential phase reflects a lower amount or frequency of this episomal form. Therefore, we hypothesized that Hfq is required to reduce the excision rate of pLP100 when the culture reaches post-exponential phase.

Hfq affects the excision/integration of pLP100

To confirm the regulation of the excision/integration of pLP100 by Hfq, the *hfq* mutant strain was complemented by transformation of a plasmid (ptacHFQ) carrying the *hfq*

Fig. 1. Genomic localization of pLP100. (a) The $\log_2$ ratio of gene expression in the *hfq* mutant strain compared to the wild-type JR32 strain is plotted against the genes coordinate. The region denoted by A refers to the 100 kb region, hereafter referred to as pLP100, showing higher expression in the *hfq* mutant strain, and the region denoted by B represents the 65 kb LpPI-1 locus absent in the JR32 strain. (b) Shows the %GC content of the *L. pneumophila* genome: outside, higher than average; inside, lower than average. The overexpressed region, identified in panel (a), is marked by a thick grey line (bp 1 067 616–1 167 117). (c) Schematic representation of the integration/excision of pLP100 in the chromosome of *L. pneumophila* strain Philadelphia-1. The primers used in this study are 0971F, 0973R, intF and tnpR (from left to right) and are depicted by black arrows. The *att* sites are shown in grey. The gene organization of pLP100 is shown in panel (d). The circles, from outside to inside, represent: (1) ORF encoded in the forward direction, (2) ORF encoded in the reverse direction, (3) position of the *int* gene (box), *attP* (vertical line) and the putative conjugative machinery (arrow) and (4) %GC content (outside, higher than average; inside, lower than average).

gene under the transcriptional control of the inducible *Ptac* promoter. Strains were grown to post-exponential phase and PCR was used to investigate the presence of the integrated form (IN), the episomal form (OUT), and the empty chromosome (EMPTY) as shown in Fig. 2. Amplification of the gene *rpoS* located outside the region was used as a control. According to Fig. 2c, the very high signal detected indicates that the integrated form seems to be predominant in the cell population during the post-exponential phase, regardless of the strain tested.

Amplification of the episomal form resulted in a higher signal in the *hfq* mutant strain than in the wild-type strain and was abrogated in the complemented strain grown with IPTG. The amplification of the episomal form from the complemented strain grown without IPTG resulted in a higher signal than in the wild-type strain. This is probably because of partial complementation due to leakage of the *Ptac* promoter (Morales *et al.*, 1991). Amplification of the empty site in the chromosome was stronger in the *hfq* mutant strain and in the complemented strain grown

Fig. 2. Differential excision of pLP100 in the *hfq* mutant strain. PCR amplification was used to investigate the presence of (a) the integrated form (IN) and (b) the excised form (OUT). (b) Shows an additional PCR using 20 ng of DNA from PE. (c) A complementation study was performed to validate the phenotype of the *hfq* mutant strain in the post-exponential phase. *L. pneumophila* strains were grown to exponential (E) and post-exponential phase (PE) and DNA was extracted. PCR was performed on 10 ng of DNA using primers intF/tnpR to amplify the integrated form (IN), intF/0973R to amplify the excised form (OUT) or 0971F/tnpR to amplify the empty chromosome (EMPTY). Amplification of *rpoS*, located outside pLP100, was used as a control to show that an equal amount of gDNA was used for each strain tested. Samples are as follows: N, negative control; 1, wild-type; 2, *hfq* mutant strain; 3, *hfq*+ptachFQ ON (grown with 1 mM IPTG); 4, *hfq*+ptachFQ OFF (grown without IPTG); L, 1 kb Plus DNA ladder (Life Technologies).

without IPTG than in the complemented strain induced with IPTG, thus confirming the excision of pLP100 from the chromosome. Taken together, these results strongly

support the hypothesis that Hfq represses the excision of pLP100 from the chromosome, at least during the post-exponential phase of growth.

Sequence of the *att* sites

The junction region in the episomal form of pLP100 was sequenced to precisely identify the site of excision and recombination. Primers intF and 0973R were used to amplify a fragment from the DNA of the *hfq* mutant strain grown to post-exponential phase. The sequence of the resulting fragment was determined in six different reactions, half using the primer intF and the other half using primer 0973R. The sequence contained a fragment homologous to bp 1 167 047 to 1 167 343 located between *int* and *tnp* genes and a fragment homologous to bp 1 067 101 to 1 067 256 comprising 42 bp of *lpg0972* (*tRNA^{thr}*) and 113 bp upstream of *lpg0973* (Fig. 1c). The two homologous sequences are overlapping by 56 bp, which is likely to be the recombination site. Thus, the episomal form is composed of bp 1 067 101 to 1 167 287.

The sequence of the junction in the empty chromosome was also determined by using primers 0971F and tnpR as described above for the episomal junction region. The sequence contains a complete *tRNA^{thr}* gene followed by 103 bp downstream of *tnp* (*lpg1071*). Analysis of the sequenced junction regions, and the sequences to the left and right side junction region of the integrated episome derived from the published genome, revealed a fragment containing 42 nucleotides of the 3'-end of the tRNA and 14 nt downstream of it, delimited by bp 1 067 101 to 1 067 156, that was found in all sequenced junction regions and represents the *att* sites of recombination of the episome. The site found in the plasmid was named *attP*, the site in the empty chromosome, *attB* and the sites flanking the integrated form, *attL* and *attR* (Fig. 1c).

pLP100 does not integrate in an alternative integration site

BLAST analysis was used to search the genome for alternative insertion sites. Only one putative additional site for insertion of pLP100 was found, which is hereafter referred to as Site 2. It is located between bp 1 181 696 and 1 181 750, 15 kb downstream of the *attR* site, and has 87.5% shared identity with the *att* sequence. This site is located upstream of a putative phage integrase in a region that also contains a reverse transcriptase. PCR was used to investigate a possible integration of pLP100 into Site 2 (data not shown). Amplification of the empty Site 2 resulted in a strong band; however, amplification of the putative integrated form, using primers site2F/0973R did not produce any amplicon. Therefore, it seems that Site 2 is not an integration site and if it is occupied at all, the frequency is not high enough to allow detection by PCR.

Excision of pLP100 does not affect its copy number

Self-replication of pLP100 after excision could lead to overexpression of the genes it encodes by a mechanism of gene dosage. We used a qPCR approach to evaluate the copy number of two genes encoded in pLP100, *copA* and *int*, and one gene encoded outside of it, *sodB*. No change in copy number was detected by qPCR in the *hfq* mutant strain compared to the wild-type (Fig. 3). Therefore, it is unlikely that gene dosage is the mechanism that causes overexpression of the pLP100 genes.

Overexpression is genetically linked to excision of pLP100

At this point, our hypothesis was that the excision of pLP100 in the *hfq* mutant strain causes overexpression of the genes it encodes. Therefore, a mutation that would prevent pLP100 excision would restore wild-type level expression of the pLP100 genes in an *hfq* mutant background. To test this hypothesis, we constructed an *attR* mutant in which the *attR* site was replaced with a gentamicin-resistance cassette. In this mutant, pLP100 seems to remain in its integrated form because no episomal form could be detected (Fig. 4a, lane 5). We also constructed a mutant strain carrying both *hfq* and *attR* mutations in which pLP100 is also locked in the chromosome (Fig. 4a, lane 6). We used qPCR analysis to monitor the expression of *copA*, a previously characterized gene carried on pLP100 (Kim *et al.*, 2009). In agreement with the microarray analysis, *copA* was overexpressed in the *hfq* mutant and complementation restored the wild-type expression level (Fig. 4b). Expression of *copA* was similar in the *attR hfq* double mutant strain and in the wild-type suggesting that its overexpression in the *hfq* mutant is dependent upon excision of pLP100. This was further confirmed by a similar expression level of *copA* in the single *attR* mutant compared to the wild-type strain (Fig. 4b). Expression of *lpg1613* was used as an Hfq-independent control located outside pLP100 and showed no change in expression in the strains studied (Fig. 4c). Taken together, these observations strongly support our hypothesis that Hfq-dependent overexpression of pLP100 genes in post-exponential phase is linked with excision.

The *hfq* mutant is more resistant to CuCl₂

Since the *copA* gene encodes a copper efflux pump and is overexpressed in the *hfq* mutant strain, we hypothesized that the *hfq* mutant strain might be more resistant to copper than the wild-type strain after growth to post-exponential phase. Resistance to CuCl₂ was investigated by incubating the strains, grown to post-exponential phase, with 4 mM CuCl₂ for 8 h. Fig. 4(d) shows that the *hfq* mutant strain is significantly more resistant than its wild-type counterpart, and that the expression of *hfq* from the *P_{tac}* promoter in the *hfq* mutant strain resulted in the

Fig. 3. The copy number of pLP100 is unchanged in the *hfq* mutant strain. DNA was extracted from the wild-type, *hfq* mutant and complemented strains, grown to post-exponential phase. qPCR was used to determine the relative copy number of two genes encoded in pLP100, *int* (a) and *copA* (b), and a gene encoded outside of it, *sodB* (c). Data are presented as the mean of three replicates and the error bars represent SD.

Fig. 4. Overexpression of genes carried by pLP100 depends on excision. (a) DNA was extracted from strains grown to post-exponential phase and 20 ng of gDNA was subjected to PCR amplification as described in Fig. 2. Note that the mutant allele of *attR* carries a gentamicin-resistance cassette, which produces a larger amplicon for the integrated form than in the wild-type strain. RT-qPCR was used to investigate expression of *copA* (b), located on pLP100, and *lpg1613* (c), located outside of it and whose expression was not affected by *hfq* mutation. Data are presented as the mean of three replicates and the error bars represent the standard deviation. Since the data represent a ratio against the wild-type strain, we used the non-parametric Mann–Whitney test to assess statistical significance (*, $P \leq 0.05$). (d) *L. pneumophila* strains were grown to post-exponential phase and CuCl₂ was added to a final concentration of 4 mM. C.f.u. were determined after 8 h and the ratio to the c.f.u. in untreated cultures was calculated. The complemented strain was grown with (ON) and without (OFF) 1 mM IPTG. Data are presented as a ratio between treated and untreated control. The mean of three replicates is shown and the error bars represent sd. Since the data represent a ratio, we used the non-parametric Mann–Whitney test to assess statistical significance, compared to the wild-type (*, $P \leq 0.05$).

wild-type phenotype. The mutant strain harbouring the plasmid for complementation but not induced with IPTG was as resistant to CuCl_2 as the *hfq* mutant strain. This phenotype was dependent on *copA*, since strains carrying a *copA* mutation or mutations for both *hfq* and *copA* were most sensitive to copper (Fig. 4d). The *attR* mutant strain and the double *attR hfq* mutant strain were as sensitive as the wild-type to copper, which supports a genetic link between excision and overexpression of the genes encoded on pLP100.

DISCUSSION

This study reports the identification of a new genomic island in *L. pneumophila* that can excise itself from the chromosome and exist as an episomal form. Due to its size of 100 kb, this element was named pLP100. A conjugation system was not readily identifiable on pLP100 based on the actual annotation of the genes encoded therein. However, the left side of the insert contains a long series of genes encoded in the reverse orientation, including *lpg0983* and *lpg0989* respectively annotated as 'conjugative coupling factor TraD' and 'Type IV secretory protein VirB4 components'. Re-annotation of the neighbouring genes was performed by BLASTP analysis and revealed that many of the gene products have homology to, or possess conserved domains related to proteins involved in conjugation systems. Therefore, it seems that pLP100 contains a putative conjugation system encoded from *lpg1005* to *lpg0983* in the reverse direction (Fig. 1d). The functionality of this conjugative system is presently unknown and will require further analysis. The Icm/Dot type IVb secretion is able to mobilize plasmid RSF1010, a process that is partially dependent on the *lvh* type IV secretion system encoded by pLP45 (Segal *et al.*, 1999; Vogel *et al.*, 1998). This suggests that mobilization and transfer of pLP100 from *L. pneumophila* to other bacterial species could be mediated by other transfer systems or could partially require transfer proteins encoded on other genomic islands, if the system encoded within pLP100 is not fully functional.

Sequence analysis revealed that the *attB* integration site of pLP100 is at the 3'-end of *tRNA^{thr}* (*lpg0972*). A search for the conserved domain in the predicted integrase (*lpg1070*) encoded on pLP100 revealed a specific hit for bacteriophage P4 integrase (*E*-value of 3.76×10^{-102}). This class of integrase is known to mediate integration into the 3'-ends of tRNA genes, which is consistent with the integration site of pLP100 (Wozniak & Waldor, 2010). There are two other predicted *tRNA^{thr}* genes (*lpg0310* and *lpg0313*) annotated in the *L. pneumophila* genome but the sequence homology with *lpg0972* is weak and it is unlikely that they can serve as integration sites for pLP100. Another integration site (Site 2) was predicted by BLAST analysis, but PCR analysis did not detect integration of pLP100 into this site. This result supports the idea that the integration of pLP100 requires high sequence homology between *attP* and *attB*.

The small RNA-binding protein Hfq seems to play a role in preventing excision of pLP100 from the chromosome during the post-exponential phase since mutation of *hfq* promotes its excision, and this phenotype can be complemented *in trans* (Fig. 2c). No difference was observed between the wild-type strain and the *hfq* mutant during exponential growth (Fig. 2). In the Paris strain, excision of pP36 from the chromosome was observed only during the post-exponential phase (Doléans-Jordheim *et al.*, 2006), which suggests, together with the results presented here, that transcriptional programmes active during post-exponential phase might have an effect on the excision of integrated elements in *L. pneumophila*. Presumably, stress encountered in the post-exponential phase, such as starvation, decrease in pH or accumulation of reactive oxygen species and waste could affect excision of the ICE from the chromosome for subsequent transfer to another cell. It is known that transfer of the SXT element of *Vibrio cholerae* is increased upon induction of the SOS response by DNA-damaging agents in a RecA-dependent manner (Beaber *et al.*, 2004). In contrast to pP36, our results suggest that in the wild-type strain, pLP100 excision during exponential phase is more frequent than during post-exponential phase and that this repression of excision, when the bacteria reach post-exponential phase is Hfq-dependent. Yet another possibility is that the excision rate is not different between growth phases, but that the episomal form of pLP100 has the ability to replicate by itself more effectively during exponential growth, resulting in more copies of the genes it encodes. This second model would suggest that during the post-exponential phase, the plasmid replication rate decreases in an Hfq-dependent manner, thus resulting in an increase in the number of the episomal form in the *hfq* mutant. However, this second model is challenged by the observation that the copy number of genes encoded on pLP100 is the same in the wild-type and *hfq* mutant strains (Fig. 3). In addition, the observation that the empty chromosomal junction region is amplified more efficiently from the *hfq* mutant strain supports our first model in which Hfq regulates excision of pLP100 (Fig. 2c). Additional investigation is required to determine the exact mechanism underlying this phenotype. To our knowledge, this is the first time that Hfq involvement in the regulation of excision of an ICE has been demonstrated. However, Hfq was previously shown to regulate the transposition of IS10 by affecting the expression of the transposase (Ross *et al.*, 2010). It is possible that a similar mechanism could affect the excision/integration rate of pLP100 by affecting the expression of the integrase *int* either directly or indirectly by interacting with an unidentified sRNA. A recent study showed that the pLP45 possess a sRNA (*lpr0035*) encoded in the *attL* site that regulates a few virulence-related genes encoded within it (Jayakumar *et al.*, 2012). No sRNA was found in the *attL* site of pLP100 (Faucher *et al.*, 2010; Weissenmayer *et al.*, 2011); however, pLP100 possesses a putative sRNA, *lpr0030*, located between *lpg1009* and *lpg1010*, whose function is currently being investigated.

Two hypotheses were considered to explain the increased expression of genes encoded on pLP100 in the *hfq* mutant strain. First, a mechanism of gene dosage was proposed, but it was rejected because similar copy numbers of *copA* and *int* genes were detected in the wild-type and *hfq* mutant strains (Fig. 3). This is further supported by a similar increase in the signal of the episomal form and of the empty insertion site in the *hfq* mutant strain, suggesting that the total copy number stays the same (Fig. 2c). Second, a genetic link between excision and overexpression was considered. To test this hypothesis, we constructed strains harbouring a mutation in *attR* and mutations in both *attR* and *hfq*, where pLP100 is locked in the chromosome (Fig. 4). Overexpression of *copA*, encoded in pLP100, was observed in the *hfq* mutant strain but not in *attR* or *hfq attR* mutant strains. Increased resistance to copper in the *hfq* mutant strain, abrogated in the *hfq attR* mutant strain, validates these observations (Fig. 4d). Taken together, these observations strongly support the hypothesis that the overexpression of pLP100 genes is dependent on excision and is therefore indirectly regulated by Hfq. Since the episomal form was detected in the wild-type strains, albeit at low level, but not in the *attR* and double *attR hfq* mutant strains, one could expect that *copA* expression and resistance to copper would be lower in these strains compared to the wild-type. This is not what we observed and the reason for this discrepancy is currently unknown. One possibility is that the level of excision in the wild-type strain compared to the *attR* and double *attR hfq* mutant strains is not enough to result in a difference in gene expression at the population level.

The mechanism resulting in increased expression from the episomal form is not known, but it may be due to differences in DNA topology between the integrated and episomal forms, which can affect gene expression (Drolet, 2006). In addition, Hfq affects the degree of supercoiling of plasmid DNA in *E. coli* (Tsui *et al.*, 1994). Similarly, the overexpression of pLP100 genes could be dependent upon both differential excision and differential supercoiling in the absence of Hfq. It is unclear how the supercoiling of pLP100 can be investigated given that the episomal form is not efficiently amplified from the wild-type and is, therefore, suspected to be present only in minute amounts. There is also the possibility that overexpression of pLP100 genes correlates with the presence of the episome but is independent of excision. For example, Hfq could also regulate the expression of a transcriptional regulator of pLP100 genes; however, the regulation of all the genes on pLP100 by a single regulator seems unlikely. Tiaden *et al.* (2010) reported that the *lqs* quorum-sensing system of *L. pneumophila* regulates the genes encoded on pLP100. More precisely, deletion of the sensor kinase *lqsS* leads to overexpression of a large island (*lpg0973–lpg1096*) that includes pLP100. In contrast, deletion of the orphan sensor kinase gene *lqsT* results in the repression of the genes *lpg0973–lpg1096* (Kessler *et al.*, 2013). The authors did not investigate the excision of the island in the *lqs* mutant

strains, and it is therefore unknown if *lqs* regulates excision of this island. In our study, the expression of genes *lpg1072–lpg1096* was not affected by deletion of *hfq*, which suggests that the quorum-sensing system and Hfq affect expression of genes encoded within this region by different mechanisms.

Copper–silver ionization is a method of choice for the disinfection of hospital water systems (Lin *et al.*, 2011). The data presented here show a potential evolutionary strategy for increased resistance to copper in *L. pneumophila*. A mutation could arise that increases the amount of the excised form of pLP100 which would, in turn, increase copper resistance and allow such a mutant strain to survive better in water systems where copper–silver ionization is used as a disinfection method. This raises the possibility that copper could induce excision of pLP100; however, the transcriptome of *L. pneumophila* in response to copper nanoparticles revealed that the expression of pLP100 genes are not affected by the presence of copper (Lu *et al.*, 2013). A heavy metal resistance operon seems to have been acquired by a number of pathogenic bacterial species, and new emergent strains identified from recent outbreaks also contain heavy metal operons (Reva & Bezuidt, 2012). These operons seem to have been acquired by horizontal gene transfer events and the authors postulated that it could be the result of an increase in heavy metal pollution followed by a transfer of a mobile genetic element from environmental microflora to pathogenic micro-organisms. The acquisition of pLP100 by *L. pneumophila* could be an example of such an event. The genome comparison tool available from the BioCyc website (<http://www.biocyc.org>), BLAST analysis (<http://blast.ncbi.nlm.nih.gov>) and the Legiolist database (<http://genolist.pasteur.fr/LegioList>) were used to investigate the presence of pLP100 in other *L. pneumophila* strains. A similar island was found in strains Alcoy and Corby, neighboured by a homologue of *lpg0973* and *int*, but some genes seem to be deleted from the element. In strains Paris, Lens, 570-CO-H, HL060414, Lorraine, 130b and *Legionella longbeachae* NSW150, the putative conjugation system is present, but the rest of the island seems to have suffered from major gene reorganization, and it is unclear if the island can still excise itself from the chromosome in these strains. Therefore, it seems that this element was acquired some time in the past, before divergence of these strains as previously suggested (Tiaden *et al.*, 2010). Since then, pLP100 has undergone some genetic reorganization, which suggests that it comes from a relatively old horizontal gene transfer event.

CONCLUSION

In conclusion, we have identified the precise boundaries of pLP100, a mobile genetic element that partially contains the previously identified metal efflux island. Hfq is required for repression of its excision in post-exponential phase. Overexpression of the pLP100 genes during the post-exponential phase in the *hfq* mutant strain is

genetically linked with excision of this element from the chromosome. In addition, excision of the island results in induction of the *copA* gene, which increases resistance of *L. pneumophila* to copper. To our knowledge, this is the first time that Hfq is implicated in regulating the excision of a mobile genetic element.

ACKNOWLEDGEMENTS

This work was supported by a FRQNT Établissement de nouveaux chercheurs grant 2013-NC-164725 and an NSERC Discovery grant 418289-2012 to S.P.F., and NIH grants AI064481 and AI23549 to H.A.S. We would like to thank Nilmini Mendis for critical reading of the manuscript.

REFERENCES

- Aiba, H. (2007). Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr Opin Microbiol* **10**, 134–139.
- Altman, E. & Segal, G. (2008). The response regulator CpxR directly regulates expression of several *Legionella pneumophila* *icm/dot* components as well as new translocated substrates. *J Bacteriol* **190**, 1985–1996.
- Amaro, F., Gilbert, J. A., Owens, S., Trimble, W. & Shuman, H. A. (2012). Whole-genome sequence of the human pathogen *Legionella pneumophila* serogroup 12 strain 570-CO-H. *J Bacteriol* **194**, 1613–1614.
- Beaber, J. W., Hochhut, B. & Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**, 72–74.
- Brassinga, A. K. C., Hiltz, M. F., Sisson, G. R., Morash, M. G., Hill, N., Garduno, E., Edelstein, P. H., Garduno, R. A. & Hoffman, P. S. (2003). A 65-kilobase pathogenicity island is unique to Philadelphia-1 strains of *Legionella pneumophila*. *J Bacteriol* **185**, 4630–4637.
- Cazalet, C., Rusniok, C., Brüggemann, H., Zidane, N., Magnier, A., Ma, L., Tichit, M., Jarraud, S., Bouchier, C. & other authors (2004). Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nat Genet* **36**, 1165–1173.
- Charpentier, X., Faucher, S. P., Kalachikov, S. & Shuman, H. A. (2008). Loss of RNase R induces competence development in *Legionella pneumophila*. *J Bacteriol* **190**, 8126–8136.
- Chen, D.-Q., Huang, S.-S. & Lu, Y.-J. (2006). Efficient transformation of *Legionella pneumophila* by high-voltage electroporation. *Microbiol Res* **161**, 246–251.
- Chien, M., Morozova, I., Shi, S., Sheng, H., Chen, J., Gomez, S. M., Asamani, G., Hill, K., Nuara, J. & other authors (2004). The genomic sequence of the accidental pathogen *Legionella pneumophila*. *Science* **305**, 1966–1968.
- D'Auria, G., Jiménez-Hernández, N., Peris-Bondia, F., Moya, A. & Latorre, A. (2010). *Legionella pneumophila* pangenome reveals strain-specific virulence factors. *BMC Genomics* **11**, 181.
- Dagan, T., Artzy-Randrup, Y. & Martin, W. (2008). Modular networks and cumulative impact of lateral transfer in prokaryote genome evolution. *Proc Natl Acad Sci U S A* **105**, 10039–10044.
- de Felipe, K. S., Glover, R. T., Charpentier, X., Anderson, O. R., Reyes, M., Pericone, C. D. & Shuman, H. A. (2008). *Legionella* eukaryotic-like type IV substrates interfere with organelle trafficking. *PLoS Pathog* **4**, e1000117.
- Doléans-Jordheim, A., Akermi, M., Ginevra, C., Cazalet, C., Kay, E., Schneider, D., Buchrieser, C., Atlan, D., Vandenesch, F. & other authors (2006). Growth-phase-dependent mobility of the *lvh*-encoding region in *Legionella pneumophila* strain Paris. *Microbiology* **152**, 3561–3568.
- Drolet, M. (2006). Growth inhibition mediated by excess negative supercoiling: the interplay between transcription elongation, R-loop formation and DNA topology. *Mol Microbiol* **59**, 723–730.
- Faucher, S. P. & Shuman, H. A. (2011). Small regulatory RNA and *Legionella pneumophila*. *Front Microbiol* **2**, 98.
- Faucher, S. P. & Shuman, H. A. (2012). Methods to study *Legionella* transcriptome *in vitro* and *in vivo*. *Methods in Molecular Biology* **954**, 567–582.
- Faucher, S. P., Porwollik, S., Dozois, C. M., McClelland, M. & Daigle, F. (2006). Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. *Proc Natl Acad Sci U S A* **103**, 1906–1911.
- Faucher, S. P., Friedlander, G., Livny, J., Margalit, H. & Shuman, H. A. (2010). *Legionella pneumophila* 6S RNA optimizes intracellular multiplication. *Proc Natl Acad Sci U S A* **107**, 7533–7538.
- Faucher, S., Mueller, C. & Shuman, H. (2011). *Legionella pneumophila* transcriptome during intracellular multiplication in human macrophages **2**, 60.
- Franco, I. S., Shuman, H. A. & Charpentier, X. (2009). The perplexing functions and surprising origins of *Legionella pneumophila* type IV secretion effectors. *Cell Microbiol* **11**, 1435–1443.
- Franze de Fernandez, M. T., Eoyang, L. & August, J. T. (1968). Factor fraction required for the synthesis of bacteriophage Q β -RNA. *Nature* **219**, 588–590.
- Gal-Mor, O. & Segal, G. (2003). The *Legionella pneumophila* GacA homolog (LetA) is involved in the regulation of *icm* virulence genes and is required for intracellular multiplication in *Acanthamoeba castellanii*. *Microb Pathog* **34**, 187–194.
- Glöckner, G., Albert-Weissenberger, C., Weinmann, E., Jacobi, S., Schunder, E., Steinert, M., Hacker, J. & Heuner, K. (2008). Identification and characterization of a new conjugation/type IVA secretion system (*trb/tra*) of *Legionella pneumophila* Corby localized on two mobile genomic islands. *Int J Med Microbiol* **298**, 411–428.
- Gomez-Valero, L., Rusniok, C., Jarraud, S., Vacherie, B., Rouy, Z., Barbe, V., Medigue, C., Etienne, J. & Buchrieser, C. (2011). Extensive recombination events and horizontal gene transfer shaped the *Legionella pneumophila* genomes. *BMC Genomics* **12**, 536.
- Hovel-Miner, G., Pampou, S., Faucher, S. P., Clarke, M., Morozova, I., Morozov, P., Russo, J. J., Shuman, H. A. & Kalachikov, S. (2009). SigmaS controls multiple pathways associated with intracellular multiplication of *Legionella pneumophila*. *J Bacteriol* **191**, 2461–2473.
- Jayakumar, D., Early, J. V. & Steinman, H. M. (2012). Virulence phenotypes of *Legionella pneumophila* associated with noncoding RNA lpr0035. *Infect Immun* **80**, 4143–4153.
- Kessler, A., Schell, U., Sahr, T., Tiaden, A., Harrison, C., Buchrieser, C. & Hilbi, H. (2013). The *Legionella pneumophila* orphan sensor kinase LqsT regulates competence and pathogen-host interactions as a component of the LAI-1 circuit. *Environ Microbiol* **15**, 646–662.
- Kim, E.-H., Charpentier, X., Torres-Urquidy, O., McEvoy, M. M. & Rensing, C. (2009). The metal efflux island of *Legionella pneumophila* is not required for survival in macrophages and amoebas. *FEMS Microbiol Lett* **301**, 164–170.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.

- Lin, Y. E., Stout, J. E. & Yu, V. L. (2011). Controlling *Legionella* in hospital drinking water: an evidence-based review of disinfection methods. *Infect Control Hosp Epidemiol* **32**, 166–173.
- Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
- Lu, J., Struewing, I., Buse, H. Y., Kou, J., Shuman, H. A., Faucher, S. P. & Ashbolt, N. J. (2013). *Legionella pneumophila* transcriptional response following exposure to CuO nanoparticles. *Appl Environ Microbiol* **79**, 2713–2720.
- Lüneberg, E., Mayer, B., Daryab, N., Kooistra, O., Zähringer, U., Rohde, M., Swanson, J. & Frosch, M. (2001). Chromosomal insertion and excision of a 30 kb unstable genetic element is responsible for phase variation of lipopolysaccharide and other virulence determinants in *Legionella pneumophila*. *Mol Microbiol* **39**, 1259–1271.
- McNealy, T. L., Forsbach-Birk, V., Shi, C. & Marre, R. (2005). The Hfq homolog in *Legionella pneumophila* demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA. *J Bacteriol* **187**, 1527–1532.
- Molofsky, A. B. & Swanson, M. S. (2003). *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. *Mol Microbiol* **50**, 445–461.
- Morales, V. M., Bäckman, A. & Bagdasarian, M. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* **97**, 39–47.
- Rasis, M. & Segal, G. (2009). The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of *Legionella pneumophila* Icm/Dot effectors. *Mol Microbiol* **72**, 995–1010.
- Reva, O. & Bezuidt, O. (2012). Distribution of horizontally transferred heavy metal resistance operons in recent outbreak bacteria. *Mobile Genet Elements* **2**, 96–100.
- Ross, J. A., Wardle, S. J. & Haniford, D. B. (2010). Tn10/IS10 transposition is downregulated at the level of transposase expression by the RNA-binding protein Hfq. *Mol Microbiol* **78**, 607–621.
- Sadosky, A. B., Wiater, L. A. & Shuman, H. A. (1993). Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect Immun* **61**, 5361–5373.
- Sahr, T., Brüggemann, H., Jules, M., Lomma, M., Albert-Weissenberger, C., Cazalet, C. & Buchrieser, C. (2009). Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. *Mol Microbiol* **72**, 741–762.
- Segal, G., Russo, J. J. & Shuman, H. A. (1999). Relationships between a new type IV secretion system and the *icm/dot* virulence system of *Legionella pneumophila*. *Mol Microbiol* **34**, 799–809.
- Tiaden, A., Spirig, T., Sahr, T., Wälti, M. A., Boucke, K., Buchrieser, C. & Hilbi, H. (2010). The autoinducer synthase LqsA and putative sensor kinase LqsS regulate phagocyte interactions, extracellular filaments and a genomic island of *Legionella pneumophila*. *Environ Microbiol* **12**, 1243–1259.
- Tsui, H. C., Leung, H. C. & Winkler, M. E. (1994). Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. *Mol Microbiol* **13**, 35–49.
- Vogel, J. P., Andrews, H. L., Wong, S. K. & Isberg, R. R. (1998). Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* **279**, 873–876.
- Weissenmayer, B. A., Prendergast, J. G. D., Lohan, A. J. & Loftus, B. J. (2011). Sequencing illustrates the transcriptional response of *Legionella pneumophila* during infection and identifies seventy novel small non-coding RNAs. *PLoS ONE* **6**, e17570.
- Wozniak, R. A. F. & Waldor, M. K. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol* **8**, 552–563.
- Zusman, T., Aloni, G., Halperin, E., Kotzer, H., Degtyar, E., Feldman, M. & Segal, G. (2007). The response regulator PmrA is a major regulator of the *icm/dot* type IV secretion system in *Legionella pneumophila* and *Coxiella burnetii*. *Mol Microbiol* **63**, 1508–1523.

Edited by: H. Hilbi