Growth-phase-dependent mobility of the *lvh*-encoding region in *Legionella pneumophila* strain Paris

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The *lvh* region of the *Legionella pneumophila* genome, which encodes a type IV secretion system, is located on a plasmid-like element in strains Paris (pP36) and Philadelphia (pLP45). The pP36 element has been described either integrated in the chromosome or excised as a multi-copy plasmid, in a similar manner to pLP45. In this paper, the chromosomal integration of pP36 in the Paris strain genome was described, occurring through site-specific recombination at the 3' end of a transfer-messenger RNA gene by recombination between attachment sites, in a similar manner to pathogenicity islands. This integration was growth-phase dependent, occurring during the exponential phase. Several pP36-borne genes were expressed during the lag phase of bacterial growth, coinciding with the peak amount of the episomal form of pP36. Expression of the same genes decreased during the exponential and stationary phases, owing to the integration phenomenon and a loss of episomal copies of pP36. A similar plasmid-like element was described in the Lens strain genome, suggesting that the mobility of the *lvh* region is a phenomenon widespread among *Legionella* sp.

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INTRODUCTION

About 90 % of clinical cases of legionellosis are due to *Legionella pneumophila* (Lp) and more than 84 % are due to *L. pneumophila* serogroup 1 (Lp1) (Doleans *et al.*, 2004; Yu *et al.*, 2002). The Lp1 Paris strain is an endemic strain, having been isolated from unrelated patients throughout Europe (Aurell *et al.*, 2003). The Lp1 Paris strain accounts for more than 12 % of clinical Lp strains isolated in France (Aurell *et al.*, 2003), suggesting a particular adaptation of this strain to its environment and/or host. All Lp1 Paris isolates have identical PFGE profiles and sequence-based types (SBTs) (Aurell *et al.*, 2003; Gaia *et al.*, 2003). The recently published genome sequences of strains Paris, Lens and Philadelphia (Cazalet *et al.*, 2004; Chien *et al.*, 2004) show a marked genome plasticity, with 9–13 % of

strain-specific genes. Moreover, each strain harbours distinct plasmids or plasmid-like elements: a plasmid and a plasmid-like element of 132 and 36 kb, respectively, are found in the Paris strain (Cazalet *et al.*, 2004); a 45 kb plasmid-like element (pLP45) is found in the Philadelphia strain (Chien *et al.*, 2004); and the Lens strain harbours a 60 kb plasmid (Cazalet *et al.*, 2004).

The 36 kb plasmid-like element found in the Paris strain can exist as a multi-copy circular episome and be integrated into the bacterial chromosome (Cazalet *et al.*, 2004). The G+C content of this plasmid-like element is higher (43 %) than that of the rest of the genome (38 %) and the presence of phage-related genes suggests that horizontal acquisition of this genomic region may occur (Cazalet *et al.*, 2004). The 36 kb element contains a locus called *lvh* (*Legionella vir* homologues) that encodes a type IV secretion system involved in conjugation and, potentially, in virulence (Ridenour *et al.*, 2003; Segal *et al.*, 1999). Other genes

Abbreviations: Ct, cycle threshold; Tm, melting curve peak; tmRNA, transfer-messenger RNA.

contained in this region include *prpA* (encoding a putative phage repressor) and the *Legionella vir* region (*lvr*) containing the genes *lvrA*, *lvrB*, *lvrE* and *lvrC* (encoding a CsrA homologue) (Molofsky & Swanson, 2003).

A large number of plasmids have been identified in clinical and environmental *Legionella* isolates, some of which are conjugative (Johnson & Schalla, 1982; Maher *et al.*, 1983; Mintz, 1999). These plasmids may influence the ecological fitness of *Legionella* (Ott, 1994), as some plasmid-possessing strains can persist longer in the environment than other strains (Brown *et al.*, 1982). For example, plasmid carriage in the Dodge strain of *L. pneumophila* confers resistance to UV radiation (Brown *et al.*, 1982; Tully, 1991). Other plasmids appear to influence strain virulence (Bezanson *et al.*, 1994; Daaka *et al.*, 1994; Doyle & Heuzenroeder, 2002). For example, a 30 kb unstable element present in the Olda strain can either be integrated in the chromosome (leading to LPS expression) or exist as an excised form (leading to altered LPS expression and loss of virulence) (Luneberg *et al.*, 1998).

Here we screened a collection of *Legionella* isolates for the 36 kb plasmid-like element (pP36) initially identified in the Paris strain. We suggest that pP36 can precisely integrate into the chromosome in a growth-phase-dependent manner through a site-specific recombination mechanism that could occur in other *Legionella* strains for other mobile elements. We also examined the physical form of pP36 (integrated or episomal) according to the growth phase, together with the expression dynamics of genes harboured by this element.

METHODS

Bacterial strains. We studied a collection of 203 *Legionella* strains initially grown on BCYE agar (Oxoid). The collection consisted of: (i) 32 reference strains, including the three genome-sequenced strains (Paris CIP 107629^T, Philadelphia ATCC 33152 and Lens CIP

108286); and (ii) 171 clinical and environmental isolates collected between 1997 and 2004 in different parts of France. These 171 isolates consisted of 121 serogroup 1 isolates, 31 non-serogroup 1 isolates and 19 isolates belonging to species other than *L. pneumophila*. Among the 121 Lp1 isolates, 31 belonged to the Paris clone, as shown by PFGE (Aurell *et al.*, 2003). The physical state of the pP36 element was studied during liquid growth of the reference Paris strain (CIP 107629^T). The detection of the Lens strain episomal element was performed using the Lens reference strain (CIP 108286).

Media and growth conditions. We used liquid growth medium (LGM) containing 12 g yeast extract l⁻¹ (Euromedex), 1 g 2-oxoglutarate l⁻¹ (Sigma-Aldrich), 0.5 g L-cysteine HCl.H₂O l⁻¹ (Merck) and 3 g iron pyrophosphate l⁻¹ (Pfaltz and Bauer), adjusted to pH 7.2. Buffered charcoal yeast extract (BCYE) agar was used as a solid medium.

The physical state of pP36 (episomal or integrated) was determined by inoculating LGM (initial OD₅₅₀ 0.15) with the Paris strain after prior growth on BCYE agar for 2 days. The cultures were incubated at 30 °C with shaking (115 r.p.m.). At the stationary phase of growth, the bacteria were pelleted by centrifugation and inoculated into fresh LGM (OD₅₅₀ 0.15). Samples were removed at various times, pelleted by centrifugation and stored at -80 °C until use.

DNA and RNA extraction. DNA and RNA were extracted from bacterial pellets by using the High-Pure PCR Template Preparation kit (Roche Diagnostics) and the High-Pure RNA isolation kit (Roche Diagnostics), respectively, according to the manufacturer's instructions.

pP36 detection and location. The integrated and episomal forms of pP36 and its chromosomal integration site were detected by PCR with four primers (MGW Biotech AG) named LEG1, LEG2, LEG3 and LEG4 (sequences available on www.lyon.inserm.fr/Primers%20pP36.pdf). The vacant integration site was amplified with primers LEG1 and LEG4, which were complementary to chromosomal sequences adjacent to the integration site. The episomal form was amplified with primers LEG2 and LEG3, and the integrated form was amplified with the LEG1/LEG2 and LEG3/LEG4 primer pairs (Fig. 1). All PCR experiments were done with a LightCycler apparatus (Roche Diagnostics) in a final reaction volume of 11 µl containing 1 µl LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), 0.45 nM forward and reverse

Fig. 1. Schematic representation of the integration/excision of element pP36 at the chromosomal integration site of *L. pneumophila* Paris. Chromosomal DNA is depicted by a thin line and the plasmid-like element pP36 by a thick line. The att sites are represented by boxes, relevant genes by hatched lines, and primers by grey arrows. The different parts (A1, B1 and B2) of the att sites are shown and described in the text. The tmRNA-encoding gene encompassing attL is shown by a thick horizontal line.

primer, 3.7 mM MgCl₂ and 2 µl DNA. Amplification consisted of 45 cycles of denaturation (95 °C for 15 s), hybridization (55 °C for 5 s) and elongation (72 °C for 35 s), followed by a fusion phase with a slow temperature increase from 65 °C to 95 °C. Fluorescence was measured at the end of the elongation step [for cycle threshold (Ct) determination] and during the fusion phase [for melting curve peak (T_m) determination]. The element in the Lens strain (episomal and integrated forms) was detected in the same way as pP36, using four primers (MGW Biotech): LEG1'/LEG4' for the vacant integration site, LEG1'/LEG2' and LEG3'/LEG4' for the integrated form, and LEG2'/LEG3' for the episomal form.

To study the interaction between the physical form of element pP36 and the bacterial growth phase, PCR detection of the episomal form (primer pair LEG2/LEG3) and the vacant site (LEG1/LEG4) was applied to DNA extracted at various times during *L. pneumophila* Paris culture. We also amplified the *lvhB9* gene with the *lvhB9* primers. Values were normalized by parallel PCR amplification of part of the 16S rRNA-encoding chromosomal gene in the same DNA samples, using primers 16S-F and 16S-R. Amplification was performed as described above, but with hybridization at 51 °C for 5 s.

Characterization of the chromosomal integration site of pP36. PCR products obtained from the Paris strain by using primer pairs LEG1/LEG4 and LEG2/LEG3, were sequenced by Genome Express.

Expression analysis of plasmid-like element pP36 genes and chromosomal gene. RT-PCR was performed for 1 h at 37 °C in a final reaction mix of 10 µl containing 4 µl RNA, 2 µl M-MLV 5 × concentrated buffer (Invitrogen), 0.01 µM DTT (Invitrogen), 25 nM dNTP mix, 0.1 µl universal primers (Invitrogen) and 20 U M-MLV enzyme (Invitrogen). Levels of DNA in the resulting cDNA samples were first normalized with respect to the chromosomal gene *gyrB* (encoding the constitutively expressed gyrase subunit B of *L. pneumophila*) with primers *gyrB1* and *gyrB2* (Broich *et al.*, 2006). We amplified eight genes on pP36 (*prpA*, *lvhB2*, *lvhB9*, *lvrC*, *lpp0182*, *lpp0183*, *lpp0193* and *lpp0194*) and also the chromosomal gene *rpoS*. Amplification was performed as described above, with hybridization for 5 s at appropriate temperatures.

Data analysis. The presence and location of plasmid-like element pP36 were determined by comparing the PCR Ct and T_m with those of positive and negative controls.

The relative amounts of amplicons specific for the integration site, the episomal form, the plasmid-like element genes and the chromosomal gene were determined by quantitative PCR relative to an internal standard (either the 16S rRNA-encoding gene or *gyrB*). The expression levels were expressed as ratios in arbitrary units (AU) calculated with RealQuant software (Roche Diagnostics).

RESULTS AND DISCUSSION

Distribution of pP36 in *Legionella* isolates

Genome sequencing of the Paris strain of *L. pneumophila* has identified a plasmid-like genomic island, designated pP36, that is capable of integrating the bacterial chromosome and of being excised from it (Cazalet *et al.*, 2004). Here we determined the distribution of pP36 in a collection of 203 *Legionella* isolates using PCR and found that it is always present in the Paris strains collected in different parts of France (Table 1). pP36 was not specific to the Paris strains, since five (5.6 %) of the 90 non-Paris Lp1 isolates harboured it. pP36 was also detected in three non-serogroup 1 isolates

(two clinical Lp8 and one environmental Lp9) and in two reference strains (CIP 107629^T, the Paris reference strain and ATCC 35289, an Lp9 reference strain) (These results are available from www.lyon.inserm.fr/distribution%20pP36.pdf). The plasmid-like element pP36 seems to be specific to the *L. pneumophila* species, since it was absent from all 19 non-*pneumophila* isolates. Nonetheless, our PCR only detected a portion of the pP36 element, so we cannot conclude that all strains positive by our method actually harboured identical elements.

Growth-phase-dependent mobility of pP36

In all these strains positive for pP36, we determined the physical state (integrated and/or episomal) in which pP36 was present, using the four primers LEG 1, LEG 2, LEG 3 and LEG 4. We demonstrated that pP36 was always present in both physical states (data not shown). We demonstrated that the integration event is dependent on the bacterial growth phase by determination of the physical state of pP36 in the Paris strain during growth in liquid medium. The episomal and the integrated form of pP36, as well as the vacant integration site, were detected at all time points studied. The episomal form and the vacant integration site were abundant during the first 10 h of culture corresponding to the lag phase (Fig. 2). The episomal form and the vacant integration site then decreased markedly at the beginning of the exponential phase (10–33 h), suggesting that pP36 had been integrated into the bacterial chromosome. This integration persisted during the stationary phase (33–48 h), although the episomal form and the vacant integration site were still detected at very low levels (Fig. 2). To detect changes in the total amount of pP36 (integrated form and episomal form), we amplified the *lvhB9* gene and expressed its relative amount using the 16S RNA gene amount as reference. *lvhB9* DNA levels were stable during the lag phase and fell during the exponential phase, suggesting a decrease in the total amount of pP36. This coincided with the increase in the integrated form, suggesting that the decrease in total pP36 DNA was due to a loss of the episomal form. This loss could be due to the absence of self-replication of pP36, or to a self-replication that is slower than bacterial multiplication. Markedly, these results (integration during exponential phase) were similar when stationary-phase bacteria were reinoculated into fresh LGM (data not shown). The excision was observed when stationary-phase bacteria were grown for 24–48 h on BCYE agar, but not in LGM media (data not shown). This excision event may therefore be more strongly promoted during multiplication under conditions other than liquid growth, such as during intracellular replication or within biofilms.

Unstable mobile elements that are capable of switching between an integrated chromosomal stage and an episomal form have already been identified for other bacteria. Their mobility might be involved in conjugation, as for the integrative and conjugative elements (ICEs) (Burrus *et al.*, 2002; Burrus & Waldor, 2003; Doublet *et al.*, 2005), or correspond to the regulation of a bacterial characteristic

Table 1. Distribution of pP36 in 203 *Legionella* isolates

Strain	Total	Positive strains (%)
<i>L. pneumophila</i> serogroup 1		
Paris strains		
Clinical isolates	20	20 (100·0)
Environmental isolates	11	11 (100·0)
Subtotal	31	31 (100·0)
Non-Paris Lp1 strains		
Clinical isolates	75	3 (4·0)
Environmental isolates	15	2 (13·3)
Subtotal	90	5* (5·6)
Total	121	36 (29·8)
<i>L. pneumophila</i> serogroup non-1 strains		
Clinical isolates	11	2† (18·2)
Environmental isolates	20	1‡ (5·0)
Total	31	3 (9·7)
<i>Legionella</i> non-<i>pneumophila</i> strains		
Clinical isolates	7	0 (0·0)
Environmental isolates	12	0 (0·0)
Total	19	0 (0·0)
Reference strains		
<i>L. pneumophila</i>	17	2§ (11·8)
<i>Legionella</i> non- <i>pneumophila</i>	15	0 (0·0)

*Non-epidemic strains.

†Lp8 strains.

‡Lp9 strain.

§CIP107629^T (Paris strain) and ATCC 35289 (Lp9 strain).

such as virulence (Dorman & Porter, 1998). It might also represent a safeguard for the element. In this way, the switch from the episomal form to the integrated form may serve to preserve pP36 during bacterial growth. This could explain the absence of the episomal form of pP36 in isolates lacking an integration site.

Expression of pP36 genes

To determine the function of pP36, we used quantitative RT-PCR to examine the expression of the genes it harbours, in the different phases of bacterial growth. *lvhB2* expression

is known to enhance *L. pneumophila* infection of mammalian cells at low temperatures such as 30 °C, increasing entry into the host cell and intracellular replication by a factor of about 100 (Ridenour *et al.*, 2003). Therefore, we analysed the expression of eight plasmid-like-element genes during growth of the Paris strain at 30 °C: *lvhB2* and *lvhB9*, belonging to the *lvh* locus; *prpA* and *lvrC*, frequently associated with the *lvh* region (Samrakandi *et al.*, 2002; Segal *et al.*, 1999); *lpp0193* and *lpp0194*, resembling an excisionase and an integrase, respectively; and *lpp0182* and *lpp0183* (unknown functions). The expression of all eight genes was similar when normalized to the *gyrB* gene expression

Fig. 2. Quantitative PCR amplification of the episomal form of the plasmid-like element pP36 (black bars), the vacant integration site (grey bars) and total pP36 (hatched bars) during growth in liquid medium monitored by OD₅₅₀ measurement (curve). The amounts of target DNA were normalized to that of the 16S rRNA-encoding chromosomal gene, as described in Methods; the results are expressed in arbitrary units (AU) and are means of three experiments.

Fig. 3(a). Maximal induction of gene expression occurred during the lag phase and was followed by a gradual decline. No significant expression was detected at the end of the exponential phase (33 h). We studied in parallel the expression of a chromosomal gene, *rpoS*, and we demonstrated that this expression was different from that of the plasmid-like-element genes. Indeed, *rpoS* expression was induced in the middle of the lag phase and was maximal during the exponential phase Fig. 3(b). Similar results have been reported by Bachman & Swanson (2004). The plasmid-like genes were expressed in the lag phase of the Paris strain at 30 °C, when the episomal form of pP36 was most abundant. We did not determine whether this phenomenon is temperature sensitive. In addition to their mobility, the genes located on the pP36 element, and particularly the *lvh* locus, might potentially interfere with phenotypic features according to the growth phase, and then confer a selective advantage upon the Paris strain linked to environmental survival and adaptation, such as adherence and survival in biofilms, DNA transfer between bacteria by conjugation, and protection from environmental stress. Gene expression and plasmid-like-element dynamics might also be involved in virulence, as described by Luneberg *et al.* (1998). The latter authors report a 30 kb unstable plasmid responsible for a phase-variation mechanism controlling the expression of virulence-associated LPS. Indeed, this 30 kb plasmid can exist either integrated in the chromosome of the *L. pneumophila* strain Olda, leading to expression of the LPS, or as an excised form corresponding to an alteration of

this LPS expression concomitant to a loss of virulence (Luneberg *et al.*, 1998).

Recombination site of pP36

We obtained evidence that pP36 integrates in the bacterial chromosome in a site-specific manner by recombination between two 92 bp *att* sites, one shared by the plasmid-free chromosomal integration site (*attB*) and the other by the episomal form of pP36 (*attP*). This recombination event would generate two chromosome–plasmid junctions, called *attL* and *attR* (Figs 1, 4 and 5). We detected *attB* in the 3' end of a transfer-messenger RNA (tmRNA)-encoding gene, between the *prlC* gene (which encodes oligopeptidase A) and the *lpp0197* gene. The location of the *attB* site at the 3' end of a tmRNA-encoding gene suggests that it is an attachment site of a pathogenicity island (Hacker *et al.*, 1997). *attP* is located on pP36, between the genes *lpp0158* and *lpp0196*. *attL*, which is located in the 3' end of the tmRNA-encoding gene, and *attR* lie between the chromosomal gene *prlC* and the pP36 genes *lpp0158* and *lpp0196*, respectively. Each *att* site consists of two domains. One, 31 bp long and designated A1, is shared by all the *att* sites. The second domain, which is 61 bp long, differs between these sites by 10 mismatches: *attB* and *attR* harbour the domain B1 whereas *attP* and *attL* consist of the domain B2 (Figs 1 and 4). A third sequence, called B3, was also detected between genes *lpp0207* and *lpp0208* (Fig. 5). It is 61 nucleotides long and differs from domains B1 and B2 by, respectively, 24 and

Fig. 3. Relative expression levels of the *lvhB2* and *lpp0193* pP36 genes, which are representative of expression levels of all pP36 genes analysed (a), and the chromosomal gene *rpoS* (b) (solid lines) during growth in liquid medium at 30 °C monitored by OD₅₅₀ measurement (dotted lines). The expression levels were normalized to the expression of *gyrB*, as described in Methods, and are expressed in arbitrary units (AU).

	31 nucleotides	61 nucleotides
Paris strain	<i>attB</i> (A1/B1)	<i>cgcgggttcgattcccgccgctccaccaat/cattaaaaccattcctgaccaccaaggaccatgataaaccttataatcaaggagtaaa</i>
	<i>attP</i> (A1/B2)	<i>cgcgggttcgattcccgccgctccaccaat/tatcaaaaccattcctgaccaccaagaccatcataaacctttagaacaacgagtaaa</i>
	<i>attL</i> (A1/B2)	<i>cgcgggttcgattcccgccgctccaccaat/tatcaaaaccattcctgaccaccaagaccatcataaacctttagaacaacgagtaaa</i>
	<i>attR</i> (A1/B1)	<i>cgcgggttcgattcccgccgctccaccaat/cattaaaaccattcctgaccaccaaggaccatgataaaccttataatcaaggagtaaa</i>
Lens strain	B3	<i>/caagatctgaccctctaggtgtaaagaccatcataaacctttagatcaacgagtaaa</i>
	A1/B4	<i>cgcgggttcgattcccgccgctccaccaat/cataaaaaccattttcgaccactaaagaccatcataaacctttagatcaacgagtaaa</i>
	A2	<i>cgtgggttcgattcccgccgctccaccaat/</i>
Philadelphia strain	A3	<i>agcgggttcgattcccgccgctccaccaat/</i>
	A1	<i>cgcgggttcgattcccgccgctccaccaat/</i>

Fig. 4. Sequences of *att* sites. The *att* sites from strains Paris, Lens and Philadelphia are aligned, with conserved nucleotides shaded grey and non-conserved nucleotides in bold type. The 3' end of the tmRNA-encoding gene, which overlaps with 29 bp of the *attB* and *attL* sites, is underlined.

20 nucleotides. This sequence seems to be incomplete and might correspond to a secondary or vestigial integration site.

Similar plasmid-like element in Lens and Philadelphia strains

To determine if this element mobility is specific to the Paris strain, we analysed the genome of the other sequenced strains. In the Lens strain, we identified a plasmid-like element similar to the pP36 element that integrated the chromosome in a sequence similar to the Paris *att* sites. Indeed, the genome of this strain contained a complete *att*-like sequence, including the 31 bp A1 domain [the 3' end of the same tmRNA-encoding gene] and a 61 bp domain (designated B4) homologous to B1 and B2 and located between the *prlC* and *lpl0143* genes (Figs 4 and 5). Domain

B4 showed 10 and six mismatches when compared to domains B1 and B2, respectively. This chromosomal region of strain Lens contained a portion of the plasmid-like element pP36. Downstream of the Lens *att* site, we found the *prpA* gene, the *lvr* and *lvh* loci, and seven other genes (*lpl0169*, *lpl0170*, *lpl0171*, *lpl0178*, *lpl0179*, *lpl0184* and *lpl0185*) sharing significant nucleotide and deduced amino acid sequence homology with the corresponding genes in the Paris strain (Fig. 5). Based on the deduced amino acid sequences, the proteins encoded by six of these genes are similar to mobility factors or enzymes: a TraA-like protein (*lpl0169*), a TraD-like protein (*lpl0170*), a phage excisionase (*lpl0178*), a phage integrase (*lpl0179*) and two transposases (*lpl0184* and *lpl0185*). These results suggest the existence of a similar element related to the pP36 element in the Lens strain. We detected both the integrated and the episomal

Fig. 5. Schematic representation of the integrated form of the pP36 element in the Paris strain, and similarities in strains Lens and Philadelphia.

form of this element, and its vacant integration site (data not shown), suggesting that a dynamic of the element encoding the *lvh* system occurs also in the Lens strain.

The Philadelphia strain harbours the A1 domain between genes *prlC* and *lpg0142*, and this putatively encodes a transposase (<http://genome3.cpmc.columbia.edu/~legion/>) (Figs 4 and 5). The region located close to this site is totally different in sequence from the Paris and Lens elements, and does not contain the *lvh* system. However, as in the Paris and Lens strains, this system is located on a plasmid-like element (pLP45), which is also mobile (Chien *et al.*, 2004). The *att* integration sites of pLP45, and the flanking regions of the *lvh* locus, are different from those of the Paris and Lens strains (data not shown), suggesting that they may have different origins. However, the mobility event of the *lvh* region seems to be common and therefore not specific to the Paris strain. These results suggest that this phenomenon could be widespread in many *Legionella* strains and may play a role in the *lvh* system activity.

In conclusion, we demonstrated that pP36 is integrated in the chromosome during the exponential and post-exponential phases, and seems to be capable of excision from it under other conditions not yet clearly defined. The level of the expression of the genes harboured by pP36 seems to be linked to the episomal pP36 copy number. To the best of our knowledge, this original phenomenon of integration/excision according to growth phase has not so far been detected in other bacteria. Further investigations will greatly improve our understanding of this plasmid-like element and should reveal the mechanism of its recombination, notably by determining the enzyme involved. In the same way, the benefit of the genetic flexibility of this *lvh*-encoding region, which is also observed for other *L. pneumophila* strains, has to be determined.

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