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1 **Transposon-insertion sequencing in a clinical isolate of *Legionella pneumophila* identifies essential**
2 **genes and determinants of natural transformation**

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8 # Abstract

9 *Legionella pneumophila* is a Gram-negative bacterium ubiquitous in freshwater environments which, if
10 inhaled, can cause a severe pneumonia in humans. The emergence of *L. pneumophila* is linked to several
11 traits selected in the environment, the acquisition of some of which involved intra- and interkingdown
12 horizontal gene transfer events. Transposon-insertion sequencing (TIS) is a powerful method to identify the
13 genetic basis of selectable traits as well as to identify fitness determinants and essential genes, possible
14 antibiotic targets. TIS has not yet been used to its full power in *L. pneumophila*, possibly because of difficulty
15 to obtain a high-saturation transposon insertion library. Indeed, we found that ST1 isolates, to which belong
16 the commonly used laboratory strains, are poorly permissive to saturating mutagenesis by conjugation-
17 mediated transposon delivery. In contrast, we obtained high-saturation libraries in non-ST1 clinical isolates,
18 offering the prospect of using TIS on unaltered *L. pneumophila* strains. Focusing on one of them, we
19 therefore used TIS to identify essential genes in *L. pneumophila*. We also revealed that TIS could be used to
20 identify genes controlling vertical transmission of mobile genetic elements. We then applied TIS to identify all
21 the genes required for *L. pneumophila* to develop competence and undergo natural transformation, defining
22 the set of major and minor Type IV pilins that are engaged in DNA uptake. This work paves the way for the
23 functional exploration of the *L. pneumophila* genome by TIS and the identification of the genetic basis of
24 other life traits of this species.

25 # Importance

26 *Legionella pneumophila* is the etiologic agent of a severe form of nosocomial and community-acquired
27 pneumonia in humans. *L. pneumophila* is found in man-made and freshwater environments which are the
28 causing source of the infection. The environmental life traits of *L. pneumophila,* such as its abilities to form
29 biofilms, resist biocides and unicellular predators, are essential to its ability to accidentally infect humans. A
30 comprehensive identification of the genetic basis of these life traits could be obtained through the use of
31 transposon-insertion sequencing. Yet, this powerful approach, had not been fully implemented in *L.*
32 *pneumophila*. Here we described the successful implementation of the transposon-sequencing approach in a
33 clinical isolate of *L. pneumophila*. We identify essential genes, potential drug targets, and genes required for
34 horizontal gene transfer by natural transformation. This work represents an important step towards
35 identifying the genetic basis of the many life traits of this environmental and pathogenic species.

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36 # Introduction

37 *Legionella pneumophila* is a Gram-negative bacterium, ubiquitous in freshwater environments where it can
38 be found in planktonic form, in biofilm communities or associated to amoebic protozoa which constitute its
39 natural host (1). *L. pneumophila* can resist predation by amoeba and even establish an intracellular vacuole
40 in which it can multiply, while being protected from external environment (2). Man-made water systems have
41 offered a new breeding-ground for the development of *L. pneumophila*. Inhalation by humans of aerosols
42 produced by these systems and contaminated by *L. pneumophila* can cause Legionnaires' disease (3). This
43 community-acquired disease, which is most often characterized by a severe pneumonia, occurs when *L.*
44 *pneumophila* infects alveolar macrophages (4). In both macrophages and its natural amoebal hosts, *L.*
45 *pneumophila* replicates intracellularly by hijacking the host cellular machinery (5). This requires the Icm/Dot
46 type IV system (6, 7), a conjugative system that can secrete up to 300 effector proteins (8, 9). The first
47 genome sequences of the original Philadelphia outbreak strain Philadelphia-1 (10) and the endemic strain
48 Paris (11) provided early evidence of genes encoding eukaryotic-like proteins, some of which are the effector
49 proteins substrates of the Icm/Dot system. Phylogenetic analyses suggest that these genes would have been
50 acquired by inter-kingdom horizontal gene transfer (HGT) during co-evolution of *Legionella* and its natural
51 host for millions of years (12). Hundreds of genome sequences of *L. pneumophila* clinical isolates have now
52 revealed that recombination events are common in this species (13–15). Thus, intra-specific and inter-
53 kingdom HGT events are playing a major role in the evolution and adaptation of this species. The high
54 plasticity of the genomes of *L. pneumophila* is consistent with the fact that it is competent for natural
55 transformation (16). Natural transformation refers to the ability of certain bacteria to capture exogenous DNA
56 and integrate it into their genome by homologous recombination (17). It is one of the driving forces for
57 bacterial evolution that can lead to the emergence of new pathogenic bacteria and new antibiotic-resistant
58 recombinants. It is a widespread mechanism of HGT in bacteria, with more than 80 experimentally-confirmed
59 transformable species (18). The DNA uptake mechanisms and associated proteins constituting the so-called
60 “DNA uptake machinery” are highly conserved (17), suggesting that most species are potentially
61 transformable. DNA uptake first involves a type IV pilus (T4P) (19) whose direct observation supports a
62 model in which it binds DNA via its tip, and its retraction allows the internalization of DNA into the periplasm
63 (20). The periplasmic DNA-binding protein ComEA serves as a ratchet (21, 22) and large amounts of DNA
64 can accumulate in the periplasm before being converted into single-stranded DNA (ssDNA) and translocated
65 across the cytoplasmic membrane through the ComEC inner membrane channel (23). In the cytoplasm, the
66 ssDNA is protected by the transformation-dedicated protein DprA (24) and the single-stranded binding
67 protein SsbB (25). If the internalized ssDNA possesses homologous regions with the bacterial chromosome,
68 it is integrated by homologous recombination mediated by the recombinase RecA which interacts with DprA
69 (26). In Gram-negative bacteria, the newly discovered ComM helicase is also involved in this recombination
70 process (27).

71 In most transformable species, these proteins are not expressed constitutively but only when the bacterium
72 is in a genetically programmed and transient state called “competence” (28). *L. pneumophila* was first
73 reported competent when grown at 37°C under some form of stress, such under microaerophilic conditions
74 (16) or exposure to DNA-damaging agents (29). In the absence of any stress, *L. pneumophila* becomes
75 transiently competent when grown at 30°C at the transition between the exponential and stationary growth

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76 phases (30, 31). *L. pneumophila* is unique in that the regulation of competence does not involve
77 transcriptional activation of the competence regulon. Rather, the core genes encoding the DNA uptake
78 system (*comEC*, *comEA*, *comFC*, *comM*) are subjected to post-transcriptional repression by a
79 ribonucleoprotein complex consisting of a small RNA, RocR, and an RNA chaperone, RocC (31). At the
80 onset of the stationary phase, the expression of RocR decreases and the translation of the mRNAs encoding
81 the DNA uptake system allows *L. pneumophila* to take up and recombine extracellular DNA. Most of *L.*
82 *pneumophila* clinical isolates do transform under these conditions, yet some isolates fail to develop
83 competence and in some instance, this is due to the presence of a mobile genetic element (MGE) that
84 encodes a RocR homolog that acts as a substitute of the chromosome-encoded RocR (32). Competence is
85 further repressed in stationary phase by the quorum-sensing system (33). The regulation of competence in
86 *L. pneumophila* still remains poorly understood (34).

87 Regulation of competence is best understood in the Gram-positive *Streptococcus pneumoniae* in which the
88 comprehensive genetic approach of transposon-insertion sequencing has recapitulated decades of findings
89 (35). Beyond the identification of additional regulatory or functional elements of natural transformation, such
90 approach allowed for a better understanding of the biology of this bacterium by identifying genes involved in
91 virulence and in resistance against stresses. Transposon-insertion sequencing (TIS) approaches encompass
92 a number of similar methods (Tn-seq, TraDIS, INseq, HITS) (36–39) that have been used for the
93 identification of essential genes on a genome-wide scale in a number of species (40). TIS relies on the
94 mapping and quantification of transposon insertion mutants by high-throughput DNA sequencing and a
95 critical factor is to obtain high-saturation libraries of transposition mutants (41). TIS has recently been applied
96 to *L. pneumophila* with a focus on effector-encoding genes and their conditional involvement in intracellular
97 replication (42, 43). However, the libraries of mutants were either targeted for effectors (42) or of low
98 coverage (43). Thus, the full power of TIS has not yet been harnessed to understand fundamental or specific
99 aspects of the biology of *L. pneumophila*, possibly because of the difficulty of obtaining high-saturation
100 mutant libraries. In addition, the current libraries were constructed in the lp02 strain which has lost
101 competence regulation during its laboratory domestication (44). Here we sought to obtain a high coverage
102 library for Tn-seq in *L. pneumophila* that could be used to apprehend the genetic basis the many life traits of
103 this species. We found that some clinical isolates of *L. pneumophila* are more permissive to transposon
104 mutagenesis than the commonly used laboratory strains. We obtained a high coverage Tn-seq library in an
105 unaltered clinical isolate, and identified genes essential for fitness and growth in axenic medium. We then
106 applied Tn-seq to identify the genes involved in competence and natural transformation.

107 Results and discussion

108 *A high-saturation Tn-seq library of L. pneumophila*

109 With the objective of obtaining a Tn-seq library of *L. pneumophila*, we tested the conjugative delivery of the
110 Himar1-based transposon encoded by the *pir*-dependent mobilizable plasmid pBT20 to the commonly used
111 strain Paris. Conjugation assays with the MFDpir donor strain only produced a handful of insertional mutants.
112 We hypothesized that the Paris strain was particularly resistant and tested 12 other clinical isolates
113 belonging to the sequence type (ST) 1. Similarly to the Paris strain, none of the ST1 isolates generated a
114 meaningful number of mutants. We concluded that for an unknown reason the ST1 isolates (which would

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115 include the Philadelphia-1 derived laboratory strains lp02 and JR32) were poorly permissive to conjugative
116 transfer and/or to transposition by Himar1. We thus tested 8 other non-ST1 clinical isolates. We obtained
117 several thousands mutants for 5 of these. We decided to purse with isolate HL-0709-3014, for which we
118 obtained a complete genome composed of a circular chromosome of 3,405 kb and a plasmid of 106 kb (see
119 Material and Methods). 3,183 open-reading frames were detected, 2,791 and 2,741 of which have orthologs
120 in the Paris and Philadelphia-1 strains, respectively (Dataset S1). HL-0709-3014 belongs to the ST18
121 lineage, which is closely related to the ST1 lineage. Hence, it is phenotypically similar to the Paris strain, it is
122 naturally transformable and shows similar intracellular replication rates in amoebae (Fig. S1). It also
123 effectively replicates in human and murine macrophages (>2 log growth in 72 h) (Fig. S1). We isolated
124 HL77S, a spontaneous streptomycin-resistant mutant of HL-0709-3014, and subjected it to mutagenesis with
125 the transposon of pBT20. This mariner-based transposon inserts at TA sites and includes an outward facing
126 Ptac promoter that can minimize possible polar effects on operon and downstream genes. About 250,000
127 colonies of mutants were isolated on CYE plates and collected (initial isolation). The library was then cultured
128 in rich medium at 30°C and re-isolated on CYE (second isolation). Sequencing of the transposon insertion
129 sites revealed a maximum of 110,679 unique insertion out of 255,021 possible TA sites (43% saturation) and
130 an average of one insertion site every 31 bp. This represents a significant improvement over the previously
131 published library in the ST1 lp02 strain which consisted in 17,781 unique insertions sites (7% saturation)
132 (43). Thus, we obtained a high saturation Tn-seq library in an *L. pneumophila* clinical isolate that can be
133 used as a surrogate to the commonly used laboratory strains (Paris, JR32, lp02, AA100).

134 *Analysis of gene essentiality*

135 The high saturation allowed the identification of genes essential for growth. To do so, we used two statistical
136 methods; the Gumbel method (45), a Bayesian model based on longest consecutive sequence of TA sites
137 without insertion in the genes, and the HMM method based on the detection of genes with unusually low
138 read counts (46). Both methods gave similar results with 401 (Gumbel) and 500 genes (HMM) identified as
139 essential, 382 of which were identified as essential by both methods (Dataset S1). This is consistent with the
140 average number (391) of essential genes identified in other bacterial species (47). The data confirmed our
141 previous observation of the essentiality of the actin-like protein MreB (48) but also of MreC and MreD, while
142 intergenic insertions between *mreC* and *mreD* are tolerated (Fig. 1A). Comparative analysis of the second
143 and initial isolation, identified 181 genes non-essential at the initial isolation but whose inactivation impaired
144 fitness (log2FC<-2, P<0.05). These include the gene encoding the exoribonuclease R, whose growth defect
145 was previously reported (49), the RNA chaperone Hfq, but also more surprisingly the substrates of the
146 Icm/Dot Type IV secretion AnkQ and SdbB (Dataset S1). Presumably because their inactivation lowered the
147 fitness so dramatically, 61 genes not essential after the initial isolation were deemed essential on the second
148 isolation. For instance, these include the phosphoenolpyruvate synthase-endoding gene *ppsa* (Fig. 1A), the
149 genes encoding the sigma factor RpoS, the RecA recombinase, the tyrosine recombinase XerC involved in
150 chromosome dimers resolution and the tmRNA-binding protein SmpB involved in trans-translation. Indeed,
151 this is consistent with our previous demonstration that trans-translation is essential in *L. pneumophila* (50)
152 and that no insertions are observed in the tmRNA-encoding gene (Fig. 1B). The vast majority of the essential
153 genes have orthologs in the Paris and Philadelphia-1 genome, as expected for genes that encodes proteins
154 involved in the fundamental processes of the cell. However, of all genes found to be essential either on initial

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155 or second isolation, 14 have no orthologs in the Paris and Philadelphia-1 strains. How could strain-specific
156 genes be essential? Three of these genes (HL77S_01135, HL77S_01141, HL77S_01146) encode antitoxin
157 component of toxin-antitoxin (TA) modules clustered within a 4 kb segment. HL77S_01068 is located next to
158 a gene encoding a toxin of type II TA system, suggesting that it also encodes an antitoxin. Others have no
159 known function, such as HL77S_02141 which encodes a protein with a predicted helix-turn-helix (HTH) motif
160 or HL77S_00079 encoding a protein with a conserved domain of unknown function (DUF3800). Consistently
161 with being part of the accessory genome, genomic comparison with other complete genome of *L.*
162 *pneumophila* indicate that all of these genes reside in highly variable regions, often in proximity to putative
163 transposase and prophage integrase. Yet, no genetic structure corresponding to a complete mobile genetic
164 element (MGE) could be detected. In contrast, two essential genes (HL77S_00162, HL77S_00189) are
165 within a recognizable MGE corresponding to an integrative conjugative element (ICE) inserted at the 3' end
166 of the tmRNA-encoding gene and carrying a conjugative system homologous to the Lvh system (Fig. 1B).
167 HL77S_00162 is an HTH-type regulator and HL77S_00189 is predicted to encode a DNA-cytosine
168 methyltransferase. The ICE shows a third essential gene (HL77S_00187, *prpA*), conserved in the lvh ICE of
169 the Paris strain and encoding a LexA/CI-like repressor homolog (Fig. 1B). Another strain-specific essential
170 gene (HL77S_00197) is located just downstream of the ICE, in a unique region that may represent a
171 remnant of another MGE. Another three of the 14 strain-specific essential genes (HL77S_03181,
172 HL77S_03182, HL77S_03183) are part of the 106-kb conjugative plasmid and clustered with another
173 essential gene (HL77S_03071) which has a homolog on the pLPP plasmid of the Paris strain (Fig. 1C).
174 Encoding Rep or Par homologs, these genes are involved in replication/partition of the plasmid and their
175 inactivation likely resulted in plasmid loss. On this plasmid, two other genes of unknown function also appear
176 essential, one that is unique and with no conserved domain (HL77S_03140) and one with a homolog on
177 pLPP (HL77S_03116, *plpp0094*) containing an N-terminal HTH motif and a C-terminal nucleotidyltransferase
178 (NT) domain also found in DNA polymerase beta (Fig. 1C). Insertions in two divergently oriented genes
179 (HL77S_03176, HL77S_03177) are also associated with strong fitness defects and are of unknown function
180 (Fig. 1C). Overall, we found that many essential genes can be found within MGEs. Insertion in genes
181 controlling vertical transmission can result in the loss of the MGE (and thus of transposon-insertions making
182 the corresponding gene seemingly essential). This might be the case for repressor of excision of ICE or
183 genes required for replication/partition of plasmids. Other genes might be required to limit the cost of the
184 MGE on the fitness of their host. This might be the case for the LexA/CI-like repressor of ICE, as exemplified
185 by the *Vibrio cholerae* SXT ICE, for which inactivation of the LexA/CI-like repressor SetR is deleterious to its
186 host (51). Whatever the mechanism, the genes characterized as essential in MGEs ensure their vertical
187 transmission. Thus, our result indicates that in addition to identifying genes required for the fundamental
188 functions of the cell, Tn-seq analyses can also reveal novel genes that contribute to vertical transmission of
189 MGEs, representing an untapped resource to study the bacteria-MGE co-evolution.

190 *Tn-seq analysis of natural transformation in L. pneumophila*

191 We seek to use the Tn-seq library to identify all genes required for competence and subsequent natural
192 transformation. Mutants defective for expression of competence, DNA uptake, protection or recombination
193 would not be able to undergo transformation and would thus be missing in the transformed population. We
194 subjected the Tn-seq library to natural transformation with two distinct transforming DNA carrying a

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195 kanamycin resistance cassette inserted in the *legk2* gene (encoding an Icm/Dot substrate) or in the *ihfB*
196 gene (encoding the B subunit of the integration host factor IHF). This strategy should limit false positive
197 arising from epistatic interactions between the gene in which the selected resistance cassette is inserted
198 (*legk2* or *ihfB*) and the transposon-disrupted genes. Transformation frequencies of the HL77S Tn-seq library
199 were in the range of $1x10^{-5}$-$6x10^{-4}$ and to avoid a bottleneck effect (41) we collected over $5x10^{6}$
200 transformants. The control, non-transformed populations were sub-sampled to obtain a similar number of
201 isolated colonies. We observed 28 genes in which insertions cause a decrease (log2FC<2, P<0.05) of the
202 mutants in the population transformed with either the *legK2*::kan or the *ihfB*::kan DNA (Fig. 2A). As expected,
203 these include the gene encoding the periplasmic DNA receptor ComEA, the genes required for DNA
204 transport across the inner membrane (*comEC*, *comFC*), for ssDNA protection in the cytoplasm (*dprA*) and
205 recombination (*comM*). Many of the other genes encode factors known to be involved in Type IV pilus
206 assembly, confirming the role of this system in natural transformation of *L. pneumophila* (16). These include
207 the retraction ATPase PilT (lpp1995), the extension ATPase PilB, the PilQ secretin, the PilC platform protein
208 and the proteins of the PilMNOP complex. Interestingly, we observed no transformation defect for insertions
209 in the gene encoding another putative pilus retraction ATPase (lpp2271). Two putative pilins, pilE (lpp0681)
210 and pilA_2 (lpp1890) were also identified as essential for transformation. Other genes potentially involved in
211 natural transformation or regulation of competence were also identified (*lpp1976*, *lpp1977*, *lpp1978*, *lpp3030*,
212 *lpp2632*, *djlA*, *letA*, *letS*). In order to confirm their role and disentangle their involvement in DNA uptake or in
213 regulation, we constructed gene deletion mutants in a Paris strain with a premature stop codon of the RocC
214 chaperone (Paris $rocC_{TAA}$) which is defective for repression of competence and constitutively transformable
215 (31). Deletion mutants corresponding to genes known to be involved in natural transformation were defective
216 for transformation as expected (Fig. 2B). The *comEC* and *comFC* mutants were totally defective for
217 transformation, the *comM* and *comEA* mutants showed a ~100-fold decrease in transformation frequencies
218 as observed for other species (27, 52). Similar partial transformation defects were observed for mutants of
219 *djlA*, encoding a DnaJ-like protein required for intracellular replication in *Legionella dumoffii (53), a*nd
220 *lpp3030*, a *Legionellaceae*-specific gene encoding an uncharacterized protein with a putative signal peptide.
221 However, in this constitutively competent background we could not confirm the involvement of *lpp2632* which
222 encodes a glutaryl-CoA dehydrogenase, indicating that this gene is dispensable for the transformation
223 process (Fig. 2B). Mutants of this gene show a reduced fitness (log2FC=-1.99, P<0.01) (Dataset S1),
224 suggesting that the transformation defect observed in the Tn-seq analysis is an indirect consequence of the
225 mutants limited growth that could prevent entry in the competence state at the onset of the stationary phase.
226 Intriguingly, in this constitutively competent strain, a deletion mutant of *letS* also showed no transformation
227 defect (Fig. 2B). LetS is the sensor of the LetA/LetS two-component system (TCS) homologous to the BarA/
228 UvrY system in *Escherichia coli* (54) and GacS/GacA in *Pseudomonas* spp (55). In *L. pneumophila*, the LetA/
229 LetS system has been identified for the first time in a screen of mutants deficient in the expression of flagellin
230 (56) and has since been shown to be involved in the activation of various virulence traits as well as
231 intracellular growth in amoeba (57–60). One of the major roles of the LetA/S TCS is to enable the transition
232 from the transmissive to the replicative phase (61). The facts that both LetA and LetS output together in the
233 transformation screens while the $rocC_{TAA}$ Δ*letS* mutant is not defective for transformation suggests that this
234 TCS is involved in the regulation of competence in *L. pneumophila*. To test this, we reconstructed an
235 insertion mutant of the *letA* gene encoding the activator of this TCS in the Paris strain and $rocC_{TAA}$ genetic
236 backgrounds and tested them for their ability to undergo transformation. Consistent with the Tn-seq data,

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237 inactivation of LetA in the Paris strain reduced transformability by over 500-fold (Fig. 2C). In contrast, like the
238 Δ*letS* mutant, the Δ*letA* mutant in the constitutively competent strain $rocC_{TAA}$ is only marginally affected for
239 natural transformation (Fig. 2C). These data suggest that the LetA/S TCS is involved in the regulation of
240 competence upstream of the regulation controlled by the RocC/RocR system. Further work will be needed to
241 determine the precise role of this TCS and the associated regulatory cascades in the regulation of *L.*
242 *pneumophila* competence.

243 *Major and minor pilins required for natural transformation*

244 With the remarkable exception of *Helicobacter pylori* (62), in all Gram-negative bacteria DNA uptake requires
245 type IV pili (19). Type IV pili are extracellular filaments resulting from the assembly of thousands copies of an
246 abundant major pilin but also of less abundant minor pilins that could be embedded in the filaments (core
247 minor pilins) or at its tip (non-core minor pilins) (63, 64). The nomenclature of pilins is relatively confusing but
248 the major pilin is generally called PilA, although in *Neisseria sp.* that protein is called PilE (64). In addition,
249 some species carry multiple copy of pilins and at least in *Thermus thermophilus* two major pilins (PilA4 and
250 PilA5) are assembled into distinct filaments respectively required for natural transformation and twitching
251 motility (65). The *L. pneumophila* genomes show two putative PilA homologs encoded by two consecutive
252 genes (*pilA_1*, lpp1889; *pilA_2*, lpp1890) in a locus away from any other genes encoding Type IV pilus
253 components. Both the Tn-seq data and reconstructed mutants show that PilA_2 is required for natural
254 transformation while PilA_1 is dispensable (Fig. 2A and 2B). The two copies of PilA-encoding genes may
255 have resulted from a gene duplication event, followed by the loss of function of one of the two copies. The
256 Tn-seq data show that a putative pilin PilE (Lpp0681) appears required for transformation, while five genes
257 upstream of pilE (*lpp0686-lpp0682*) and respectively annotated as PilC/PilY1 and minor pilins PilX, PilW, PilV
258 and GspH/FimT appears dispensable. Targeted gene deletion also confirmed the Tn-seq result that PilE is
259 required for natural transformation, corroborating an initial observation that a mutant of the *pilE* gene (then
260 denoted $pilE_L$) is not competent for transformation (16). Based on sequence comparison with PilA from *P.*
261 *aeruginosa*, $pilE_L$ was then proposed to encode a type IV pilin structural gene (66). We thus investigated
262 which of PilE and PilA_2 constitute the major pilin in *L. pneumophila*. We tested the complementation of the
263 Δ*pilE* and Δ*pilA_2* deletion mutants obtained in the constitutively competent strain $rocC_{TAA}$. Both *pilE*
264 (*lpp0681*) and *pilA_2* (*lpp1890*) were ectopically expressed from an IPTG-inducible promoter to produce
265 fusion proteins with a C-terminal FLAG epitope. Western-blot analysis showed that both PilE-FLAG and
266 PilA_2-FLAG could be expressed in an IPTG-dependent manner with PilE-FLAG always expressed at a
267 higher level than PilA_2-FLAG, likely reflecting the efficiency of their ribosome-binding site (Fig. 3A). Data
268 show that a low expression of PilE-FLAG is sufficient to restore natural transformation in the $rocC_{TAA}$ Δ*pilE*
269 mutant as full complementation of the transformation phenotype is obtained even in the absence of IPTG
270 (Fig. 3B). In contrast, a higher concentration of IPTG and thus a higher expression of PilA_2-FLAG is
271 required to obtain a functional complementation of the $rocC_{TAA}$ Δ*pilA_2* mutant (Fig. 3B). The results are
272 consistent with a model in which PilA_2 is the major pilin while PilE is a low-abundance minor pilin. In
273 addition, when expressed ectopically in the $rocC_{TAA}$ strain, PilA_2-FLAG assembles in long extracellular
274 filaments (Fig. 4A and 4B). In the $rocC_{TAA}$ Δ*pilE* strain, fewer PilA_2-FLAG filaments are observed by
275 microscopy and western-blot confirmed a lower abundance of extracellular PilA_2-FLAG (Fig. 4A and 4B).
276 This indicates that PilE, while not strictly essential still plays a role in pilus formation. Minor pilins have been

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277 proposed to localized at the tip of the pilus and stabilize it (63). In *Vibrio cholerae*, DNA binding has been
278 observed to occur at the tip of the pilus (20). Because PilE is not strictly essential for pilus assembly but
279 required for transformation and DNA internalisation (Fig. 3B and 4C), we propose that PilE is the DNA
280 receptor at the tip of a pilus composed of PilA_2 subunits.

281 *Genes of unknown function and pilZ*

282 In addition to the pilin mutants that were strongly defective for natural transformation, we investigated the
283 underlying reason for the strong transformation defect of the mutant deleted of the operon *lpp1976-lpp1977-*
284 *lpp1978* (Fig. 2B). Consistent with being important for natural transformation this operon was found to be up-
285 regulated in the constitutively transformable mutant *rocC*$_{TAA}$ (31). Automated annotation did not assign a
286 predicted function for the three genes and blast search failed to identify homologs outside of the *Legionella*
287 genus. The deletion mutant of the entire operon Δ*lpp1976-8* was found unable to take up DNA (Fig. 4C),
288 indicative of a defect in Type IV pilus-mediated DNA import. Indeed, in this mutant, the levels of extracellular
289 PilA_2-FLAG were strongly reduced (Fig. 4B). The mutant produced few, and short, PilA_2-FLAG filaments
290 (Fig. 4A) revealing a major defect in Type IV pilus assembly or stability. A search for conserved domains in
291 the three predicted proteins only identified, in the 268 aa-long Lpp1977, a partial homology with the N-
292 terminal part of the Tfp pilus assembly protein PilW. This suggested that *lpp1976-lpp1977-lpp1978* would
293 encode a set of minor pilins. Indeed, PilFind (67) identified an N-terminal transmembrane segment in all
294 three predicted proteins and a type III signal in Lpp1977 and Lpp1978. The operonic organization of these
295 three genes is reminiscent of the operon encoding four minor pilins of the type IV pilus of *N. meningitidis*
296 (*pilHIJK*) and *P. aeruginosa* (*fimU-pilVWX*) and of the Type II secretion system (T2SS) of enterotoxigenic *E.*
297 *coli* (*gspHIJK*). In the latter system, the last three genes (*gspIJK*) encode minor pseudopilins which
298 assemble into a stable complex (68). This complex of minor pilins would form in the inner membrane to
299 establish a platform for the assembly of the major pilin (69), and remain at the tip of the pilus, stabilizing it
300 (63). Such heterotrimeric complex may be formed by minor pilins of limited homology but displaying
301 structural similarity (70). Altogether, this supports the hypothesis that in *Legionella* species, the initiation
302 complex of the transformation pilus is formed by Lpp1976, Lpp1977 and Lpp1978 which serve as a scaffold
303 for assembly of the major pilin PilA_2.

304 Another gene whose deletion resulted in strong deficiency in natural transformation is *pilZ*. The Δ*pilZ* mutant
305 is defective for DNA uptake and is totally unable to produce extracellular PilA_2 or assemble PilA_2 filaments
306 (Fig. 4). PilZ was originally identified in *P. aeruginosa* as required for the secretion of PilA polymers, pilus
307 genesis and Type IV pilus-dependent motility (71). Although the *P. aeruginosa* PilZ served as the founding
308 member of a diverse family of proteins with PilZ domains (72), some of which bind the cyclic-di-GMP second
309 messenger, it itself does not bind c-di-GMP (73). A *pilZ* mutant in *Xanthomonas campestris* pv. campestris
310 displays a minor defect in Type IV pilus-dependent motility (74) and this PilZ ortholog directly binds to the
311 PilB ATPase and the c-di-GMP interacting FimX protein (75). Yet, no homolog for FimX could be identified in
312 *L. pneumophila* and Tn-seq did not reveal any c-di-GMP synthesis enzyme required for natural
313 transformation. However, Tn-seq did show that PilB was important for transformation (Fig. 2A). We thus
314 speculate that, in *L. pneumophila*, PilZ controls Type IV pilus assembly independently of c-di-GMP signaling
315 and through a direct interaction with PilB.

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316 # Conclusion

317 We report on a clinical isolate of *L. pneumophila*, which displays phenotypes (intracellular replication,
318 competence for natural transformation) similar to commonly used laboratory strains. In contrast to laboratory
319 strains, a high-saturation Tn-seq library could be obtained and allowed to define essential genes, including
320 strain-specific genes in MGEs. Tn-seq analyses of transformation, with follow-up work performed in the Paris
321 strain, defined the set of major and minor Type IV pilins that are engaged in DNA uptake. While we here
322 focused on mutants that were strongly deficient for natural transformation, Tn-seq also identified potential
323 regulators of competence as well as genes of unknown function that also participate in natural transformation
324 (for instance *djlA* and *lpp3030*). We exemplify here that strain HL77S could represent a surrogate for the
325 commonly used lab strains to perform Tn-seq analysis. Unleashing the full power of Tn-seq is a major step
326 toward the identification of the genetic basis of traits that turned *L. pneumophila* into a successful pathogen,
327 such as its ability to form biofilms, resist biocides and unicellular predators.

328 # Material and methods

329 *Bacterial strains and growth conditions*

330 *Legionella pneumophila* strains were grown in liquid medium ACES [N-(2-acetamido)-2-aminoethanesulfonic
331 acid]-buffered yeast extract (AYE) or on solid medium ACES-buffered charcoal yeast extract (CYE) plates.
332 When appropriate kanamycin, gentamycin and streptomycin were added respectively at 15 µg/mL, 10 µg/mL
333 and 50 µg/mL. Clinical isolates of *L. pneumophila*, including HL-0709-3014 ("HL77"), were provided by the
334 Centre National de Référence des Légionelles, Lyon, France. A streptomycin-resistant of HL-0709-3014
335 mutant was obtained by plating 1 mL of culture on streptomycin-containing CYE plate and named HL77S.
336 The *Escherichia coli* MFDpir (76) with chromosome-integrated RP4 conjugative system was used as donor
337 strain for conjugative transfer of the mutagenesis system pBT20 (77) that carries a Himar1 transposon
338 bearing a gentamycin resistance gene and an outward-facing promoter. MFDpir is auxotrophic for
339 diaminopimelic acid (DAP) and thus was always cultivated with 1% DAP. Axenic *Acanthamoeba castellanii*
340 cells were grown in PYG medium (Proteose yeast extract glucose medium) at 30°C and split once or twice a
341 week. Human U937 cells were maintained in RPMI 1640 with 10 % heat-inactivated fetal calf serum and 1 %
342 penicillin/streptomycin at 37°C and 5 % CO_2. Differentiation into macrophages was induced by the addition
343 of PMA (phorbol 12-myristate 13-acetate) at a final concentration of 100 ng/ml. Murine macrophages RAW
344 264.7 were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C
345 and 5 % CO_2.

346 *Intracellular growth experiments*

347 The ability of HL77S strain to infect host cells was compared to the Paris strain. Paris Δ*dotA* was used as a
348 negative control as this mutant is unable to multiply in host cells. The ability of HL77S to replicate in the
349 amoeba *Acanthamoeba castellanii* was determined as follows. Amoebas were resuspended in PYG medium

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350 at a concentration of 1.10^6 amoebas/mL. The suspension was distributed in a flat-bottom 6-well plate (2 mL
351 per well, 2.10^6 amoebas per well) and incubated 3 h at 30°C to allow amoebas to settle and adhere to the
352 plate. 1 mL of a PY medium suspension containing 2.10^6 bacteria (from a culture in stationary phase OD~5)
353 were added in each well to obtain the multiplicity of infection (MOI) of 1. The plate was centrifuged 10 min at
354 650 g and incubated at 30°C for 72 h. At T=0, 48 h and 72 h, 250 µL of supernatant of each well were serial-
355 diluted and spotted onto CYE plates and incubated at 37°C for 72 h to determine the number of colony-
356 forming units (CFU) per mL. The ability of *L. pneumophila* strains to infect macrophages was determined as
357 follows. Overnight cultures of bacterial strains (OD~5 in AYE medium) were diluted (1:10) in the appropriate
358 cell culture media (DMEM for RAW 264.7 and RPMI 1640 for U937) and incubated for 1 h at 37°C. Host cells
359 (differentiated U937 and RAW 264.7) were seeded in 24-well plates, 3 wells per condition. Cells were
360 washed and were infected with bacteria at a MOI of 1 or 10. Plates were centrifuged at 500 g for 5 min to
361 promote bacteria-cell contact and incubated at 37°C and 5% CO2 for 24 h to 72 h. Every 24 h, the content of
362 one well per condition was transferred to an 1.5 mL tube and centrifuged at 16,000 g for 5 min. The pellet
363 containing the infected macrophages was resuspended in sterile distilled water to lyse the macrophages and
364 release the bacteria. The suspension was serial-diluted and spotted onto CYE plates and incubated at 37°C
365 for 72 h to determine the titer in CFU/mL.

366 *Plasmid and strains constructions*

367 Plasmid pJET1.2-legk2::kan, used for natural transformation experiments, was constructed by cloning a 6
368 kb-long fragment consisting of the *legk2::kan* gene (78) and 2 kb of its flanking regions in pJET1.2/blunt
369 cloning vector (ThermoFisher) according to the manufacturer's instructions. All the mutants generated in this
370 study are derived from *L. pneumophila* Paris or *L. pneumophila* Paris *rocC*$_{TAA}$. All the genes suspected to be
371 involved in natural transformation were deleted by replacement with a kanamycin resistance gene. To do so,
372 the upstream (PCRA, 2 kB) and downstream (PCRC, 2 kB) regions of each suspected genes were amplified
373 respectively with the primers pairs X_P1/X_P2-tail-pKD4 and X_P3-tail-pKD4/X_P4 (where X designated the
374 genes to be deleted). X_P2-tail-pKD4 and X_P3-tail-pKD4 carrying 30-nucleotide sequences complementary
375 to the ends of the kanamycin cassette. This complementarity was used to assemble PCRA and PCRC to the
376 kanamycin resistance cassette (PCRB, 1,490 kB amplified from plasmid pGEMPKD4 (31) with primers pair
377 pKD4_P1/pKD4_P2) by overlap extension PCR. Overlapping PCRs were naturally transformed in the
378 desired strain. Transformants were selected on CYE supplemented with kanamycin (15 µg/mL). Integration
379 of the kan cassette at the correct locus was finally verified by colony PCR. Plasmids p1890F and p0681F,
380 encoding the FLAG-tagged PilA_2 and PilE, were constructed by amplifying *lpp1890* (pilA_2) and *lpp0681*
381 (pilE) with primers lpp1890-F/lpp1890F-R and lpp0681-F/lpp0681F-R, respectively. The PCR products and
382 the recipient plasmid pMMB207C were digested with *Hin*dIII/*Bam*HI and ligated to place the genes under the
383 Ptac promoter. All strains, plasmids and oligonucleotides are listed in Table S1.

384 *Generation of transposon insertion mutants library of Legionella pneumophila*

385 Transposon-based random mutagenesis was performed as previously described (79) by conjugative delivery
386 of the Himar1-based transposon suicide vector pBT20 from the donor strain *E. coli* MFDpir to the recipient
387 strain of *L. pneumophila* to be mutagenized. To do so, both bacteria were cultivated overnight at 37°C with

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388 shaking in their corresponding liquid media : 7.5 mL LB broth containing 100 µg/mL ampicillin and 1% DAP
389 for *E. coli* and 15 mL standard AYE medium for *L. pneumophila*. Once in stationary phase (i.e; DO~5), the *L.*
390 *pneumophila* and *E. coli* cultures were concentrated by centrifugation (5,000 g, 10 min) and cell pellets were
391 resuspended respectively in 1.5 mL sterile water and 0.750 mL sterile PBS. To promote cell-to-cell contacts
392 and the subsequent conjugation, both concentrated cultures were mixed together by pipetting, and spotted
393 on CYE plates without iron and cystein but supplemented with DAP (CYED) (79) until the sample was
394 exhausted. Plates were incubated at 37°C for 5 to 6 h. All the spots were resuspended in sterile water and
395 used to inoculate transconjugants-selective plates (*i.e.,* CYE plates supplemented with 10 µg/mL of
396 gentamicin). In parallel, the suspension was ten-fold diluted and spotted onto transconjugants-selective
397 plates to evaluate the number of mutants in the library. After 72 h of incubation at 37°C, mutant library was
398 obtained by collecting all colonies from the plates and resuspending them in AYE-15% glycerol. The
399 suspension was aliquoted and stored at -80°C until its use for a Tn-seq screen. This library, called "initial
400 isolation" is named sample XRCR13.

401 *Natural transformation Tn-seq screen*

402 Transposon mutants of *L. pneumophila* HL77S were screened for their ability to undergo transformation. To
403 avoid a bottleneck effect, a volume of the -80°C frozen library containing ten times the number of mutants in
404 the library was spotted on a CYE plate supplemented with gentamycin (10 µg/mL) and streptomycin (50 µg/
405 mL) to obtain exponentially growing cells. After 24 h of incubation at 37°C, fresh bacteria from the spot were
406 resuspended in AYE to an OD~0.2. This suspension was used to perform transformation assays using 2 µg/
407 mL of either pGEM-ihfB-kan or pGET1.2-legK2-kan as transforming DNA, both conferring resistance to
408 kanamycin. For both transforming DNA, the transformation screen was conducted in duplicate. The
409 suspensions were cultivated at 30°C with shaking for 40 h to ensure that bacteria undergo transformation
410 and achieve an OD~5. These conditions were expected to give about ~10^5 transformants/mL for the “DNA”
411 conditions. Regarding the “no DNA” condition, cultures were diluted in AYE to obtain the same number of
412 CFUs on non-selective plates than the output condition on selective plates. Each sample (“+DNA” and “no
413 DNA”) was used to inoculate respectively nonselective (*i.e.,* CYE) and transformants-selective (*i.e.,* CYE
414 supplemented with 20 µg/mL of kanamycin) plates. In parallel, “no DNA” samples were tenfold diluted and
415 spotted on nonselective plates to determine transformation frequencies as mentioned above. After 72 h of
416 incubation at 37°C, colonies were collected from the plates and resuspended in AYE-15% glycerol until the
417 preparation of DNA libraries. The "no DNA" condition is also referred as the "second isolation" used in the
418 fitness analysis.

419 *DNA library preparation and sequencing*

420 Libraries were prepared as previously described (79). Mutant libraries from the -80°C frozen stock were
421 thawed and centrifuged at maximum speed to pellet them. gDNA extraction was carried out directly on the
422 pellet cells with the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer’s
423 instructions. ~30 µg of DNA were mechanically sheared by sonication using a Branson sonifier for 4 min (1
424 sec on and 11 sec off; 20 % intensity) in 0,5-mL PCR tubes kept on ice. Small gDNA molecules were
425 removed by mixing sonicated gDNA with 0.6X Agencourt Ampure XL magnetic beads (Beckman Coulter)

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426 according to manufacturer's instructions. These treatments led to gDNA fragments being between 300 and
427 1000 pb. Homopolymeric cytosine-tails (C-tail) were then added to the 3' ends of all fragments by incubation
428 of 3 µg of size-selected DNA fragments with the recombinant terminal deoxynucleotidyl transferase (rTdT, 30
429 U/µL, Promega) at 37°C for 1H, followed by heat inactivation at 75°C for 20 min. TdT reagents were then
430 removed by purifying the TdT reaction mixture with 1X of Ampure XL beads. To amplify transposon junctions,
431 a first-round of PCR (PCR1) was performed in a final volume of 50 µL by mixing 500 ng C-tailed DNA, 1 µL
432 biotinylated pBT20-PCR1 primer (30 µM), 3 µL olj376 primer (30 µM), 2.5 µL dNTPs (10 mM), 10 µL Q5
433 reaction buffer and 0.75 µL Q5 High-Fidelity DNA Polymerase (New England Biolabs). PCR1 products were
434 purified using 1X Ampure beads. Biotinylated and purified PCR1 products were then selectively captured
435 using Dynabeads M-280 Streptavidin (Invitrogen) according to manufacturer's instructions. A second-round
436 of PCR was carried out in a final volume of 50 µL by resuspending Dynabeads (which have PCR1 products
437 bound to them) in the pre-prepared PCR2 reaction mix constituted of 1 µL pBT20-PCR2 primer (30 µM), 1µL
438 TdT_index_X primer (30 µM), 2.5 µL dNTPs (10 mM), 10 µL Q5 reaction buffer and 0.75 µL Q5 High-Fidelity
439 DNA Polymerase. PCR2 products were purified with 1X Ampure XL beads. The obtained libraries were
440 sequenced on an Illumina HiSeq 4000 in single-end 50 pb using the custom sequencing primer Read1TnLp.
441 Samples and conditions are listed in Dataset S1. Essentiality analysis was performed using reads from
442 sample XRCR13. Fitness analysis was performed by comparing reads from samples XRCR24, 26, 36 and
443 38 (no DNA conditions from the transformation screen) versus XRCR13. Analysis of transformation was
444 performed by comparing samples XRCR27, 39 (legK2::kan transforming DNA) vs samples XRCR26, 38 (no
445 DNA control) and XRCR25, 37 (ihfB::kan transforming DNA) to samples XRCR24, 36 (no DNA control). Raw
446 sequencing reads were deposited to the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under the
447 study accession number PRJEB40244.

448 *Tn-seq data analysis*

449 For each condition, 10-50 million reads were obtained and trimmed with tools from the Galaxy's project
450 public server. Fastx_clipper was used to cut poly-C tails and remove short reads (<15 pb after polyC
451 clipping). Then, reads were filtered by quality using trimmomatic and quality checked with FastQC. Trimmed
452 reads in the fastq output file were mapped to the reference genome using Tn-seq software TPP (Tn-seq pre-
453 processor) (80). Output wig files from TPP were used to perform essentiality analysis using Transit (81).
454 Single-condition essentiality analysis was performed with the hmm (46) or Gumbel (45) methods. Conditional
455 essentiality analysis was performed with the "resampling" method according to the Transit software
456 documentation. Complete genome sequence of HL-0709-3014 was obtained (see *Genome sequence and*
457 *accession numbers*) and annotated with Prokka (82). An orthology search was carried out between the
458 strains of *L. pneumophila* HL77S, Paris and Philadelphia-1 using the orthology detection eggNOG mapper
459 (83) and COG and KEGG number were assigned to each gene.

460 *Transformation assays*

461 Natural transformation assays were conducted differently depending on the genetic background of *L.*
462 *pneumophila strain* used: (1) For the constitutively transformable *rocC*$_{TAA}$ strains, natural transformation was
463 conducted on solid medium at 37°C as follows. The strains were streaked on CYE solid medium from a

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464 frozen stock culture and incubated for 72 h at 37°C. The strains were then restreaked on a new CYE plate
465 and incubated overnight at 37°C to obtain freshly growing cells. Bacteria were resuspended in sterile water
466 to an OD600 of 1 to obtain a suspension of 1.10^9 colony forming units (CFUs) per milliliter. 10 µL of the
467 suspensions (~1.10^7 CFU) were spotted on CYE with 1.5 µg of transforming DNA. Once the spots are
468 absorbed by the agar, plates were incubated at 37°C for 24 h. Each spot was resuspended in 200 µL sterile
469 water and used to perform tenfold serial dilutions which were then plated on nonselective medium and
470 selective medium. Plates were incubated at 37°C for 72 h. Finally, transformation frequencies were
471 calculated as the ratio of the number of CFUs counted on selective medium divided by the number of CFUs
472 counted on nonselective medium. For all the *rocC*$_{TAA}$ strains, "rpsL" PCR product was used as transforming
473 DNA. This transforming DNA is obtained by amplificating the 2-kB regions upstream and downstream the
474 *rpsL* single point mutation conferring resistance to streptomycin (PCR primers pairs rpsL_F/rpsL_R).
475 Transformation experiments on strains bearing the p0681F and p1890F plasmids were performed the same
476 way, using CYE plates containing different concentration of IPTG. (2) For the non-constitutively
477 transformable strains of *L. pneumophila*, transformation was realized in liquid medium at 30°C as follows:
478 strains were streaked on CYE solid medium from a frozen stock culture and incubated for 72 h at 37°C and
479 then restreaked on a new CYE plate and incubated overnight at 37°C. Fresh bacteria were resuspended in 3
480 mL of AYE in 13-mL tubes to an OD~0.2 with 2 µg of transforming DNA and cultivated at 30°C with shaking
481 for 24 h. Tenfold serial dilution of each culture was then performed and plated on nonselective medium and
482 selective medium and incubated at 37°C for 72 h. Finally, transformation frequencies were determined as
483 described above. (3) For *letA* mutants of constitutively and non-constitutively transformable strains of *L.*
484 *pneumophila*: strains were streaked on CYE solid medium from a frozen stock culture and incubated for 72 h
485 at 37°C and then restreaked on a new CYE plate and incubated overnight at 37°C. Fresh bacteria were
486 resuspended in 3 mL of AYE in 13-mL tubes to an OD~0.2 and cultivated at 30°C with shaking until OD~2-4
487 (corresponding to the competence phase of *L. pneumophila*). A volume corresponding to 1.10^8 bacteria was
488 spotted on CYE plates with 1.5 µg the *rpsL* PCR product. The following steps were the same as for the
489 transformation of constitutively transformable *rocC*$_{TAA}$ strains as mentioned in (1).

490 *Detection of extracellular pilin by Western-Blot*

491 Strains bearing plasmid p1890F were grown overnight at 37°C on CYE containing 500 µM IPTG, and were
492 then resuspended in 2 mL AYE at an OD600~1.5. 1 mL of the suspension was then submitted to max-speed
493 vortex agitation for 1 min, and centrifuged 15 min at 21,000 g and 4°C. Supernatants were recovered in a
494 new tube and centrifuged again, while pellets were saved on ice. After centrifugation, 900 µL of supernatants
495 were recovered and proteins were precipitated by adding 100 µL of Trichloroacetic Acid (TCA, final
496 concentration of 10%). After 30 min of incubation on ice, a 15 min centrifugation at 21,000 g and 4°C was
497 performed. Pellets were washed three times with acetone, dried at room temperature and resuspended with
498 100 µL of Laemmli Sample Buffer 1X. Pellets previously saved on ice were resuspended with 150 µL of
499 Sample Buffer 1X. Samples were then analyzed by Western-blot. Aliquots were boiled for 5 min and
500 subjected to denaturing polyacrylamide gel electrophoresis. Proteins from SDS-polyacrylamide gels were
501 electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell) and subsequently
502 stained with Ponceau S (Sigma) to check the loading of the lanes. Membranes were incubated with
503 monoclonal Anti-FLAG antibody (dilution 1:1000, Sigma F1804) as a primary antibody and an anti-mouse

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504 peroxidase conjugate (dilution 1:50000, Sigma A0168) as secondary antibody. Nitrocellulose membranes
505 were revealed with the SuperSignal® West Dura detection system (Pierce) and an imaging workstation
506 equipped with a charge-coupled device camera (Thermo).

507 *Determination of the DNA uptake ability*

508 The ability of the transformation-deficient mutants to uptake DNA was determined as follows: strains were
509 inoculated in AYE media at an OD600 = 0.05 and tubes were incubated overnight under constant shaking at
510 30°C. When OD600 = 0.9 was reached, 1 mL of each culture was centrifuged 3 min at 5000g, and pellets
511 were resuspended in 200 µL ultrapure water containing 2 µg of pGEM-HYG1. This plasmid is non-replicative
512 plasmid in *L. pneumophila* and, as it contains no homology with *L. pneumophila* genome, it cannot integrate
513 by recombination either.. After 20 min of incubation at 37°C, tubes were centrifuged 3 min at 5000g and
514 pellets were resuspended in 200 µL AYE liquid medium containing 10 Units of DNase I (Sigma). After 20 min
515 of incubation at 37°C, DNase I was removed and bacteria were washed by two successive centrifugation 3
516 min at 5000 g and resuspension in 1 mL of water. Pellets were finally resuspended in 100 µL ultrapure water,
517 and incubate 30 min at 65°C to complete DNAse I inactivation and kill bacteria. DNA uptake ability of each
518 mutants was then determined by PCR, using two couples of primers amplifying on the one hand the
519 chromosomal *mreB* gene (*lpp0873*) and on the other hand a part of pGEM-HYG1, giving respectively PCR
520 products of 1194 pb (mreBseqF/mreBseqR) and 1657 pb (M13F(-47)/M13R(-48)).

521 *Microscopy*

522 Bacteria expressing the FLAG-tagged pilins were grown as spots on CYE plates with 0.5 mM IPTG for 24 h
523 at 37°C. Bacteria were gently collected with a pipette tip. In order to limit shearing and breaking of the pilus,
524 the pipette tip was left standing an eppendorf tube with 1 mL of water for a few minutes. Once the collected
525 bacterial culture is starting to dissociate and falling off from the tip, the bacterial pellet is resuspended gently
526 by slowly pipetting up and down. The collected 1 mL suspensions were centrifuged 3 min at 5000 g and
527 pellets were gently resuspended in 300 µL PBS Formaldehyde 3.7% and incubated at room temperature for
528 30 minutes. Acid-washed (ethanol/HCl 1M) glass coverslips were coated with poly-L-lysine by immersion in a
529 poly-L-lysine 0.01% solution in distilled water (Sigma-Aldrich) for 5 minutes. Fixed bacteria in PBS
530 Formaldehyde 3.7% were pipetted (250 µl) on the air-dried coverslips and let to settle and stick to the
531 coverslips for about 30 minutes. Coverslips were then washed twice with PBS, and incubated with
532 monoclonal anti-FLAG M2 fluorescein conjugates at 1/200 in PBS for 1h. Coverslips were then washed twice
533 with PBS and DNA was labeled using Hoechst 33288 (12 µg/mL in PBS) 1 h. Coverslips were washed twice
534 in PBS and mounted using 8 µL of mounting solution (DAPCO). After an overnight incubation at 4°C slides
535 were observed and imaged with an epifluorescence microscope (Zeiss Axioplan 2).

536 *Genome sequence and raw reads accession numbers*

537 The complete genome of isolate HL-0709-3014 was obtained using Illumina MiSeq paired end reads from
538 previously available SRA sample ERS1305867 and long reads from Oxford Nanopore sequencing on a

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539 MinION sequencer according to manufacturer's instructions (Oxford Nanopore). Illumina and Nanopore
540 reads were then used for short reads/long reads hybrid assembly using Unicycler v0.4.6 (84). The complete
541 genome of isolate HL-0709-3014 is available under accession numbers CP048618.1 (chromosome) and
542 CP048619.1 (plasmid). The strain is listed under the name *Legionella pneumophila* strain ERS1305867
543 (BioProject: PRJEB15241; BioSample: SAMEA4394418). Raw sequencing reads of the Tn-seq samples are
544 available at the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under the study accession number
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555 LH, PAJ and BCG designed and performed experiments, and analyzed data. LH and XC analyzed Tn-seq
556 data. LH and XC wrote the manuscript. XC conceptualized and supervised the project.

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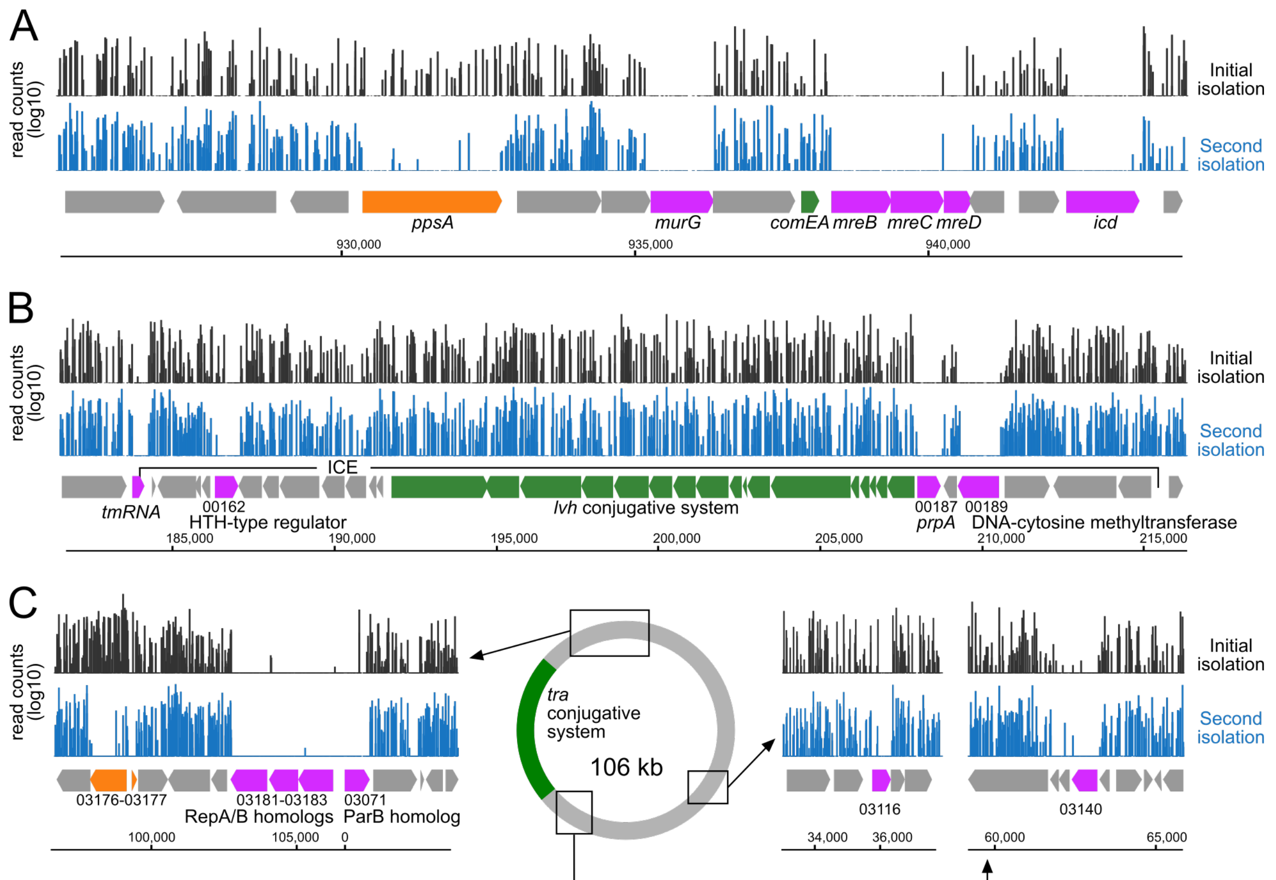
558 # Figures



559 **Figure 1**. Tn-seq analysis of *L. pneumophila* strain HL77S. A) Log10 reads counts of transposon insertions
560 after initial library isolation (black) and second isolation (blue). Genes identified as essential are colored in
561 magenta, fitness determinants are colored in orange. Other genes of interest are colored in green. B)
562 Transposon-insertion coverage in a region encompassing an integrative conjugative element (ICE) harboring
563 essential genes (magenta) and genes encoding a conjugative system (green). The duplicated sequence
564 gcgggttcgattcccgccgcctccacca of the tmRNA and located 66 kb away delineate the boundaries of the ICE. C)
565 Essential genes and fitness determinants in the conjugative plasmid of HL77S.

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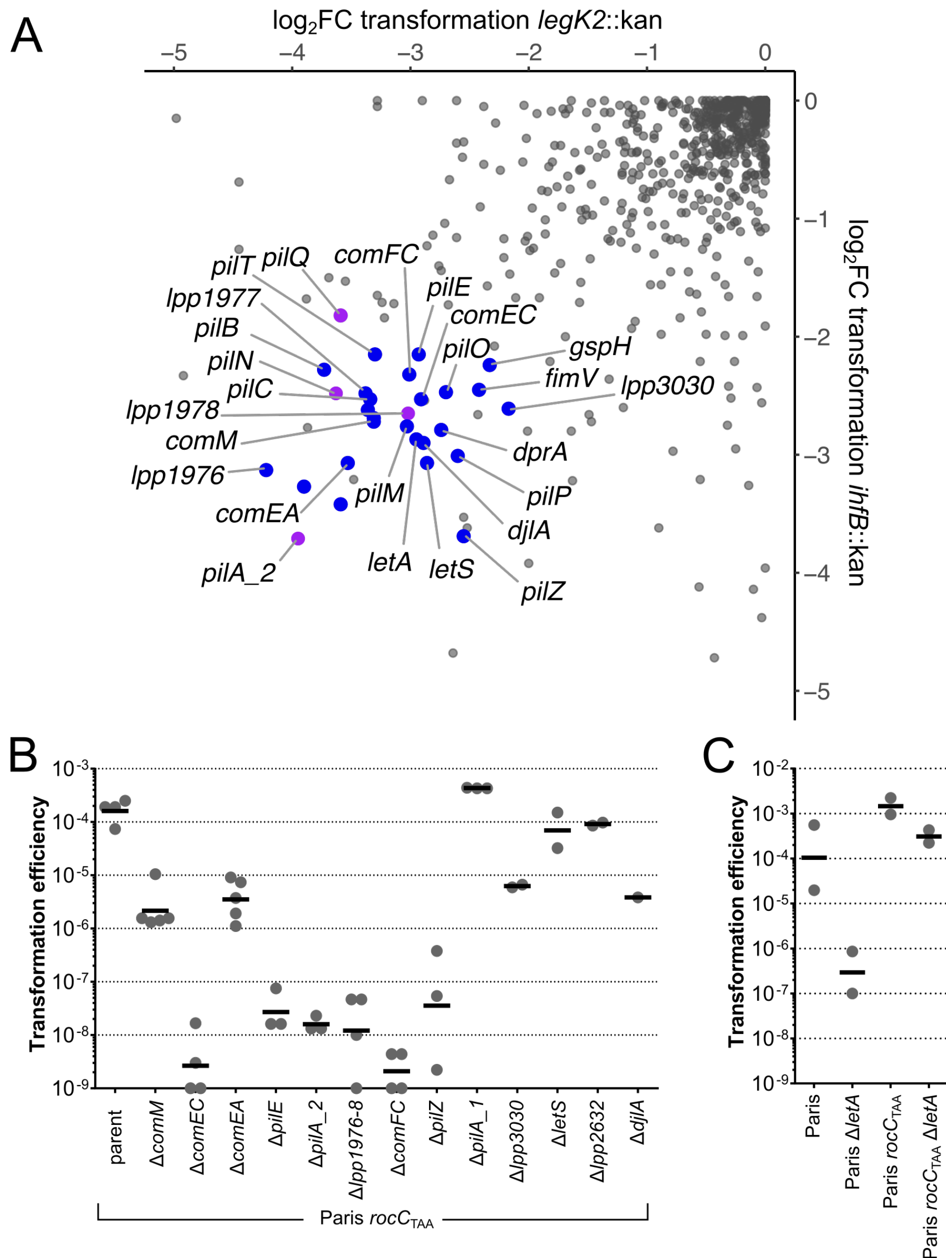

566 **Figure 2**. Identification of genes required for natural transformation by Tn-seq. A) Scatter plot of fold-change
567 (log_2) of insertions in the corresponding genes in two tested transformation conditions. HL77S was subjected
568 to natural transformation with a 4kb-PCR fragment of the *legK2* or *ihfB* genes interrupted by a kanamycin
569 resistance gene. Reads count per genes were determined and expressed as fold-change between the non-
570 transformed population and the *legK2*::*kan* or *ihfB*::*kan* transformed populations. Individual genes (gray dots)
571 were considered required for natural transformation if log_2FC was >2 or <-2 and if P<0.05 in one (magenta
572 dots) or both conditions (blue dots). B) Natural transformation efficiency of reconstructed mutants in the Paris
573 *rocC*$_{TAA}$ strain which is constitutively competent for natural transformation. Transformation experiments were
574 performed at least three times independently and transformation frequencies were plotted (grey dots) along
575 with the geometric mean (black line). C) Natural transformation efficiency of the reconstructed mutant Δ*letA*
576 in the original Paris strain and constitutively competent Paris *rocC*$_{TAA}$ strain. Transformation experiments
577 were performed twice independently and transformation frequencies were plotted (grey dots) along with the
578 geometric mean (black line).

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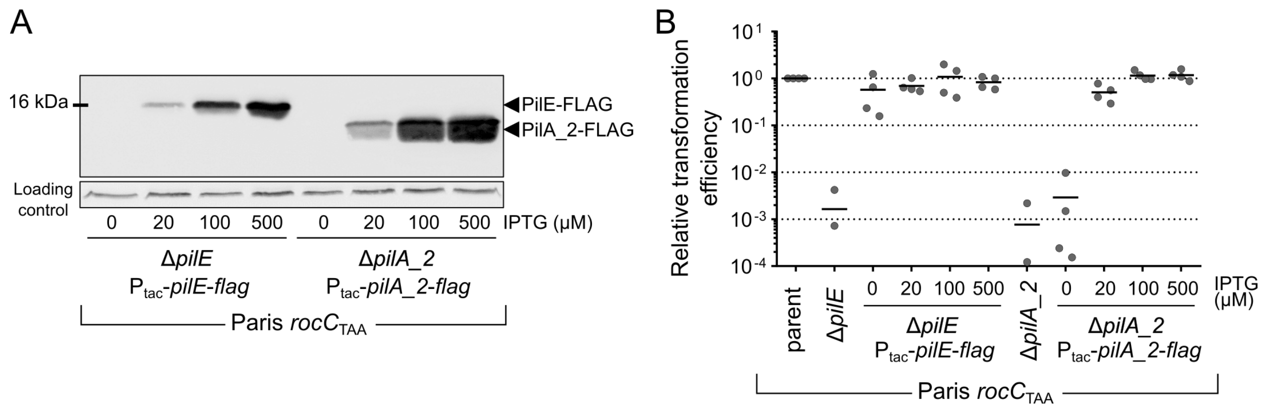
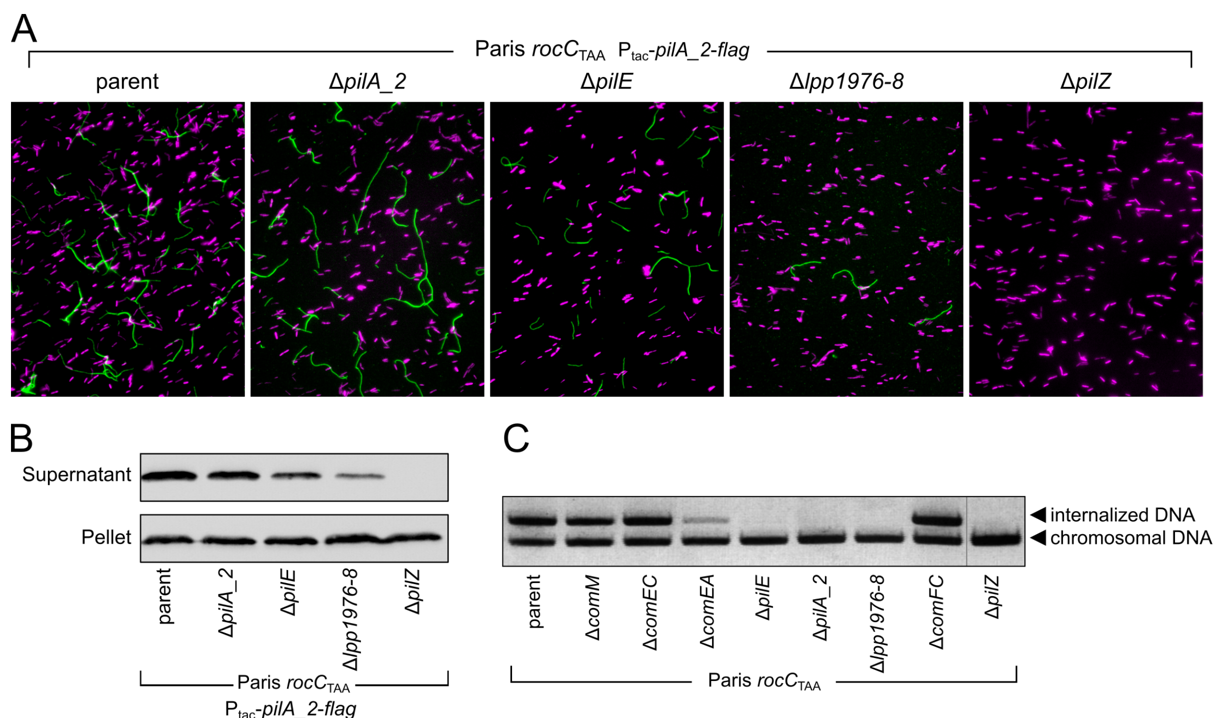


579 **Figure 3**. PilA_2 is the major pilin of the *L. pneumophila* transformation pilus. A) Western-blot analysis of
580 ectopically-expressed PilA_2-FLAG (encoded by p1890F) and PilE (encoded by p0681F) as a function of the
581 IPTG inducer. B) Complementation of the Δ*pilE* and Δ*pilA_2* mutants in the Paris *rocC*$_{TAA}$ strain by the
582 ectopic expression of PilA_2-FLAG (encoded by p1890F) and PilE (encoded by p0681F). Transformation
583 frequencies were determined four times independently as a function of the IPTG inducer, and normalized to
584 1 for the parental strain (Paris *rocC*$_{TAA}$).



585 **Figure 4**. PilA_2 assembly into extracellular filaments depends on *pilE*, the operon *lpp1976-8* and *pilZ*. A)
586 Visualization of PilA_2-FLAG filaments (green) by immunofluorescence microscopy using fluorescein-
587 conjugated anti-FLAG antibody. Bacteria were visualized by labeling DNA with Hoechst 33288 (magenta). B)
588 Western-blot detection of extracellular PilA_2. Bacteria were vortexed to release pili which were precipitated
589 from supernatants. PilA_2-FLAG was detected in supernatant and whole cell lysates (pellet) using Anti-FLAG
590 antibodies. C) DNA uptake assay of the reconstructed mutants defective for natural transformation. The
591 ability of the transformation-deficient mutants were tested for the ability to internalize pGEM-HYG1, a non-
592 replicative plasmid. Following incubation with the DNA and subsequent DNAse I treatment, the internalized
593 DNA was detected in cells by PCR for pGEM-HYG. As control, chromosomal DNA was also detected by
594 PCR. This multiplex PCR was analyzed by agarose gel electrophoresis and labeling of DNA with ethidium
595 bromide.

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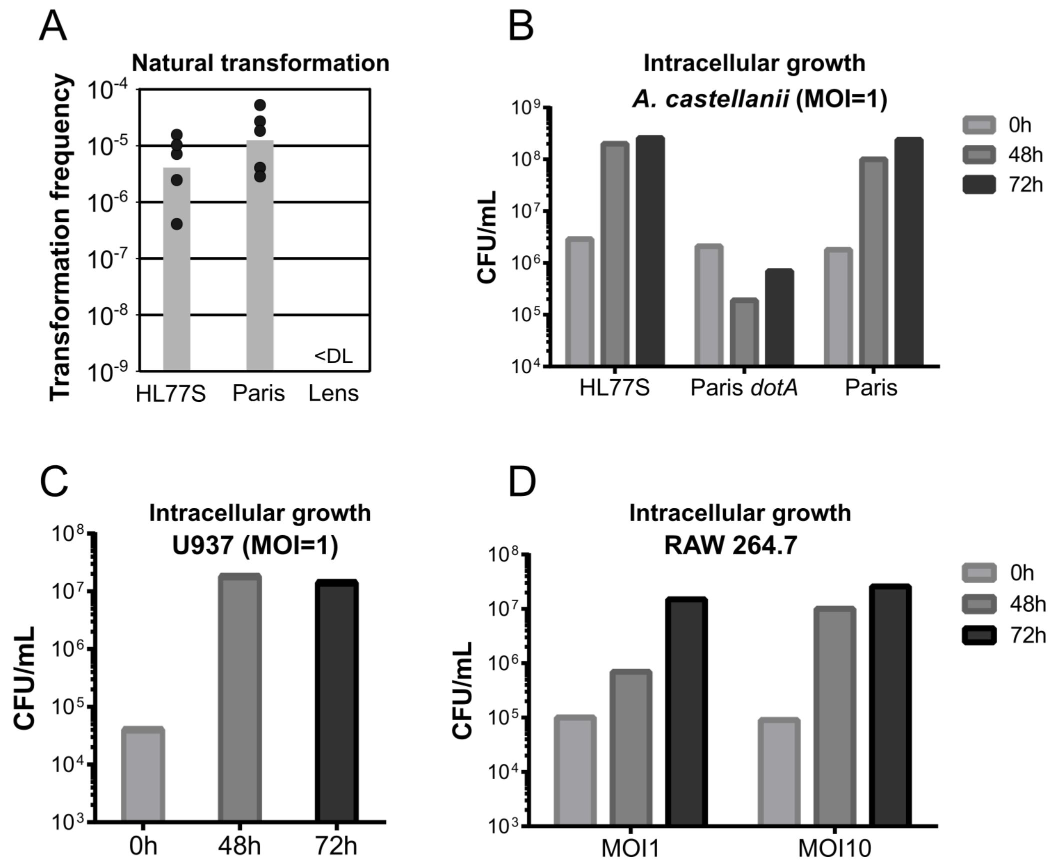

596 **Figure S1**. Phenotypic characterization of strain HL77S, a streptomycin-resistant mutant of the clinical
597 isolate HL-0709-3014. A) Natural transformability of HL77S compared to the Paris and Lens strain.
598 Transformation was tested by growing the strains in AYE at 30°C for 24h in the presence of 2 µg of
599 transformation DNA consisting of a kanamycin resistance gene interrupting the *ihfB* gene. B) Intracellular
600 replication of HL77S in the amoeba *Acanthamoeba castellanii*. Cells were infected with a suspension of
601 HL77S at a multiplicity of infection (MOI) of 1. At the initial time point, after 48 h and 72 h of culture at 30°C,
602 colony-forming units are determined by plating on CYE medium. C) Intracellular replication of HL77S in
603 differentiated human monocytes of the U937 cell line. Cells were infected with a suspension of HL77S at a
604 MOI of 1. At the initial time point, after 48 h and 72 h of culture at 30°C, colony-forming units are determined
605 by plating on CYE. D) Intracellular replication of HL77S in the murine macrophage-like cell line RAW 264.7.
606 Cells were infected with a suspension of HL77S at a MOI of 1 or 10. At the initial time point, after 48 h and 72
607 h of culture at 30°C, colony-forming units are determined by plating on CYE.

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608 **Table S1.** Strains, plasmids and oligonucleotides used in this study

Strains

Name	Genotype	Source
Paris WT	Wild-type *Legionella pneumophila*	Paris Outbreak isolate CIP107629, CNR Lyon
Paris_S	Spontaneous streptomycin-resistant mutant of Paris; StrepR	This study
HL-0709-3014	Wild-type *Legionella pneumophila*	Clinical isolate, CNR Lyon
HL77S	Spontaneous streptomycin-resistant mutant of HL-0709-3014; StrepR	This study
Paris $rocC_{TAA}$	Paris $rocC_{TAA}$ *(=lpp0148*$_{TAA}$*); rocC* allele with a premature stop codon TAA	Juan P-A, Attaiech L, Charpentier X. Scientific Reports 5:16033, 2015, https://doi.org/10.1038/srep16033
Paris $rocC_{TAA}$ *letS::kan*	Paris $rocC_{TAA}$; *lpp1887::kan; KanR*	This study
Paris $rocC_{TAA}$ *letA::kan*	Paris $rocC_{TAA}$; *lpp2699::kan; KanR*	This study
Paris $rocC_{TAA}$ *comM::kan*	Paris $rocC_{TAA}$; *lpp2632::kan; KanR*	This study
Paris $rocC_{TAA}$ *comEC::kan*	Paris $rocC_{TAA}$; *lpp0680::kan; KanR*	This study
Paris $rocC_{TAA}$ *pilE::kan*	Paris $rocC_{TAA}$; *lpp0681::kan; KanR*	This study
Paris $rocC_{TAA}$ *comEA::kan*	Paris $rocC_{TAA}$; *lpp0872::kan; KanR*	This study
Paris $rocC_{TAA}$ *pilA_1::kan*	Paris $rocC_{TAA}$; *lpp1890::kan; KanR*	This study
Paris $rocC_{TAA}$ *lpp1976-1977-1978::kan*	Paris $rocC_{TAA}$; *lpp1976-1977-1978::kan; KanR*	This study
Paris $rocC_{TAA}$ *comF::kan*	Paris $_{rocCTAA}$; *lpp2280::kan; KanR*	This study
Paris $rocC_{TAA}$ *pilA_2::kan*	Paris $rocC_{TAA}$; *lpp1889::kan; KanR*	This study
Paris $rocC_{TAA}$ *pilZ::kan*	Paris $rocC_{TAA}$; *lpp1356::kan; KanR*	This study
Paris $rocC_{TAA}$ *lpp3030::kan*	Paris $rocC_{TAA}$; *lpp3030::kan; KanR*	This study
Paris $rocC_{TAA}$ *djlA::kan*	Paris $rocC_{TAA}$; *lpp2289::kan; KanR*	This study
Paris $rocC_{TAA}$ *lpp2632::kan*	Paris $rocC_{TAA}$; *lpp2632::kan; KanR*	This study
Paris *letA::kan*	Paris *lpp2699::kan; KanR*	This study
Paris *dotA::kan*	Paris *dotA::kan; KanR*	This study
MFDpir	*Escherichia coli*; MG1655 RP4-2-Tc::[ΔMu1::aac(3)IV-ΔaphA-Δnic35-ΔMu2::zeo] ΔdapA::(erm-pir) ΔrecA	Ferrières L et al,. J Bacteriol 192:6418–6427, 2010, https://doi.org/10.1128/jb.00621-10

Plasmids

Name	Genotype	Description	source
pGEM-ihfB::kan	pGEM-T Easy:: *ihfB*::*nptII*; AmpR, KanR	plasmid bearing the *ihfB* gene of *L. pneumophila* Paris interrupted by a kanamycin cassette ; Kan R	Juan P-A, Attaiech L, Charpentier X. 2015. Scientific Reports 5:16033, 2015, https://doi.org/10.1038/srep16033
pGEM-HYG1	pGEM-T Easy::*hygR*	plasmid bearing an hygromycine cassette ; HygR	This study

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pJET1.2-legk2::kan	pJET1.2 ; *legk2*::kan from HL77 ; AmpR, KanR	plasmid bearing the *legK2* gene of L. pneumophila HL77 interrupted by a kanamycin cassette ; Kan R	This study
pBT20	Gmr; Himar1C9 transposon	Pir-dependent, mobilizable plasmid carrying the Mariner Himar1 transposon with gentamycin resistant gene	Kulasekara HD et al,. Molecular Microbiol 55:368–380, 2005, https://doi.org/10.1111/j.1365-2958.2004.04402.x
pMMB207C	RSF1010::*lacI^q-tacp mobA*	cloning vector for expression genes under the Ptac promoter, derived from RSF1010 ; CmR	Segal, G. & Shuman, H. A. Molecular Microbiology 30, 197–208, 1998, https://doi.org/10.1046/j.1365-2958.1998.01054.x
p0681F	pMMB207C ; lpp0681::FLAG	complementation plasmid for the lpp0681::kan mutant, expressing the lpp0681-FLAG (PilE-FLAG) fusion protein ; CmR	This study
p1890F	pMMB207C ; lpp1890::FLAG	complementation plasmid for the lpp1890::kan mutant, expressing the lpp1890-FLAG (PilA_2-FLAG) fusion protein ; CmR	This study

Oligonucleotides

Name	sequence	use
LH1_lpp3030_P1	ctgcatgggatattggtatcactgc	Forward primer to amplify a 2kB fragment uptstream of lpp3030
LH2_lpp3030_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCtggcgatgataaacaagcgagagcc	Reverse primer to amplify a 2kB fragment uptstream of lpp3030
LH3_lpp3030_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCgaaaatctgccgatactgtttctgc	Forward primer to amplify a 2kB fragment downstream of lpp3030
LH4_lpp3030_P4	ggtcccatctcattctccttaatcc	Reverse primer to amplify a 2kB fragment downstream of lpp3030
LH5_lpp1887_P1	gggtaatttcctgagacagtggagg	Forward primer to amplify a 2kB fragment uptstream of letS
LH6_lpp1887_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCggcaggaataagagtagtgattcttagc	Reverse primer to amplify a 2kB fragment uptstream of letS
LH7_lpp1887_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCctaatgctgataatttaaccggagcc	Forward primer to amplify a 2kB fragment downstream of letS
LH8_lpp1887_P4	ggtggaggtaccatagttatgaccc	Reverse primer to amplify a 2kB fragment downstream of letS
LH9_lpp2699_P1	ggtatcctgtctgacagtctaaacc	Forward primer to amplify a 2kB fragment uptstream of letA
LH10_lpp2699_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCtcccattctaaccaatgcatggtc	Reverse primer to amplify a 2kB fragment uptstream of letA
LH11_lpp2699_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCcgcattattgaacaccccaatg	Forward primer to amplify a 2kB fragment downstream of letA
LH12_lpp2699_P4	caggggaaatcaaagacattgcc	Reverse primer to amplify a 2kB fragment downstream of letA
LH21_lpp2632_P1	cggcttcttgatattattgcgagac	Forward primer to amplify a 2kB fragment uptstream of lpp2632
LH22_lpp2632_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCcgagacacactatctcgtatcatgc	Reverse primer to amplify a 2kB fragment uptstream of lpp2632
LH23_lpp2632_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCgtgcatactttggtcttgggaagac	Forward primer to amplify a 2kB fragment downstream of lpp2632
LH24_lpp2632_P4	gatggagaaatttttccgctcatcc	Reverse primer to amplify a 2kB fragment downstream of lpp2632
LH29_lpp2289_P1	caggaatgttcacactgaattttcc	Forward primer to amplify a 2kB fragment uptstream of djlA
LH30_lpp2289_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCccccaccaggttgttattacaaag	Reverse primer to amplify a 2kB fragment uptstream of djlA

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LH31_lpp2289_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCgctcaaggtttacccgaagaaatg	Forward primer to amplify a 2kB fragment downstream of djlA
LH32_lpp2289_P4	cgagtcagggaagtgtttacagg	Reverse primer to amplify a 2kB fragment downstream of djlA
rpsL_Fw	GCAGCTCCAGATGGCTCAATC	Forward primer to amplify a 2kB fragment downstream of rpsL
rpsL_Rv	CAACCATACATGTCCATATTGACCAC	Reverse primer to amplify a 2kB fragment uptstream of rpsL
lpp0640_P1	TCATCCAACTCATCTCGCAATCG	Forward primer to amplify a 2kB fragment uptstream of comM
lpp0640_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCACTGCTGTTTCAGCAAGTCCT ACC	Reverse primer to amplify a 2kB fragment uptstream of comM
lpp0640_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCGTCTGCTCGTGGCTACCATCGC	Forward primer to amplify a 2kB fragment downstream of comM
lpp0640_P4	GCTGTAGGACAGCGGCTAACTTG	Reverse primer to amplify a 2kB fragment downstream of comM
lpp0681_P1	ATGGGAGCTGGCGTAGATCCTG	Reverse primer to amplify a 2kB fragment downstream of pilE
lpp0681_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCGGCAATTGAAACCAGAATGC CC	Reverse primer to amplify a 2kB fragment downstream of pilE
lpp0681_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCAAACAACGCCGAACGGGTAC	Reverse primer to amplify a 2kB fragment downstream of pilE
lpp0681_P4	CATTGCCATTGCGGGTATGAATG	Reverse primer to amplify a 2kB fragment downstream of pilE
lpp1889_P1	GTGAACTGCAGCAAGCTCCATCC	Reverse primer to amplify a 2kB fragment downstream of pilA_1
lpp1889_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCGATGGCAACCAGAATCCCAA G	Reverse primer to amplify a 2kB fragment downstream of pilA_1
lpp1889_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCTGCAAACGCAGGTAATGGCAC	Reverse primer to amplify a 2kB fragment downstream of pilA_1
lpp1889_P4	GCGAAATGGCCGTTACTGCTTG	Reverse primer to amplify a 2kB fragment downstream of pilA_1
lpp1890_P1	GTCAGGTAATAACCGGGTTTGCC	Reverse primer to amplify a 2kB fragment downstream of pilA_2
lpp1890_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCGCGATTGCTGCCAAAATACCG	Reverse primer to amplify a 2kB fragment downstream of pilA_2
lpp1890_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCAATTGCTATTGGTGCGAACGG	Reverse primer to amplify a 2kB fragment downstream of pilA_2
lpp1890_P4	CAGTCTGGTGGTGTGACCGCTG	Reverse primer to amplify a 2kB fragment downstream of pilA_2
lpp2280_P1	CCGGCAACTGGAAAAGGGAG	Reverse primer to amplify a 2kB fragment downstream of comF
lpp2280_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCTCCATGCAATTAGAGCAAACT GC	Reverse primer to amplify a 2kB fragment downstream of comF
lpp2280_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCAAGCCAACCTTGATGGAGAGC	Reverse primer to amplify a 2kB fragment downstream of comF
lpp2280_P4	GCCCGCAAGCTACAAATACCATAG	Reverse primer to amplify a 2kB fragment downstream of comF
lpp1976-1978_P1	CAATCAATCAAACTCTCTCAAGAACG	Reverse primer to amplify a 2kB fragment downstream of the operon of unknown function
lpp1976-1978_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCGCCAGATAAGATGATTGATTA GATCTC	Reverse primer to amplify a 2kB fragment downstream of the operon of unknown function
lpp1976-1978_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCGGTGAAGGAAGGGGTAAGCA GC	Reverse primer to amplify a 2kB fragment downstream of the operon of unknown function
lpp1976-1978_P4	GAAAAATGAATGGGAGCTTCTGG	Reverse primer to amplify a 2kB fragment downstream of the operon of

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		unknown function
lpp0872_P1	GTAGTGGGAGGAAGCTTAGG	Reverse primer to amplify a 2kB fragment downstream of comEA
lpp0872_P2-tail-pKD4	GGAACTTCGAAGCAGCTCCAGCCTAC ACAATCGAGGCTCGTTCTCAGCTTGA GAAGAG	Reverse primer to amplify a 2kB fragment downstream of comEA
lpp0872_P3-tail-pKD4	GAACTAAGGAGGATATTCATATGGACC ATGGCGCGGAAGTCAAGGGTATAGG	Reverse primer to amplify a 2kB fragment downstream of comEA
lpp0872_P4	GCGACAGGGCTAACTGTAAC	Reverse primer to amplify a 2kB fragment downstream of comEA
olj376	GTGACTGGAGTTCAGACGTGTGCTCT TCCGATCTGGGGGGGGGGGGGGGG	Primer for Tn-seq to amplify transposon junction and add polyC-tail during PCR1
pBT20-PCR1	Biotin-TCGTATAATGTGTGGAATTGTGAGCGG	Biotinylated primer for Tn-seq to amplify transposon junction during PCR1
pBT20-PCR2	AATGATACGGCGACCACCGAGATCTAC ACTCTTTGGACTCTAGAGGATCACCCA GCTTTCTTG	Primer for Tn-seq to amplify transposon junction during PCR2
TdT_Index_X	CAAGCAGAAGACGGCATACGAGATXX XXXXGTGACTGGAGTTCAGACGTGTG CTCTTCCGATCT	HPLC-purified primer for Tn-seq to amplify transposon junction and add index for multiplexing during PCR2
Read1TnLp	CTAGAGACCGGGGACTTATCAGCCAA CCTGTTA	HPLC-purified custom sequencing primer for Tn-seq library sequencing
lpp0681-F	CCGGGGATCCGCAAATTCAATAGAGG ATACCCAAATG	cloning of lpp0681 in pMM207C
lpp0681F-R	AGCCAAGCTTTTACTTGTCATCGTCGT CCTTGTAATCAGCGCTACCGGGATTCC AGCATTCTGGTTG	cloning of lpp0681 in pMM207C
lpp1890-F	CCGGGGATCCGTTAACTATGGAGATGG TCATGAGAC	cloning of lpp1890 in pMM207C
lpp1890F-R	AGCCAAGCTTTTACTTGTCATCGTCGT CCTTGTAATCAGCGCTTGGTCTGCAGC TGGCAGGTCG	cloning of lpp1890 in pMM207C
mreBseqF	TCCGGTTAAAAGCAGTTGTCTGG	Forward primer to amplify *mreB*, chromosomal control for uptake test
mreBseqR	CCCAGAGAACTGTGTCCGCCC	Reverse primer to amplify *mreB*, chromosomal control for uptake test
M13F(-47)	CGCCAGGGTTTTCCCAGTCACGAC	Forward primer to amplify a 1657 pb fragment of the pGEM-HYG1
M13R(-48)	AGCGGATAACAATTTCACACAGGA	Reverse primer to amplify a 1657 pb fragment of the pGEM-HYG1