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Abstract

Background: *Legionella pneumophila* is an intracellular pathogen of environmental protozoa. When humans inhale contaminated aerosols this bacterium may cause a severe pneumonia called Legionnaires' disease. Despite the abundance of dozens of *Legionella* species in aquatic reservoirs, the vast majority of human disease is caused by a single serogroup (Sg) of a single species, namely *L. pneumophila* Sg1. To get further insights into genome dynamics and evolution of Sg1 strains, we sequenced strains Lorraine and HL 0604 1035 (Sg1) and compared them to the available sequences of Sg1 strains Paris, Lens, Corby and Philadelphia, resulting in a comprehensive multigenome analysis.

Results: We show that *L. pneumophila* Sg1 has a highly conserved and syntenic core genome that comprises the many eukaryotic like proteins and a conserved repertoire of over 200 Dot/Icm type IV secreted substrates. However, recombination events and horizontal gene transfer are frequent. In particular the analyses of the distribution of nucleotide polymorphisms suggests that large chromosomal fragments of over 200 kbs are exchanged between *L. pneumophila* strains and contribute to the genome dynamics in the natural population. The many secretion systems present might be implicated in exchange of these fragments by conjugal transfer. Plasmids also play a role in genome diversification and are exchanged among strains and circulate between different *Legionella* species.

Conclusion: Horizontal gene transfer among bacteria and from eukaryotes to *L. pneumophila* as well as recombination between strains allows different clones to evolve into predominant disease clones and others to replace them subsequently within relatively short periods of time.

Background

Legionella pneumophila is the etiologic agent of Legionnaires' disease, an atypical pneumonia, which is often fatal if not treated promptly. However, it is principally an environmental bacterium that inhabits fresh water reservoirs worldwide where it parasitizes within free-living protozoa but also survives in biofilms [1-3]. Since *L. pneumophila* does not spread from person-to-person, humans have been inconsequential for the evolution of this pathogen. Instead, the virulence strategies of *L. pneumophila* have been shaped by selective pressures in

aquatic ecosystems. Indeed, the co-evolution of *L. pneumophila* with fresh-water amoebae is reflected in its genome sequence. The analysis of two *L. pneumophila* genomes identified the presence of an unexpected high number and variety of eukaryotic-like proteins and proteins containing motifs mainly found in eukaryotes [4]. These proteins were predicted to interfere in different steps of the infectious cycle by mimicking functions of eukaryotic proteins [4]. For several of these eukaryotic like proteins it has been shown recently that they are secreted effectors that help *L. pneumophila* to subvert host functions to allow intracellular replication [5,6]. The possibility that *L. pneumophila* has acquired at least some of these genes through horizontal gene transfer from eukaryotes has been suggested by two studies [7,8].

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Plasticity is another specific feature of the *L. pneumophila* genomes as integrative plasmids, putative conjugation elements and genomic islands were identified. In addition to DNA interchange between different bacterial genera and even domains of life, horizontal gene transfer within the genus *Legionella* and within the species *L. pneumophila* has been reported. For example a 65-kb pathogenicity island described first in *L. pneumophila* strain Philadelphia [9] is present in several *L. pneumophila* strains and also in other *Legionella* species like *L. anisa* [10]. Another example is the particular lipopolysaccharide cluster of serogroup 1 strains that has been detected in *L. pneumophila* strains of different lineages and genetic backgrounds [10]. *L. pneumophila* has all necessary features for incorporating foreign DNA, as these bacteria are naturally competent and possess an intact recombination machinery [11,12]. These findings suggest that the *L. pneumophila* genomes are very dynamic and one would expect that horizontal gene transfer and recombination events play an important role in their evolution.

However, different analyses like early studies applying multilocus enzyme electrophoreses (MEE) supported a clonal population structure of *L. pneumophila* [13]. Two recent reports using genetic profiling based on six or three genetic loci, respectively concluded also that *L. pneumophila* shows a clonal populations structure [14,15] although the presence of few recombination events was not ruled out. Later the analysis of the *dotA*, *mip* and *rpoB* genes in different isolates suggested for the first time that recombination may play some role in *L. pneumophila* evolution [16-18] and a more in depth analysis using over 20 loci suggested that recombination events might be more frequent than was previously thought [19]. However, comparisons of these studies are difficult due to different sampling and different analysis methods used. Furthermore there may be a bias associated with some of the genes selected in these studies like intergenic spacer regions or genes under positive selection that may lead to artefactual effects in detecting recombination. To solve these problem efforts have been undertaken recently to homogenize the results obtained for different species to allow comparisons [20]. These authors report for *L. pneumophila* a low recombination rate like for the obligate pathogens *Bordetella pertussis* or *Bartonella henselae*. In contrast Coscolla and colleagues suggest a more important role for recombination at the intergenic level [21].

These different results and the fact that a globally distributed *L. pneumophila* clone implicated in Legionnaires' disease has been described [10] may suggest that the role of recombination is not relevant. However, the description of clonal complexes is not incompatible with high recombination rates. Transient clones may appear within a recombining population [22], in particular if clones with high disease prevalence appear, as this seems to be the case for some *L. pneumophila* strains. These clones are often vastly over-sampled due to their clinical importance and show strong clonality. Thus, this may be correct for this subgroup, but it may not be representative for the population. Indeed when analyzing over 200 clinical and environmental *L. pneumophila* strains, significantly less diversity was found among the clinical isolates [23].

In this study we investigated the genome dynamics and evolution of the species *L. pneumophila* by analyzing horizontal gene transfer, mobile genetic elements and recombination on a genome-wide level. We undertook this analysis based on six complete genome sequences four of which are the previously published reference genomes of *L. pneumophila* Paris, Lens [4], Corby [24] and Philadelphia [25] and two that were sequenced in this study. The newly sequenced strains were selected according to epidemiological features that might be reflected in their genomes and should thus allow to study genome dynamics with respect to virulence. Strain Lorraine is rarely isolated from the environment but its prevalence in human disease is increasing considerably in the last years [26]. In contrast, *L. pneumophila* strain HL 0604 1035 has been frequently isolated from a hospital water system since over 10 years but has never caused disease. Analysis of these six strains identified a highly conserved and syntenic core genome and a diverse accessory genome. Furthermore, it showed that recombination events and horizontal gene transfer are frequent in *L. pneumophila*. Horizontal gene transfer from eukaryotes as well as recombination between strains were identified suggesting that *L. pneumophila* genomes are highly dynamic, a feature allowing different clones to evolve into predominant disease clones and others to replace them subsequently within relatively short periods of time.

Results and discussion

The *L. pneumophila* core genome comprises over 2400 conserved genes that are highly syntenic

To get comprehensive insight into the genetic basis, evolution and genome dynamics of *L. pneumophila* Sg1, the strains responsible for over 90% of disease worldwide, we analyzed six completely sequenced genomes. The strains selected are all of Sg1, have endemic and/or epidemic character (*e.g.* Paris, Lorraine or Philadelphia) were isolated in different countries (France, England, Spain, US) and in different years. Two strains were newly sequenced for this study (Lorraine and HL 0604 1035), the other four *L. pneumophila* genomes (Paris, Lens, Philadelphia, Corby) have been published previously [4,24,25]. The genomes of *L. pneumophila*

Table 1 General features of the 6 *L. pneumophila* strains analyzed

L. pneumophila strains	Philadelphia	Paris	Lens	Corby	HL06041035	Lorraine
Chromosome size (bp)	3397754	3503610	3345687	3576469	3492535	3467254
G+C content (%)	38.27	38.37	38.42	38.48	38.35	38.36
N° of genes	3031	3123	2980	3237	3132	3117
N° of protein coding genes	2999	3078	2921	3193	3079	3080
Pseudogenes	55	71	84	59	73	48
tRNA	43	43	43	44	43	44
16S/23S/5S	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3
Average length CDS (nts)	1082.47	1000.85	1008.76	984.35	995.47	988.54
Average length ig (nts)	147.72	154	152.36	149.24	155.12	155.28
Coding density (%)	88.22	86.93	87.07	87.25	86.94	87.26
Plasmids	0	1	1	0	0	1

bp, base pairs; nts, nucleotides; CDS, coding sequence; ig, intergenic region

Lorraine and HL 0604 1035 consist each of a single circular chromosome of 3.4 Mb. Strain Lorraine also contains a plasmid. As shown in Table 1, the main features of the six *L. pneumophila* genomes analyzed (*e.g.* genome size, GC content and coding density), are highly conserved. The core genome of the six *L. pneumophila* genomes comprises 2434 genes, which represents about 80% of the predicted genes in each genome. Furthermore, the gene order is highly conserved as the 260 kb inversion in strain Lens with respect to the other strains is the only exception. When comparing the strains two by two, in average 90% of the genes are present in both strains (Figure 1). However, when determining the non-orthologous genes specific of each genome and not present in the remaining 5 strains, each strain contains between 136 (strain HL 0604 1035) and 222 (strain Corby) strain specific genes mainly encoded on mobile genetic elements. Taken together, the *L. pneumophila* genomes have a highly conserved and syntenic backbone and a highly dynamic accessory genome of about 300 genes each mainly formed by mobile genetic elements, genomic islands and genes of unknown function. The complete annotation of these six genomes is available in a new data base resource that we have set up, LegionellaScope https://www.genoscope.cns.fr/agc/microscope/about/collabprojects.php?P_id = 27 and at the Institut Pasteur, LegioList http://genolist.pasteur.fr/LegioList/.

The species *L. pneumophila* has a highly conserved core genome
a) Most eukaryotic like proteins are conserved in all *L. pneumophila* genomes
The presence of proteins with high similarity to eukaryotic proteins or proteins with domains preferentially or only present in eukaryotic genomes are a particular feature of *L. pneumophila* [4]. However, the criteria for identifying these proteins were never clearly defined. To analyze their evolution and possible origin in depth we

have thus developed an automatic and systematic method to identify eukaryotic like proteins according to defined criteria. Previously we had identified eukaryotic like proteins in *L. pneumophila* as proteins with the
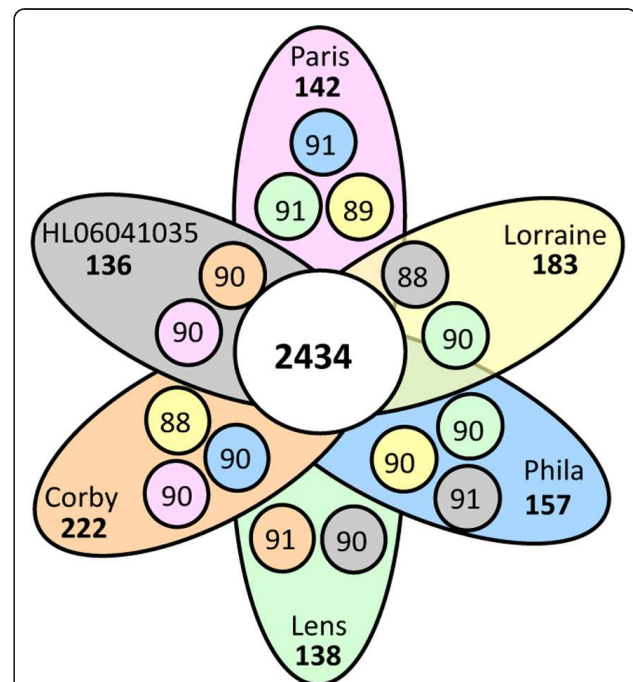
Figure 1 Shared and specific gene content of 6 *L. pneumophila* genomes. Each petal represents a genome with an associated color. The number in the center of the diagram represents the orthologous genes shared by all the genomes. The number inside of each individual petal corresponds to the specific genes of each genome with non-orthologous genes in any of the other genomes. The small circles inside of each petal represent the percentage of shared genes (total number divided by the number of genes in the smallest genome) between the genome of this petal and the genome represented by the color of the small circle. Yellow circle inside orange petal means that there are 88% of genes shared among Corby and Lorraine.

highest similarity score to eukaryotic proteins according to BLAST results, or by identifying eukaryotic domains [4]. However, due to constantly growing databases BLAST results are changing. Furthermore, recent analyses of amoeba-associated bacteria, in particular symbionts of amoeba have shown that they also contain eukaryotic like proteins, suggesting multiple origins of these proteins in prokaryotes [27]. To get a more complete picture of eukaryotic like proteins of *L. pneumophila* and also to include those proteins that might have been transferred independently to different amoeba associated bacteria we defined a eukaryotic like protein as i) a protein having a better normalized blast score against eukaryotic sequences than against prokaryotic ones and ii) a protein that did not show BLAST results against neither *Legionella* spp. nor other bacterial species for which resistance to amoeba infection has been demonstrated (see material and methods). Applying these criteria we identified 46 proteins with putative eukaryotic origin, of which 17 are described here for the first time (Table 2). Given the fact that these proteins were probably acquired by HGT one would expect high diversity in the repertoire. However, our analyses revealed a considerable conservation as more than 50% (26) are conserved in all six *L. pneumophila* strains, indicating an ancient transfer. Furthermore, they show 89-99% nucleotide identity, probably due to high selection pressure for their maintenance. Thus most of these proteins belong to the core genome, indicating that their acquisition has taken place before the speciation of *L. pneumophila*. These 26 proteins might have allowed a common *Legionella* ancestor to colonize an intracellular niche or to adapt better to the intercellular environment of a specific protozoan species leading to the evolution of the species *L. pneumophila*. Interestingly, 19 of these 26 proteins are also conserved in *L. longbeachae*, which might thus be those indispensible for intracellular replication of *Legionellae* (Table 2) [28].

b) Eukaryotic protein motifs are highly conserved among the L. pneumophila genomes

A second class of eukaryotic proteins of *L. pneumophila* is carrying domains predominantly present in eukaryotic proteins. To systematically identify these proteins we used the Interpro database comprising 10 different domain search programs [29]. This allowed to identify the *L. pneumophila* proteins carrying eukaryotic domains in the newly sequenced strains Lorraine and HL 0604 1035 as well as to identify previously not reported motifs. Similarly to the above described eukaryotic like proteins over half of the eukaryotic domain coding proteins are conserved in all six genomes and over 80% are conserved when two genomes are compared (*e.g.* 33 of the 39 proteins containing an eukaryotic motif in strain Lens are present also in strain Paris).

Moreover half of them share very high nucleotide identity of in average 98%-100% (Table 3) again suggesting high selection pressure to maintain them.

Our approach identified also new eukaryotic domains like spectrin repeats. The spectrin repeat forms a three-helix bundle and was reported primarily in the animal kingdom [30]. These repeats act as modules building long, extended molecules that also serve as a docking surface for cytoskeletal and signal transduction proteins. In *L. pneumophila* it is present in up to eight proteins of each strain (Table 3) and all spectrin repeat proteins are predicted to be secreted Dot/Icm substrates [31-33]. Another interesting domain is the RAS GEF domain that is present in two proteins encoded by strain Paris one of which (Lpp0350) is conserved in the six strains analyzed. Ras-GEFs are small GTPases typically present in eukaryotes that are involved in numerous cellular processes like gene expression, cytoskeleton re-organization, microtubule organization and vesicular and nuclear transport [34]. GEFs (GDP-GTP exchange factors) regulate Rabs, GTP-binding proteins with conserved functions in membrane trafficking [35]. Interestingly, according to the Pfam database Ras-GEF domains in bacteria are only present in *Legionella*, *Parachlamydia acanthamoebae* and *Protochlamydia amoebophila*, all of which are amoeba-associated bacteria.

Coiled-coil domains have been identified previously in the *L. pneumophila* genomes as this motif can be found in all kingdoms of life. However extended coiled-coil domains are largely absent from bacterial genomes but are typical for archaea and eukaryotes. We thus searched the *L. pneumophila* genomes and 29 other genomes of bacterial pathogens or bacteria present in the aquatic environment (Table 4) for proteins with five or more coiled coil domains. Interestingly, *Legionella* spp, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* contain the highest percentage of proteins with extended coiled-coil domains (6-11 domains) compared to the number of predicted proteins encoded in their genome and only *P. aeruginosa* and *L. pneumophila* encode proteins containing more than 10 coiled-coil domains (Table 4). Most of these *Legionella* proteins are predicted substrates of the Dot/Icm secretion system [31-33,36]. This suggests that large coiled-coil domains are specific adaptations to the eukaryotic cell probably implicated in interactions with host proteins.

c) High selection pressure acts on the Dot/Icm T4SS and its substrates

Central to the pathogenesis of *L. pneumophila* are the *dot/icm* loci, which together direct assembly of a type IV secretion apparatus [37,38]. Although all *L. pneumophila* strains investigated to date contain the complete *dot/icm* loci, sequence variations among the *dot/icm* genes among different *L. pneumophila* strains have been

Table 2 Orthologous eukaryotic like proteins present in the 6 *L. pneumophila* strains and in *L. longbeachae*

| Product | Name | *L. pneumophila* strains | | | | | | | | | | | *L. lo* |
		Paris		Lens		Corby		Lorraine		HL06041035		Philadelphia		
Glucoamylase (Glucan 1,4-alpha-glucosidase)		*lpp0489*	99.31	*lpl0465*	98.93	*lpc2921*	99.38	*lpo0482*	99.45	*lpv0523*	99.14	*lpg0422**	95.92	*llo2801*
Putative inosine-uridine nucleoside N-ribohydrolase §		*lpp0208*	98.81	*lpl0206*	98.64	*lpc0223*	98.94							
SidE protein		*lpp0304*	98.46	*lpl0288*	95.25	*lpc1602*	98.35	*lpo0273*	97.95	*lpv0315*	98.28	*lpg0234**	94.63	
Putative methyltransferase		*lpp0358*	98.06	*lpl0334*	89.28	*lpc0359*	97.93	*lpo0334*	97.29	*lpv0375*	97.80	*lpg0282*	89.28	*llo2356*
Conserved exported protein of unknown function		*lpp0379*	99.63	*lpl0354*	98.53	*lpc0380*	99.45	*lpo0358*	99.45		99.82	*lpg0301*	99.26	
Phosphatidylcholine-hydrolyzing phospholipase C §		*lpp0565*	99.37	*lpl0541*	98.66	*lpc2843*	99.34	*lpo0571*	99.21	*lpv0603*	99.29	*lpg0502*	97.87	*llo1329*
Phytanoyl-CoA dioxygenase domain-containing protein 1		*lpp0578*	99.25	*lpl0554*	98.60	*lpc2829*	99.46	*lpo0586*	98.60	*lpv0619*	99.25	*lpg0515**	99.35	*llo3224*
Leucine-rich repeat protein		*lpp1007*	97.53			*lpc2344*	97.87	*lpo1029*	93.94	*lpv1082*	97.87	*lpg0945**	97.54	
ecto-ATP diphosphohydrolase II	*map*	*lpp1033*	98.95	*lpl1000*	98.78	*lpc2316*	98.78	*lpo1060*	98.69	*lpv1110*	98.86	*lpg0971*	98.43	*llo1247*
Major acid phosphatase Map §		*lpp1120*	99.06	*lpl1124*	97.92	*lpc0577*	98.12	*lpo1121*	97.93	*lpv1267*	99.06	*lpg1119*	98.59	*llo1016*
Pyruvate decarboxylase		*lpp1157*	99.70	*lpl1162*	98.87	*lpc0618*	99.70	*lpo1168*	98.69	*lpv1308*	99.70	*lpg1155*	98.51	
SAM-dependent methyltransferase §		*lpp1192*	98.38	*lpl1198*	98.97	*lpc0657*	99.15	*lpo1205*	99.06	*lpv1346*	97.61	*lpg1190*	99.15	*llo1296*
Putative 2OG-Fe(II) oxygenase superfamily protein §		*lpp1405*	100					*lpo1449*	96.84	*lpv1569*	100,00	*lpg1450*	93.74	
Phospholipase C §		*lpp1411*	100	*lpl1573*	93.12	*lpc0870*	100,00	*lpo1455*	97.61	*lpv1576*	100,00	*lpg1455*	97.93	*llo1329*
Putative mitogen-activated protein kinase	*thi*	*lpp1439*	99.12	*lpl1545*	98.11	*lpc0898*	97.61	*lpo1483*	98.93	*lpv1609*	99.12	*lpg1483**	97.67	*llo1682*
Thiamine biosynthesis protein NMT-1		*lpp1522*	99.04	*lpl1461*	97.98	*lpc0988*	99.04	*lpo1583*	97.88	*lpv1700*	99.04	*lpg1565*	97.47	*llo0920*
Leucine-rich repeat-containing protein	*purC*	*lpp1567*	97.68			*lpc1028*	98.44			*lpv1852*	98.91	*lpg1602**	97.98	
Phosphoribosylamidoimidazole-succinocarboxamide synthase	*mvaB*	*lpp1647*	100	*lpl1640*	97.76	*lpc1106*	99.08	*lpo1715*	98.98	*lpv1936*	98.16	*lpg1675*	97.58	*llo3277*
Hydroxymethylglutaryl-CoA lyase (HMG-CoA lyase) §		*lpp1793*	99.34	*lpl1794*	97.13	*lpc1274*	98.01	*lpo1891*	99.01	*lpv2102*	98.68	*lpg1830*	99.12	*llo0113*
Putative apyrase		*lpp1880*	99.47	*lpl1869*	98.77	*lpc1359*	99.74	*lpo1975*	98.86	*lpv2179*	99.56	*lpg1905*	95.34	*llo1247*
Conserved protein of unknown function		*lpp1905*												
Leucine-rich repeat-containing protein		*lpp1940*	94.44					*lpo2043*	93.7	*lpv2255*	93.88	*lpg1958**	92.56	
ZIP metal transporter family protein §		*lpp2018*	99.60	*lpl2013*	99.07	*lpc1521*	99.47	*lpo2138*	99.34	*lpv2339*	99.47	*lpg2035*	99.07	*llo2518*
Ankyrin repeat protein		*lpp2058*	99.2	*lpl2048*	90.42	*lpc1566*	98.80	*lpo2181*	98.05					
Conserved protein of unknown function		*lpp2061*	99.6	*lpl2051*	95.95	*lpc1569*	96.80	*lpo2185*	95.38					
Sphingosine-1-phosphate lyase I		*lpp2128*	98.84	*lpl2102*	98.29	*lpc1635*	99.06	*lpo2245*	98.62	*lpv2428*	98.02	*lpg2176**	94.02	
Conserved protein of unknown function		*lpp2134*	100	*lpl2109*	98.55	*lpc1642*	100	*lpo2253*	99.60	*lpv2436*	100	*lpg2182*	96.27	
Conserved protein of unknown function		*lpp2419*	99.84	*lpl2298*	99.37	*lpc2129*	100							
Leucine rich repeat protein		*lpp2459*	98.98	*lpl2316*	86.85	*lpc2085*	90.48	*lpo2572*	99.43	*lpv2704*	99.32	*lpg2392**	97.28	
Putative unspecific monooxygenase		*lpp2468*	99.47	*lpl2326*	99.01	*lpc2075*	98.88	*lpo2586*	98.15					
Protein kinase-like		*lpp2626*	94.88	*lpl2481*	98.85	*lpc1906*	95.31	*lpo2765*	98.70	*lpv2900*	99.13	*lpg2556**	99.13	*llo2218*
Putative methyltransferase		*lpp2747*	99.25	*lpl2620*	99	*lpc0443*	99.37	*lpo2974*	97.49	*lpv3039*	99.62	*lpg2693*	99.37	*llo2356*
Phytanoyl-CoA dioxygenase, PhyH		*lpp2748*	99.76	*lpl2621*	98.91	*lpc0442*	99.15	*lpo2975*	98.67	*lpv3040*	99.76	*lpg2694**	95.44	
Sugar kinase §	*hemG*	*lpp2874*	99.38			*lpc3108*	98.89	*lpo3114*	98.15	*lpv3175*	99.14	*lpg2821*	98.52	
Protoporphyrinogen oxidase §	*cysK*	*lpp2909*	98.14	*lpl2763*	96.65	*lpc3136*	98.90	*lpo3153*	98.69	*lpv3207*	98.83	*lpg2851*	96.02	*llo0133*
Cysteine synthase A, O-acetylserine sulfhydrolase A subunit		*lpp3022*	99.26	*lpl2880*	98.95	*lpc3266*	95.99	*lpo3279*	99.16	*lpv3334*	99.26	*lpg2951*	98.52	*llo0076*
Putative methyltransferases §		*lpp3025*	98.50	*lpl2883*	97.06	*lpc3269*	99.30	*lpo3282*	97.62	*lpv3338*	97.54	*lpg2954*	97.76	*llo0074*

Table 2 Orthologous eukaryotic like proteins present in the 6 _L. pneumophila_ strains and in _L. longbeachae_ (Continued)

	lpl		lpc		lpo		lpv		lpg	
Flavanone 3-dioxygenase §					lpo1380					
Protein of unknown function §					lpo1577					
Conserved protein of unknown function with SNARE domain §			lpc2110	98.97	lpo2553	97.25	lpv2681	98.97		
(S)-2-hydroxy-acid oxidase §					lpo2960					
Protein of unknown function §					lpo3145	100,00	lpv3199	94.82		
Putative Pyridine nucleotide-disulphide oxidoreductase	lpl2845	95.59	lpc3225	97.70	lpo3239	98.47	lpv3288	97.80	lpg2917	98.28
Regulator of chromosome condensation, rcc							lpv2481	79.24	lpg2224*	99.83
Putative metallophosphoesterase §							lpv2663			
Serine carboxypeptidase							lpv3278	97.64	lpg2911	100

* Substrates of the Dot/Icm secretion system; § eukaryotic like proteins newly identified in this study; numbers, % nucleotide identity to strain Philadelphia; L.lo, _Legionella longbeachae_

Table 3 Orthologous proteins with eukaryotic motifs present in the 6 *L. pneumophila* strains and in *L. longbeachae*

Motif	*L. pneumophila* strains												*L. lo*
	Paris		Lens		Philadelphia		Lorraine		HL06041035		Corby		
ANK	lpp0037	96.30	lpl0038	97.40	lpg0038*	97.04	lpo0042	97.89	lpv0043	93.66	lpc0039	97.10	
ANK	lpp0126	98.94	lpl0111	98.48	lpg0112	94.83	lpo0119	98.79	lpv0127	93.03	lpc0131	92.16	llo1394
ANK	lpp0202												
ANK	lpp0356												
ANK	lpp0469	98.94	lpl0445	96.35	lpg0403*	95.53	lpo0463	97.64	lpv0501	98.48	lpc2941	98.67	
ANK	lpp0503	98.37	lpl0479	93.86	lpg0436*	93.31	lpo0501	98.12	lpv0537	98.37	lpc2906	98.37	
ANK	lpp0547	99.50	lpl0523	96.31	lpg0483*	96.82	lpo0551	99.83	lpv0585	98.16	lpc2861	99.16	llo2705
ANK	lpp0750	100.00	lpl0732	97.65	lpg0695*	100.00	lpo0775	99.84	lpv0817	100.00	lpc2599	98.44	
ANK	lpp1100												
ANK + SET	lpp1683	97.68	lpl1682	96.32	lpg1718*	98.41	lpo1757	97.86	lpv1985	96.91	lpc1152	97.25	
ANK	lpp1905												
ANK	lpp2058	99.20	lpl2048	90.42			lpo2181	98.05			lpc1566	98.80	
ANK	lpp2061	99.60	lpl2051	95.95			lpo2185	95.38			lpc1569	96.80	
ANK	lpp2065	99.93	lpl2055	98.56			lpo2189	98.62			lpc1573	98.03	
ANK + Fbox	lpp2082	97.40	lpl2072	98.26	lpg2144*	98.84	lpo2207	99.03	lpv2392	93.99	lpc1593	99.22	
ANK	lpp2166	99.25	lpl2140	99.12	lpg2215*	99.06	lpo2285	97.74	lpv2469	99.18	lpc1680	98.93	
ANK	lpp2248	99.50	lpl2219	99.14	lpg2300*	99.14	lpo2371	99.43	lpv2567	98.93	lpc1765	99.21	llo0584
ANK	lpp2270	99.64	lpl2242	97.97	lpg2322*	98.34	lpo2399	98.08	lpv2591	99.53	lpc1789	99.53	llo0570
ANK	lpp2517	99.60	lpl2370	97.94	lpg2452*	98.19	lpo2642	98.95	lpv2776	99.46	lpc2026	99.46	
ANK	lpp2522	98.76	lpl2375	96.90	lpg2456*	95.75	lpo2647	98.49	lpv2781	98.76	lpc2020	91.27	llo0365
ANK	plpp0098	96.00					lpop0045	96.00					
ANK			lpl1681	100.00							lpc1151	97.98	
ANK			lpl2058	86.17			lpo2193	95.37	lpv2375	94.96			
ANK			lpl2339	98.64	lpg2416*	91.21	lpo2601	99.00	lpv2736	99.28	lpc2057	98.98	
ANK					lpg0402*	100.00			lpv0500	96.01			
ANK									lpv2258				
ANK			lpl1681	100.00							lpc1151	97.98	
ANK			lpl2344	100.00			lpo2607	97.93					
F-Box	lpp0233	98.58	lpl0234	93.97	lpg0171*	96.81	lpo0202	97.87	lpv0254	98.94			
F-Box	lpp2486												
F-Box					lpg2224*	99.83			lpv2482	79.24			
F-Box									lpv2481				
RAS GEF	lpp0350	94.53	lpl0328	96.32	lpg0276*	97.33	lpo0327	97.64	lpv0368	97.64	lpc0353		llo0327
RAS GEF§									lpv2258				
Sec7	lpp1932	98.41	lpl1919	97.40	lpg1950*	92.16	lpo2033	98.32	lpv2243	98.58	lpc1423	97.57	llo1397
Sel1											lpc0165		
Sel1			lpl1059	100.00	lpg1062	99.61			lpv1209	100.00	lpc2212	99.61	
Sel-1§	lpp0957	98.93	lpl0927	98.67	lpg0896	98.93	lpo0978	98.67	lpv1030	98.67	lpc2397	99.47	llo0844
Sel-1	lpp1174	99.39	lpl1180	98.30	lpg1172	98.32	lpo1187	99.11	lpv1327	99.39	lpc0638	99.05	
Sel-1	lpp1310	97.87	lpl1307	98.40	lpg1356	98.67	lpo1345	99.02	lpv1469	97.87	lpc0770	98.76	llo1443
Sel-1	lpp2174	99.64	lpl2147	98.48	lpg2222*	99.56	lpo2292	99.47	lpv2477	99.47	lpc1689	96.27	
Sel-1	lpp2692	99.25	lpl2564	98.61	lpg2639	98.39	lpo2917	99.28	lpv2979	99.39	lpc0501	98.75	llo2649
Sel-1							lpo3233						
Spectrin	lpp1848§	99.18	lpl1845	98.77	lpg1884*	99.01	lpo1944§	98.93	lpv2158§	99.18	lpc1331	99.18	
Spectrin	lpp2246	99.29	lpl2217	98.75	lpg2298*	99.29	lpo2369	99.29	lpv2565	98.27	lpc1763	98.75	llo1707
Spectrin	lpp1930	95.11			lpg1947*	96.65	lpo2029	97.72					
Spectrin	lpp1309	100.00			lpg1355*	90.59			lpv1468	100.00			
Spectrin §	lpp1002	98.01	lpl0971	91.62	lpg0940*	97.92	lpo1024	98.05	lpv1077	97.87	lpc2349	97.15	
Spectrin §	lpp0471	97.79	lpl0447	97.45	lpg0405*	98.30	lpo0465§	98.28	lpv0504	98.28	lpc2939	97.70	llo2845
Spectrin §	lpp1843	95.45	lpl1840	97.57					lpv2151	100.00	lpc1323§	99.60	

Table 3 Orthologous proteins with eukaryotic motifs present in the 6 *L. pneumophila* strains and in *L. longbeachae* (Continued)

Spectrin §	lpp1173	98.56	lpl1179§	98.80	lpg1171*§	98.56	lpo1186	99.28	lpv1326	98.56	lpc0637	97.84	llo3114
STPK	lpp0267	96.95	lpl0262	98.72	lpg0208	93.26	lpo0242	98.92	lpv0288	95.13	lpc0283	97.26	
STPK	lpp1439	99.12	lpl1545	98.11	lpg1483*	97.67	lpo1483	98.93	lpv1609	99.12	lpc0898	97.61	llo1682
STPK	lpp2626	94.88	lpl2481	98.85	lpg2556*	99.13	lpo2765	98.70	lpv2900	99.13	lpc1906	95.31	llo2218
U-box	lpp2887	99.72			lpg2830*	97.15	lpo3124	99.58	lpv3185	98.75			

*Substrates of the Dot/Icm secretion system according to previous publications; ¶ orthologs proteins where the corresponding motif was not present in the other genome; § eukaryotic like proteins newly identified in this study; numbers, nucleotide identity with respect to the *L. pneumophila* Philadelphia gene; L.lo, *Legionella longbeachae*

reported [39]. The *dot/icm* loci of the six strains analyzed here exhibited a very high nucleotide conservation of 98-100% among orthologs except for *dotA*, *icmX* and for *icmC* of strain Corby that is shorter and more divergent (84% nucleotide identity) as compared to *icmC* of strain Paris. These results indicate that strong negative selection acts on these genes (Table 5).

Since the identification of RalF [40], numerous approaches have been used to identify Dot/Icm translocated substrates. Currently 278 proteins of *L. pneumophila* have been described as being transloctaed by the Dot/Icm T4SS system [7,31,32,41-44]. Analysis of their distribution among the six *L. pneumophila* strains reveals a very high conservation, as 206 of the 278 substrates are present in all six strains. Nearly all of them show a nucleotide similarity of 95-100% and only nine are specific to strain Philadelphia (Additional file 1, Table S1). Furthermore, only 34 of the 278 substrates of strain Philadelphia are missing in strain Paris, 30 in strain Lorraine or 25 in strain HL 0604 1035 (Additional file 1, Table S1). Thus, although high redundancy seems to be present in the repertoire of Dot/Icm effectors, the strong conservation of nearly all of them in all genomes, argues for their mutual importance for the *L. pneumophila* life cycle,

Rare exceptions are RalF and AnkB/Lpp2028. The nucleotide sequence of *ralF* of strain Philadelphia is only 85% similar to the *ralF* genes of the other strains and is 72 nts (24aa) shorter. A similar situation is seen for *lpg2144/ankB* that is 54 nts (18aa) longer in strain Philadelphia and Lens than in strain Paris and Corby. This is surprising, as the C-terminal region of AnkB of strain Philadelphia contains a eukaryotic prenylation C*AAX* motif mediating posttranslational modification of effector proteins, important for intracellular replication of *L. pneumophila*. Lipidation facilitates the localization of this effector protein to host organelles and serves as a docking platform for ubiquitinated proteins [45,46]. Thus in strain Paris and Corby other proteins might take over this function. Taken together, this analysis suggests that over 200 of the Dot/Icm substrates of *L. pneumophila* have been present or have been acquired before the speciation and that such a large repertoire of

effectors is indeed necessary for intracellular replication and adaptation to the specific protozoan hosts.

The species *L. pneumophila* has a highly dynamic accessory genome
a) A wide variety of T4ASSs and conjugative elements contribute to genome plasticity

Based on sequence comparisons, T4SSs are categorized according to their similarity to the *A. tumefaciens* VirB/D4 system into type IVA (type F and P) and type IVB secretion systems [47]. T4ASSs resemble the VirB/D4 system of *A. tumefaciens*, whereas T4BSS proteins are more distantly related to the VirB/D4 proteins [48]. T4SSs are involved in effector translocation, horizontal DNA transfer to other bacteria and eukaryotic cells, in DNA uptake from or release into the extracellular milieu or in the spread of conjugative plasmids [49]. Genome sequence analyses suggest that for *L. pneumophila* T4SSs play an important role for adaptation and virulence as each genome encodes several T4ASSs in addition to the essential T4BSS Dot/Icm discussed above. We identified in each strain either F-type or P-type T4ASSs or both. Figures 2 and Figure 3 show the organization of the structural genes encoding these systems, their organization and their localization (chromosomal or plasmid). The F-type T4ASSs are all predicted to encode a complete T4SS core as well as the essential gene products for pilus assembly and mating pair stabilization that appears to be involved in DNA transfer. They show homology and colinearity with the *tra*-region of the *E. coli* F plasmid [50] and with the recently described *tra* region of *Rickettsia belii* [51]. In *L. pneumophila* strain Philadelphia (Tra5) and *L. longbeachae* strain NSW (Tra6), where the system has a chromosomal localization, it is inserted in a tRNA gene and flanking repeats are present as well as a gene coding for an integrase, suggesting that these T4SSs are mobile (Figure 2). Furthermore, comparison of amino acid identities revealed that the Tra- region on the *L. pneumophila* strain Paris plasmid (Tra1) shows much higher identity with the Tra region located on the *L. longbeachae* plasmid (Tra4) than with those of the different *L. pneumophila* strains (Paris-Tra1, Lens-Tra3 or Lorraine-Tra2)

Table 4 Genes coding for proteins with more than 5 coiled coil domains/protein in different bacterial genomes

Organism	Coiled coil domains proteins	Gene	Product	Number of Coiled coil
B. henselae Houston-1	0			
Ch. pneumoniae J138	0			
Ch. trachomatis D UW-3	0			
C.glutamicum ATCC 13032	0			
E. coli O157:H7	1	ECH74115_2173	tail length tape measure protein	5
H. influenzae Rd KW20	0			
H. pylori 26695	1	HP0527	cag pathogenicity island protein Y	10
L. pneumophila Corby	7	lpc1130	substrate of the Dot/Icm system/Icm system	5
		lpc1131	substrate of the Dot/Icm system/Icm system	6
		lpc1452	substrate of the Dot/Icm system/Icm system	6
		lpc1611	hypothetical protein	12
		lpc1987	substrate of the Dot/Icm system, effector protein B	9
		lpc2349	substrate of the Dot/Icm system, LidA	6
		lpc3079	substrate of the Dot/Icm system, effector protein A	5
L. pneumophila HL06041035	10	lpv1077	substrate of the Dot/Icm system, LidA	6
		lpv1725	substrate of the Dot/Icm system	6
		lpv1966	substrate of the Dot/Icm system	5
		lpv1967	substrate of the Dot/Icm system	6
		lpv2269	substrate of the Dot/Icm system	7
		lpv2408	conserved protein of unknown function	5
		lpv2816	substrate of the Dot/Icm system, effector protein B	10
		lpv2959	chromosome segregation SMC protein	9
		lpv3144	substrate of the Dot/Icm system, effector protein A	5
		lpv3184	substrate of the Dot/Icm system, SidH	9
L. pneumophila Lens	7	lpl1437	substrate of the Dot/Icm system	6
		lpl1660	substrate of the Dot/Icm system	7
		lpl1661	substrate of the Dot/Icm system	6
		lpl1941	substrate of the Dot/Icm system	5
		lpl2084	substrate of the Dot/Icm system	5
		lpl2411	substrate of the Dot/Icm system, effector protein B	9
		lpl2708	substrate of the Dot/Icm system, effector protein A	5
L. pneumophila Lorraine	10	lpo1024	substrate of the Dot/Icm system, LidA	6
		lpo1608	substrate of the Dot/Icm system	6
		lpo1735	substrate of the Dot/Icm system	7
		lpo1736	substrate of the Dot/Icm system	5
		lpo2060	substrate of the Dot/Icm system	6
		lpo2216	substrate of the Dot/Icm system, SdeC	5
		lpo2680	substrate of the Dot/Icm system, effector protein B	9
		lpo2896	chromosome segregation SMC protein	9
		lpo3083	substrate of the Dot/Icm system, effector protein A	5
		lpo3123	substrate of the Dot/Icm system	9
L. pneumophila Paris	6	lpp1002	substrate of the Dot/Icm system, LidA	6
		lpp1546	substrate of the Dot/Icm system	6

Table 4 Genes coding for proteins with more than 5 coiled coil domains/protein in different bacterial genomes
(Continued)

		lpp1666	substrate of the Dot/Icm system	7
		lpp1952	substrate of the Dot/Icm system	6
		lpp2555	substrate of the Dot/Icm system, effector protein B	10
		lpp2883	substrate of the Dot/Icm system	6
L. pneumophila Philadelphia	8	lpg1355	substrate of the Dot/Icm system, SidG protein	5
		lpg1588	substrate of the Dot/Icm system	6
		lpg1701	substrate of the Dot/Icm system	5
		lpg1702	substrate of the Dot/Icm system	6
		lpg2156	protein of unknown function	5
		lpg2490	substrate of the Dot/Icm system, effector protein B	9
		lpg2793	substrate of the Dot/Icm system, effector protein A	5
		lpg2829	substrate of the Dot/Icm system	8
L. monocytogenes EGD-e	3	lmo0650	hypothetical protein	5
		lmo0955	hypothetical protein	5
		lmo1224	hypothetical protein	5
M. tuberculosis F11	1	TBFG_12936	chromosome partitioning protein Smc	10
M. tuberculosis H37Ra	1	MRA_2947	putative chromosome segregation Smc	10
N. meningitidis MC58	0			
P. aeruginosa LESB58	11	PLES_08211	putative tail length tape measure protein	7
		PLES_12531	hypothetical protein	7
		PLES_12541	hypothetical protein	5
		PLES_13581	putative tail length tape measure protein	7
		PLES_15241	electron transport complex protein RnfC	8
		PLES_15871	hypothetical protein	6
		PLES_36651	putative ClpA	_
		PLES_38011	putative chromosome segregation protein	11
		PLES_46621	putative exonuclease	13
		PLES_50721	hypothetical protein	6
		PLES_55491	putative outer membrane protein precursor	5
R. felis URRWXCal2	2	RF_0022	putative surface cell antigen sca1	7
		RF_0725	antigenic heat-stable 120 kDa protein	5
R.prowazekii Madrid E	0			
R.a typhi Wilmington	0			
S. typhimurium LT2	5	STM0395	exonuclease subunit SbcC	7
		STM0567	putative DNA repair ATPase	7
		STM0994	chromosome partition protein mukB	10
		STM1041	minor tail protein	5
		STM3199	hypothetical protein	5
S. flexneri 2a 2457T	1	S0984	fused chromosome partitioning protein	10
Synechocystis sp. PCC 6803	2	sll1772	MutS2 protein	5
		slr1301	hypothetical protein	6
S. pneumoniae D39	4	SPD_0126	exported protein of unknown function	6
		SPD_0710	putative Septation ring formation regulator EzrA	7
		SPD_1104	chromosome partition protein Smc	10
		SPD_2017	exported protein of unknown function	6
W. pipientis wMel	0			

Table 4 Genes coding for proteins with more than 5 coiled coil domains/protein in different bacterial genomes
(Continued)

X. fastidiosa 9a5c	0			
Y. pestis KIM	4	y0227	hypothetical protein	6
		y0976	ATP-dependent dsDNA exonuclease	12
		y2765	chromosome partition protein MukB	10
		yapB	autotransporter	6

(Figure 2). Thus these systems seem to be transferred horizontally via plasmids but are also able to integrate in the genome similar to what was reported for the Lvh-region [52].

The F-type T4SS encode long, flexible pili that allow donors to mate in liquid and on solid media with equal efficiencies [53]. In contrast P-type T4SS like described in *P. aeruginosa* encode short and rigid conjugative pili that allow surface mating. Homologues to this system are also present in the *Legionella* genomes. They were initially described in two genomic islands of *L. pneumophila* strain Corby (Figure 3; Trb1 and Trb2) [54]. We show here that they are also present in the chromosomes of *L. pneumophila* strain Lorraine (Trb3) and *L.*
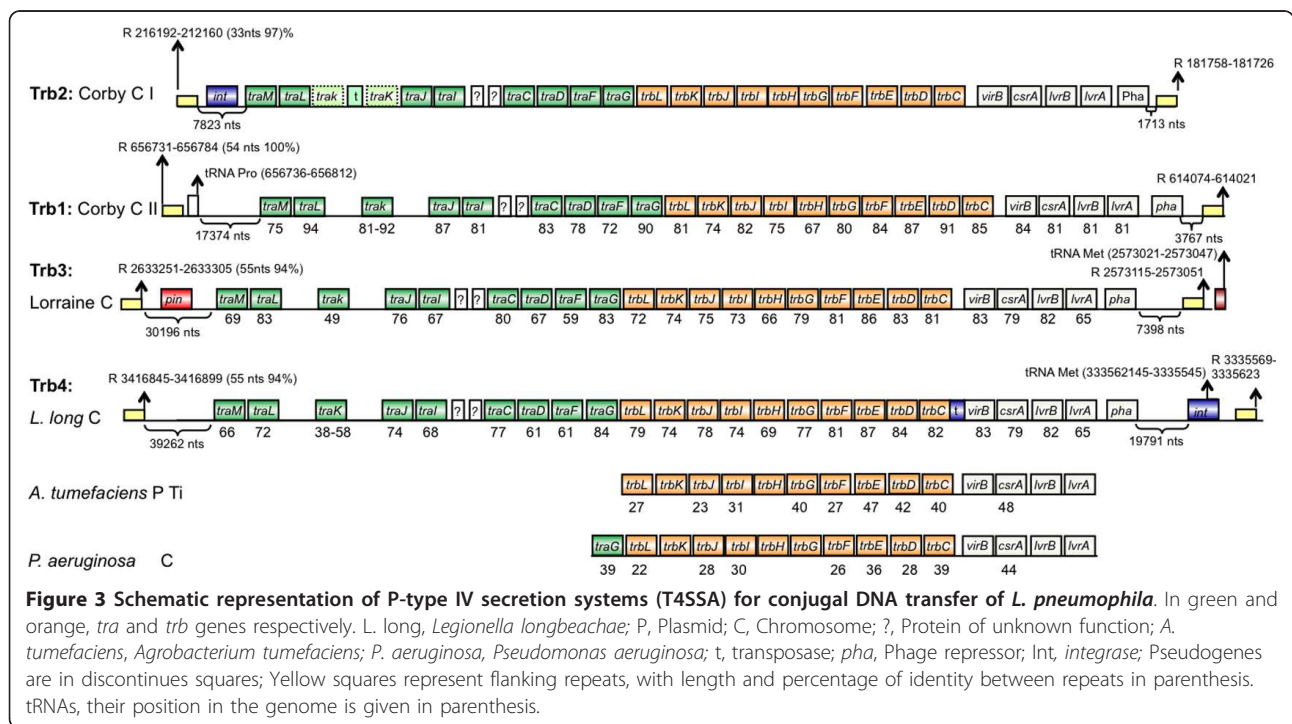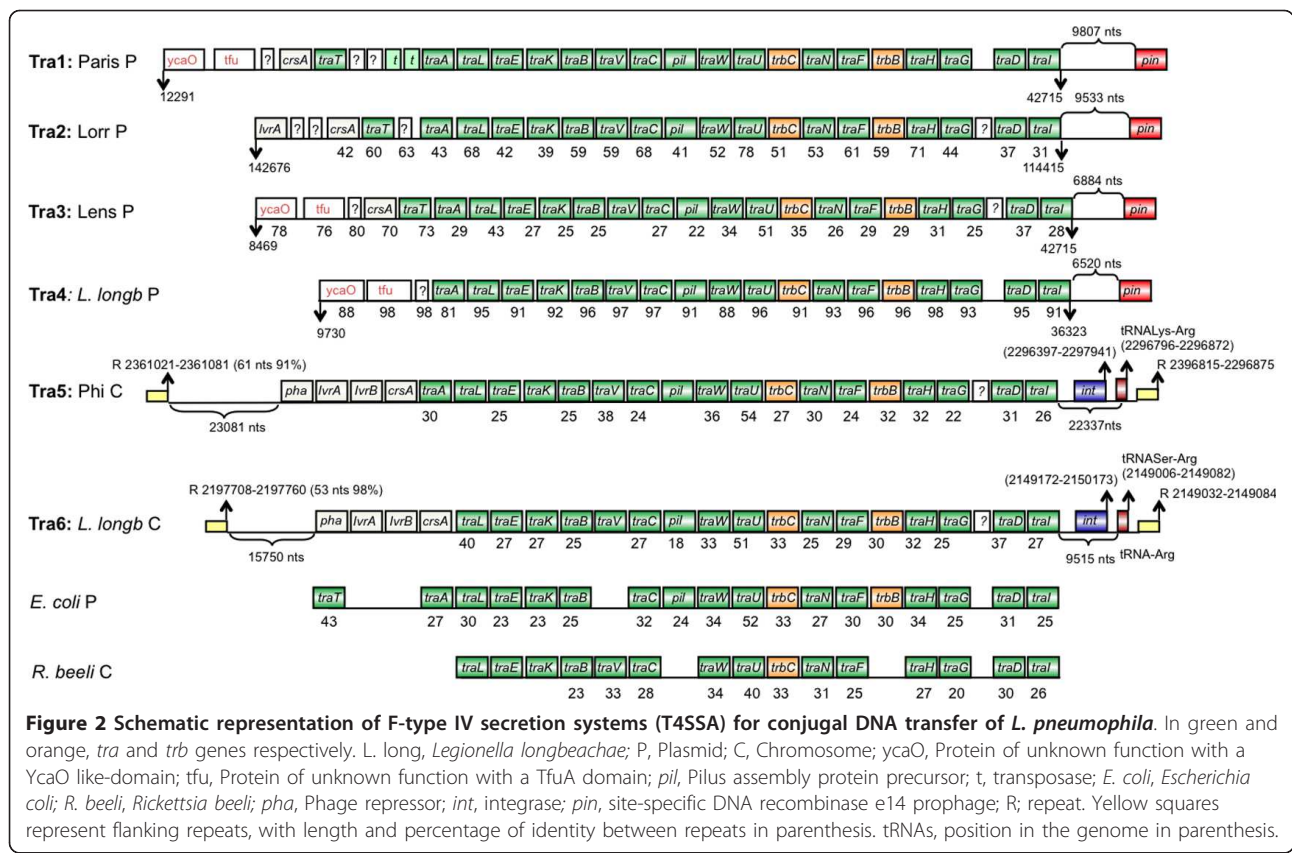
longbeachae NSW150 (Trb4) (Figure 3). Again for all T4SS regions flanking repeats are found suggesting mobility, and protein identity values and GC-content values of the *tra-trb* genes are higher than the genomic average (38%), supporting again horizontal and not vertical transmission.

Another intriguing feature of these regions is that several transposases and phage related proteins are present in each of the *tra* clusters as well as genes coding for homologues of a putative phage repressor protein (PrpA) and for homologues of LvrA, LvrB and LvrC, first described for the Lvh region of *L. pneumophila*. LvrC is a homologue of CsrA, a protein crucial for the regulation of the switch between replicative and

Table 5 Percentage of nucleotide identity of orthologous *dot/icm* genes with respect to the *L. pneumophila* Philadelphia sequence

Gene name	Length (nts)	Phila	Paris	Id	Lens	Id	Lorrain	Id	HL06041035	Id	Corby	Id	*L. long*	Id
icmT	261	lpg0441	lpp0507	99.6	lpl0483	99.1	lpo0507	100	lpv0541	96	lpc2902	99.2	llo2795	75.2
icmS	345	lpg0442	lpp0508	98.5	lpl0484	98.8	lpo0508	99.1	lpv0542	94.4	lpc2901	98.3	llo2794	76.9
icmR	363	lpg0443	lpp0509	96.9	lpl0485	98.3	lpo0509	97.8	lpv0543	97.5	lpc2900	96.9		
icmQ	576	lpg0444	lpp0510	97	lpl0486	99	lpo0510	98	lpv0544	98	lpc2899	98	llo2792	70.7
icmP/dotM	1131	lpg0445	lpp0511	98	lpl0487	99	lpo0511	98	lpv0545	98	lpc2898	99	llo2791	74.5
icmO/dotL	2352	lpg0446	lpp0512	98.4	lpl0488	97.7	lpo0512	98.1	lpv0546	98.3	lpc2897	98.3	llo2790	77.7
icmN/DotK	570	lpg0447	lpp0513	99.3	lpl0489	98.6	lpo0513	98.9	lpv0547	99.6	lpc2896	99.7	llo2789	67.3
icmM/dotJ	285	lpg0448	lpp0514	97.9	lpl0490	97.9	lpo0514	97.9	lpv0548	99.3	lpc2895	98.6	llo2788	61.7
icmL/dotI	639	lpg0449	lpp0515	99.8	lpl0491	99.4	lpo0515	99.4	lpv0549	99.8	lpc2894	99.5	llo2787	78.6
icmK/dotH	1083	lpg0450	lpp0516	94.8	lpl0492	94.3	lpo0516	95.2	lpv0550	94.4	lpc2893	94.7	llo2786	71.2
icmE/dotG	3147	lpg0451	lpp0517	93.7	lpl0493	94.0	lpo0517	94	lpv0551	94	lpc2892	94.3	llo2785	69.1
icmG/dotF	810	lpg0452	lpp0518	98	lpl0494	97	lpo0518	98	lpv0552	98	lpc2891	97	llo2784	55.7
icmC/dotE	585	lpg0453	lpp0519	99.6	lpl0495	99.1	lpo0519	99.7	lpv0553	99.3	lpc2890	54	llo2783	69.1
icmD/DotP	399	lpg0454	lpp0520	97	lpl0496	98	lpo0520	97	lpv0554	98	lpc2889	97	llo2782	77.3
icmJ/dotN	627	lpg0455	lpp0521	99	lpl0497	98	lpo0521	99	lpv0555	99	lpc2888	98	llo2781	79.4
icmB/DotO	3030	lpg0456	lpp0522	98.1	lpl0498	98.3	lpo0522	98.3	lpv0556	98.2	lpc2887	97.6	llo2780	76.4
icmF	2922	lpg0458	lpp0524	98.2	lpl0500	98.5	lpo0524	98.3	lpv0558	98.5	lpc2885	98.2	llo3075	69.5
icmH/DotU	786	lpg0459	lpp0525	99.4	lpl0501	99.5	lpo0525	99.7	lpv0559	99	lpc2884	99	llo3074	68.8
dotD	492	lpg2674	lpp2728	98	lpl2601	98	lpo2953	98	lpv3018	98	lpc0463	99	llo0369	76.5
dotC	912	lpg2675	lpp2729	98.7	lpl2602	98.5	lpo2954	98.8	lpv3019	98.6	lpc0462	99.9	llo0368	74.8
dotB	1134	lpg2676	lpp2730	99	lpl2603	98	lpo2955	98	lpv3020	98	lpc0461	99	llo0367	76
dotA	3108	lpg2686	lpp2740	83.3	lpl2613	96.8	lpo2967	83	lpv3032	83.6	lpc0450	85.8	llo0364	51.4
icmV	456	lpg2687	lpp2741	91	lpl2614	91	lpo2968	91	lpv3033	92	lpc0449	92	llo0363	64.3
icmW	456	lpg2688	lpp2742	95.1	lpl2615	97.6	lpo2969	95.1	lpv3034	95.4	lpc0448	95.1	llo0362	79.3
icmX	1419	lpg2689	lpp2743	84.3	lpl2616	85.2	lpo2970	85.6	lpv3035	85.6	lpc0447	84.1	llo0361	46.9

Id, identity

Figure 2 Schematic representation of F-type IV secretion systems (T4SSA) for conjugal DNA transfer of *L. pneumophila*. In green and orange, *tra* and *trb* genes respectively. L. long, *Legionella longbeachae*; P, Plasmid; C, Chromosome; ycaO, Protein of unknown function with a YcaO like-domain; tfu, Protein of unknown function with a TfuA domain; *pil*, Pilus assembly protein precursor; t, transposase; *E. coli, Escherichia coli; R. beeli, Rickettsia beeli; pha*, Phage repressor; *int*, integrase; *pin*, site-specific DNA recombinase e14 prophage; R; repeat. Yellow squares represent flanking repeats, with length and percentage of identity between repeats in parenthesis. tRNAs, position in the genome in parenthesis.

Figure 3 Schematic representation of P-type IV secretion systems (T4SSA) for conjugal DNA transfer of *L. pneumophila*. In green and orange, *tra* and *trb* genes respectively. L. long, *Legionella longbeachae*; P, Plasmid; C, Chromosome; ?, Protein of unknown function; *A. tumefaciens, Agrobacterium tumefaciens; P. aeruginosa, Pseudomonas aeruginosa*; t, transposase; *pha*, Phage repressor; Int, integrase; Pseudogenes are in discontinues squares; Yellow squares represent flanking repeats, with length and percentage of identity between repeats in parenthesis. tRNAs, their position in the genome is given in parenthesis.

transmissive phase of *L. pneumophila* [55]. It is tempting to assume that these CsrA homologues are implicated in the regulation of the mobility of these islands. Possibly, dependent on the growth phase and/or on metabolic cues *L. pneumophila* might excise these islands as multiple copies could be advantageous in certain conditions, or perhaps allow high frequencies of DNA transfer leading to fast and efficient adaptation to new conditions. The genomic features of these islands suggest a particular mechanism of mobility, which will be interesting to investigate.

b) The *L. pneumophila* genomes encode systems specific for protection against invading DNA and stabilization of large genomic fragments

Bacteria have developed multiple methods of protection against mobile genetic elements or bacteriophages. An example for acquired phage specific immunity is clustered regularly interspaced short palindromic repeats (CRISPR) loci [56]. Another type of protection may be conferred by toxin-antitoxin (TA) systems. Bacterial TA systems are small genetic modules composed of a toxin and antitoxin. While toxins are always proteins, antitoxins are either RNAs (type I and III) or proteins (type II) [57]. These systems were first described for being dedicated to plasmid maintenance. Several lines of research indicate that chromosomal TA systems might serve as protection against mobile genetic elements such as plasmids and phages. However, recent studies have shown that type II systems are also involved in the stabilization of large genomic fragments and of integrative conjugative elements [57]. Interestingly, type II TA systems are thought itself to be part of the mobilome and to move from one genome to another through horizontal gene transfer [57].

Genome analyses identified several TA and CRISPR systems. Interestingly, we identified only type II TA systems of which all except two are in a chromosomal location (Table 6). However, of the 18 chromosomal encoded TA systems identified at least 14 are located on putative genomic islands or mobile genetic elements. The two most frequently found TA systems in the *L. pneumophila* genomes are homologues of the HigAB and RelEB systems. HigAB was first described in the *Vibrio cholerae* superintegron where it encodes mRNA

Table 6 Genes encoding putative toxin-antitoxin systems in six *L. pneumophila* genomes

	L. pneumophila strains					
Toxin-antitoxin	Paris	Lens	Philadelphia	Corby	Lorraine	HL06041035
higA		*lpl2833* (96)*	*lpg2914* (96)			*lpv3285* (96)
higB		*lpl2834* (87)*	*lpg2915* (103)			*lpv3286* (103)
higA		*lpl1092* (93)*				
higB		*lpl1093* (107)*				
higA	*lpp0064* (434)*				*lpo0072* (432)*	
higB	*lpp0065* (79)*				*lpo0073* (79)*	
				lpc2112 (312)		
		lpl2291 (102)*	*lpg2369* (102)	*lpc2113* (37)		*lpv2676* (102)*
Similar to *hipA*	*lpp2427* (78)*	*lpl2292* (312)*	*lpg2370* (312)	*lpc2114* (65)	*lpo2551* (115)*	*lpv2677* (310)*
yhvA					*lpo1074* (168)*	
sohA					*lpo1075* (115)*	
relE	*plpp0090* (83)	*lpl1587* (82)*				
relB	*plpp0089* (95)	*lpl1588* (85)*				
relE		*lpl1084* (84)*		*lpc2177* (93)*	*lpo0120* (93)*	
relB				*lpc2178* (88)*	*lpo0119* (86)*	
parE						*lpe2361* (98)*
parD						*lpe2360* (84)*
pemK					*lpo0114* (106)	

*TA systems located on putative genomic islands; In parenthesis length of the corresponding protein

cleaving enzymes and can stabilize plasmids [58]. RelEB was shown, when introduced into the *E. coli* chromosome to prevent deletion of flanking DNA and thus to diminish large scale genome reduction [59]. The same function was shown for the ParED system of *Vibrio vulinificus*, homologues of which are also present in one of the *L. pneumophila* genomes (Table 6). Thus, the different *L. pneumophila* TA systems might be important for stabilization of plasmids and integrative conjugative elements and for protection against invasion of plasmids, phages, or other mobile genetic elements.

The CRISPR/cas system was shown to provide resistance against invading viruses and plasmids and has been identified in many bacteria and archea [60]. CRISPR/cas loci are also present in the *L. pneumophila* genomes of strains Paris, Lens, Alcoy and 130 b but are absent from strains HL06041035 and Lorraine. According to the cas genes, the CRISPR locus of Paris is closely related to that of strain 130 b. In contrast the one of strain Lens located on the plasmid is closely related to the chromosomal CRISPR locus of strain Alcoy as previously described [61]. Strain Lens carries a second CRISR locus on the chromosome; however, it does not seem to be functional like the one encoded by strain Alcoy. Probably strong protection against invading phages is not extremely important, as not all *L. pneumophila* strains contain CRISPR loci. This may be related to their intracellular life style or that despite their widespread occurrence in aquatic environments only few bacteriophages that specifically infect *Legionella* seem to exist [62].

c) Accessory genome of strains Lorraine and HL 0604 1035
In order to get insight in the genetic basis of the two newly sequenced strains, possibly implicated in their different disease frequencies (Lorraine is an newly emerging endemic clone and strain HL 0604 1035 is a *L. pneumophila* Sg1 strain never isolated from disease) we analyzed the specific gene content of each of these strains more in depth. Strain HL 0604 1035 contains 92 and strain Lorraine 148 genes without homology to any gene of the other five *L. pneumophila* strains sequenced of which the majority (60 in strain HL 0604 1035 and 73 in strain Lorraine) code for proteins of unknown function (Additional file 2, Tables S2 and additional file 3, Table S3). Among the genes in these two genomes that lack an ortholog in the other sequenced *L. pneumophila* genomes, about 50% are clustered on three large genomic islands. One genomic Island (GI-HL1) of 45 kb spans from *lpv2637* to *lpv2691*. It is bordered by a Met tRNA gene and encodes a phage related integrase. A second putative mobile element (GI-HL2) of 27 kbs contains the region from *lpv0193* to *lpv0226*. It is bordered at one side by an integrase and a reverse transcriptase (*lpv0225*) and on the other side by a prophage

Rac integrase and a phage excisionase. Strain Lorraine contains also a large genomic island (GI-Lo1) of 69 kb that spans from *lpo2442* to *lpo2531*. It is inserted in a Met tRNA gene, contains a phage related integrase and flanking repeats of 72 nts. Additional, smaller genomic islands seem to be present, however, their borders are difficult to define. Thus most of the strain specific genes seem to be acquired by HGT through mobility of genomic islands.

Only for few of the specific genes a putative function can be predicted like genes coding for proteins involved in sugar and nucleotide metabolism, for uridine diphosphoglucuronate 5'-epimerase or for an UDP-glucose 6-dehydrogenase. Furthermore a specific ANK motif containing protein and a leucine reach repeat protein are present in strain HL 0604 1035. In strain Lorraine we identified mainly specific metabolic enzymes like a putative flavanone 3-dioxygenase, an enzyme involved in flavonoids metabolism and in biosynthesis of phenylpropanoids, which are secondary metabolites of plants and algae. In addition, *lpo2614* is predicted to encode a kynurenine-oxoglutarate transaminase, an enzyme that is part of the tryptophan metabolism and *lpo2960* codes for a putative glycolate oxidase that catalyses the conversion of glycolate and oxygen to glyoxylate and hydrogen proxide. *lpo2502* codes a homologue of CsbD, a general stress response protein of *Bacillus subtilis* [63]. However, the best BLASTp hit is with the *Protochlamydia amoebophila* homologue, an *Acanthamoeba* sp. symbiont [64]. Probably this gene has been acquired by HGT between these two bacteria within their amoeba host. Quite surprisingly, we identified a gene coding a putative methyl-accepting chemotaxis sensory transducer (*lpv1770*) although all *L. pneumophila* strains analyzed to date do not encode chemotaxis systems. This gene shares 71.34% amino acid identity with Llo3301 of *L. longbeachae* a protein that is part of its chemotaxis system [28] also present in *L. drancourtii* [65]. Probably a common ancestor encoded a chemotaxis system that was lost in *L. pneumophila* through a deletion and degradation process.

d) Shared genome of the epidemic strains Paris and Lorraine
A search for genes shared by the two endemic strains but absent in all other strains identified only three genes that fulfilled these criteria and for which a function could be predicted. These encode the alpha, beta and gamma subunits of a putative thiocyanate hydrolase (*lpo1236, lpo1237, lpo1238 and lpp1219, lpp1220, lpp1221*). Most interestingly, these strains are both common in France and strain Paris is also world-wide distributed [10] suggesting a better niche adaptation. Indeed, thiocyanate compounds are used for cleaning water circuits and these strains are thus probably able to

better resist these treatments [66]. Furthermore, strain Alcoy that is responsible for several outbreaks and many cases of Legionnaires' disease in Spain, also contains these genes [61]. The genes coding the putative thiocyanate hydrolase have a GC content of 41-43%, which is significantly higher than the average G+C content of the *L. pneumophila* genome, which is 38%. When searching for the closest homologues according to BLAST searches we identified them in the genomes of *Rhodococcus opacus* strain B4 and *Nocardia farcinica* spp. These two are high G+C Gram-positive bacteria belonging to the *Actinomycetales*, which are phylogenetically not closely related to *Legionella* suggesting that *L. pneumophila* acquired these genes by horizontal gene transfer.

Taken together, the analysis of the accessory gene content showed again that *L. pneumophila* genomes show high plasticity due to mobile genetic elements and HGT. No specific virulence related genes explaining their different disease frequencies have been identified. However, the identification of a specific thiocyanate hydrolase might explain the wide distribution of strains Paris and Lorraine as it may allow them to better adapted to artificial water systems.

Evolutionary genomics
Phylogenetic reconstruction reveals extensive recombination
To analyze the relationship among the six different *L. pneumophila* strains a phylogenetic reconstruction was done based on a multilocus sequence (MLSA) approach using 31 genes selected according to Zeigler [67] (Table 7 and Additional file 4, Table S4). These 31 genes were chosen as they had been shown to be powerful for predicting the relatedness of bacterial genomes [67]. The phylogeny obtained from their concatenated alignment showed a well-resolved topology with bootstrap values

Table 7 Characteristics of the 31 genes used for phylogenetic reconstruction

Gene Name	Product	Label[a]	Function	Length (nts)[a]
uvrB	Excinuclease ABC, subunit B	lpp0086	DNA replication, recombination, and repair	1992
pgk	Phosphoglycerate kinase	lpp0152	Glycolysis/gluconeogenesis	1191
rpoA	RNA polymerase, alpha subunit	lpp0419	Transcription	993
ffh	Signal recognition particle protein, GTPase	lpp0467	Transport and binding proteins	1377
serS	Seryl tRNA synthetase	lpp0575	tRNA aminoacylation	1281
proS	Prolyl-tRNA synthase	lpp0749	tRNA aminoacylation	1710
glyA	Serine hydroxymethyltransferase	lpp0791	Glycine/serine hydroxymethyltransferase	1254
dnaB	Replicative DNA helicase	lpp0803	DNA replication, recombination, and repair	1383
gpi	Glucose-6-phosphate isomerase	lpp0825	Glycolysis/gluconeogenesis	1500
lig	DNA ligase	lpp1020	DNA replication, recombination, and repair	2022
cysS	Cysteinyl-tRNA synthetase	lpp1271	tRNA aminoacylation	1371
trpS	Tryptophanyl tRNA synthetase	lpp1399	tRNA aminoacylation	1215
aspS	Aspartyl-tRNA synthetase	lpp1434	tRNA aminoacylation	1782
ruvB	Holliday junction DNA helicase	lpp1534	tRNA aminoacylation	1011
nrdA	Ribonucleoside-diphosphate reductase, alpha subunit	lpp1738	Deoxyribonucleotide/ribonucleoside metabolism	2829
recA	Bacterial DNA recombination protein	lpp1765	DNA replication, recombination, and repair	1047
tig	Trigger factor	lpp1830	Protein folding and stabilization	1332
lepA	GTP-binding membrane protein	lpp1837	Translation	1833
metK	S-adenosylmethionine synthetase	lpp2004	tRNA aminoacylation	1149
dnaJ	Heat shock protein	lpp2006	Protein folding and stabilization	1140
argS	Arginyl tRNA synthetase	lpp2013	tRNA aminoacylation	1770
eno	Enolase	lpp2020	Glycolysis/gluconeogenesis	1269
ftsZ	Cell division protein	lpp2662	Cell division	1197
uvrC	Excinuclease ABC, subunit C	lpp2698	DNA replication, recombination, and repair	1857
dnaX	DNA polymerase III, subunits gamma and tau	lpp2802	DNA replication, recombination, and repair	1671
recN	DNA repair protein	lpp2877	DNA replication, recombination, and repair	1668
metG	Methionyl tRNA synthetase	lpp2941	tRNA aminoacylation	2013
rho	Transcription terminator factor	lpp3002	Translation	1262
atpD	ATP synthase F1, subunit beta	lpp3053	ATP-proton motive force interconversion	1377
atpA	ATP synthase, subunit alpha	lpp3055	ATP-proton motive force interconversion	1554
thdF	GTP binding protein, thiophene oxidation	lpp3073	tRNA and rRNA base modification	1341

[a] with respect to strain Paris, nts nucleotides

over 50%. To ascertain the reliability of the obtained phylogenetic tree we established individual phylogenies for each of the 31 genes. Surprisingly, the incongruence among several gene trees was high. In addition the Consense program results did not support any node to at least 50%. To further investigate these results we undertook a second analysis using a Shimodaira-Hasegawa test and compared the topologies of the individual alignments of each gene and the concatenated alignment of the 31 genes. As shown in Additional file 5, Table S5 the likelihood-based SH test for alternative tree topologies identified striking discordances. A possible explanation for the identified incongruences among the phylogenies obtained in our study is the presence of recombination events.

With the aim to explore whether recombination events are present in the selected genes we undertook an in depth analysis using the program RDP [68]. Indeed, the analysis of individual genes identified intragenic recombination in 9 of the 31 genes (Table 8). Numerous additional recombination events were detected with the concatenated alignment of the 22 genes for which no intragenic recombination had been shown (Table 8). To minimize false positive recombination events only those that were supported by at least two of the six methods used in RDP were taken into account. However, except one, all were supported by at least three methods. No artifacts resulting of positive selection should be included in this analysis since all of the genes are either informational or operational (housekeeping). Most interestingly, four of the genes in which intragenic recombination was detected are housekeeping genes (*pgk, atpD, ffh, metK*). Housekeeping genes allow to estimate the extent of recombination within bacterial species since presence of recombination in such "normally recombination free genes" is indicative of a high rate of recombination [22]. Similarly antigen-coding genes of *Legionella* were reported to show recombination events [18,69] and certain other genomic regions [17,19,70-72]. Another example of intragenic recombination in *L. pneumophila* is the *rtxA* gene that contains a long tandem repeated domain of variable copy number and sequence [4,10,73]. *rtxA* of strain Lorraine and Corby share the same repeats, whereas the other strains have unique types of repeats. However, when including the newly sequenced strains Lorraine and HL 0604 1035 we found that repeats of the same type are shared by HL 0604 1035 and Philadelphia and by Lorraine and Lens (Figure 4 and Additional file 6, Table S6), further substantiating high intragenic recombination among strains.

To reconstruct the phylogenetic history of the species *L. pneumophila* we used thus the concatenated alignment of the 31 genes described above. It gave a topology with high bootstrap support, however recombination

bias may result in high support for the wrong tree. To avoid possible bias we thus analyzed the concatenated alignment of the 31 genes using a split tree decomposition that allows a more realistic representation of the phylogenetic relationships. Furthermore we constructed a classical bifurcating tree using the highest possible number of genes [all orthologs among the six strains with (1867 genes) and without (2434 genes) *L. longbeachae* as outgroup]. As shown in Figure 5 the Splits Decomposition phylogeny is network-like suggesting incompatible partitions within sequence data, which commonly arise from recombination. Although the phylogeny based on the orthologous genes can also be affected by recombination, the high number of informative sites included in this data set, should allow recovering the correct history of the species as it has been shown previously for other closely related bacterial species [74].

Taken together, in contrast to previous studies, which reported that the species *L. pneumophila* is a clonal population [13,14] our results show clearly that a high recombination rate shapes the *L. pneumophila* genomes. This finding is in line with the natural competence of *L. pneumophila*. However, some worldwide distributed *L. pneumophila* clones have been described (*e.g.* [10]), suggesting that *L. pneumophila* is able to develop a unique genetic population structure within a particular region or environment as reported recently [72].

Recombination of large chromosomal regions of over 200 kbs among *L. pneumophila* strains

Our recombination analysis revealed not only intragenic recombination events but also intergenic recombination as recombination was detected when using the entire alignment even with only recombination free genes (Table 8). This finding may be explained by the recombination of fragments encompassing several genes or multiple recombination events involving smaller tracts along the genome. To test this hypothesis we used a method recently developed for the analysis of *Streptococcus agalactiae* genomes [75]. In order to identify patterns of recombination, nucleotide substitutions between strains were counted in sliding windows across the previously defined core chromosome representing 15 possible pair wise comparisons. Each pair wise comparison revealed highly conserved regions (<0.05% polymorphism on average) and less-conserved regions (>0.7% polymorphism), suggesting the occurrence of recombinational exchanges. When analyzing the different strains in depth we identified in each genome several regions with very low polymorphisms (below 0.05%) suggesting that DNA exchange of these fragments has occurred between the different *L. pneumophila* strains. Most interestingly, the two French strains Paris and HL 0604 1035 that are present since several years in France

Table 8 Intragenic and intergenic recombination in six *L. pneumophila* genomes predicted on individual genes and on combined data using six different methods

Data set	Event Number	Putative recombinant sequences	Detection Method					
			RDP	GENECONV	Boot scan	Max chi	Chimaera	SiSscan
metG	1	Lorraine, Lens	NS	NS	NS	Yes	Yes	Yes
dnaX	1	Philadelphia	NS	NS	Yes	Yes	Yes	Yes
	2	Lens, Lorraine	NS	NS	NS	Yes	Yes	Yes
proS	1	HL06041035	Yes	Yes	Yes	Yes	Yes	Yes
	2	Philadelphia	NS	Yes	NS	Yes	Yes	Yes
cysS	1	Philadelphia	NS	NS	NS	Yes	Yes	NS
lig	1	Lorraine	NS	Yes	Yes	NS	NS	NS
uvrC	1	Lens,Philadelphia, Lorraine	NS	NS	NS	Yes	Yes	Yes
flh	1	Lens	NS	NS	Yes	Yes	Yes	Yes
	2	Paris, HL06041035	NS	NS	NS	Yes	Yes	Yes
pgk	1	Lens	NS	NS	NS	Yes	Yes	Yes
atpD	1	Corby	NS	NS	NS	Yes	Yes	Yes
Concatenated	1	Philadelphia	Yes	Yes	Yes	Yes	Yes	Yes
	2	Philadelphia	Yes	Yes	Yes	Yes	Yes	Yes
	3	HL06041035	Yes	Yes	Yes	Yes	Yes	Yes
	4	HL06041035	Yes	Yes	Yes	Yes	Yes	Yes
	5	Philadelphia, Corby, Lorraine	Yes	Yes	Yes	Yes	Yes	Yes
	6	Lens	Yes	Yes	Yes	Yes	Yes	Yes
	7	Paris, HL06041035	Yes	NS	NS	Yes	Yes	NS
	8	Paris	Yes	Yes	Yes	Yes	Yes	Yes
	9	Lens	Yes	Yes	NS	Yes	Yes	Yes
	10	Lens	Yes	Yes	Yes	Yes	Yes	NS
	11	HL06041035	Yes	Yes	NS	Yes	NS	NS
	12	Paris, HL06041035	Yes	Yes	Yes	Yes	Yes	Yes
	13	HL06041035, Lens	NS	Yes	NS	Yes	Yes	Yes
	14	Lens, Lorraine	Yes	NS	NS	Yes	Yes	NS
	15	Paris, HL06041035	Yes	Yes	NS	Yes	Yes	NS
	16	Corby	Yes	NS	NS	Yes	Yes	NS
	17	Lens	NS	Yes	NS	Yes	Yes	NS
	18	HL06041035, Paris	Yes	NS	NS	Yes	Yes	Yes
	19	Corby	Yes	NS	NS	Yes	Yes	NS
	20	Lorraine	Yes	Yes	NS	NS	NS	Yes
	21	Lens	Yes	NS	Yes	NS	NS	Yes
	22	Corby	Yes	NS	Yes	Yes	NS	Yes
	23	Lens	NS	Yes	NS	Yes	NS	NS
	24	Lens	NS	Yes	NS	Yes	NS	Yes
	25	Philadelphia	Yes	NS	NS	Yes	Yes	Yes

NS = non significant result. Yes = significant result with p-value ≤0.05 (where P is the highest acceptable probability value of recombination occurrence).

show 15 regions of a size between 10 and 99 kbs that have very low polymorphism and thus seem to have been exchanged between them (Additional file 7, Figure S1). In contrast when comparing strain Lens with the other 5 genomes analyzed here, very few regions with low polymorphism, two with strain HL 0604 1035 and one with strain Lorraine, were detected. Furthermore, no DNA exchanges seem to have occurred with strains Corby, Philadelphia or Paris. This indicates that strains

that are frequent in the same environment (*e.g.* strain Paris and HL 0604 1035) show high rates of DNA exchange probably by conjugation as suggested for *Streptococcus agalactiae* [75] and *Enterococcus fecalis* [76]. In contrast strain Lens, which has been identified to date only twice, in Lens (France) and in Germany, very few DNA transfers with the studied *L. pneumophila* strains seem to have taken place. Furthermore, some regions may be transferred also between several strains.

Figure 4 Schematic representation of the repeat regions present in the *rtxA* gene of *L. pneumophila*. Colored squares represent repeated sequences where the same color corresponds to the same type of repeat. Discontinues lines indicate that the exact number of repeats has not been defined.
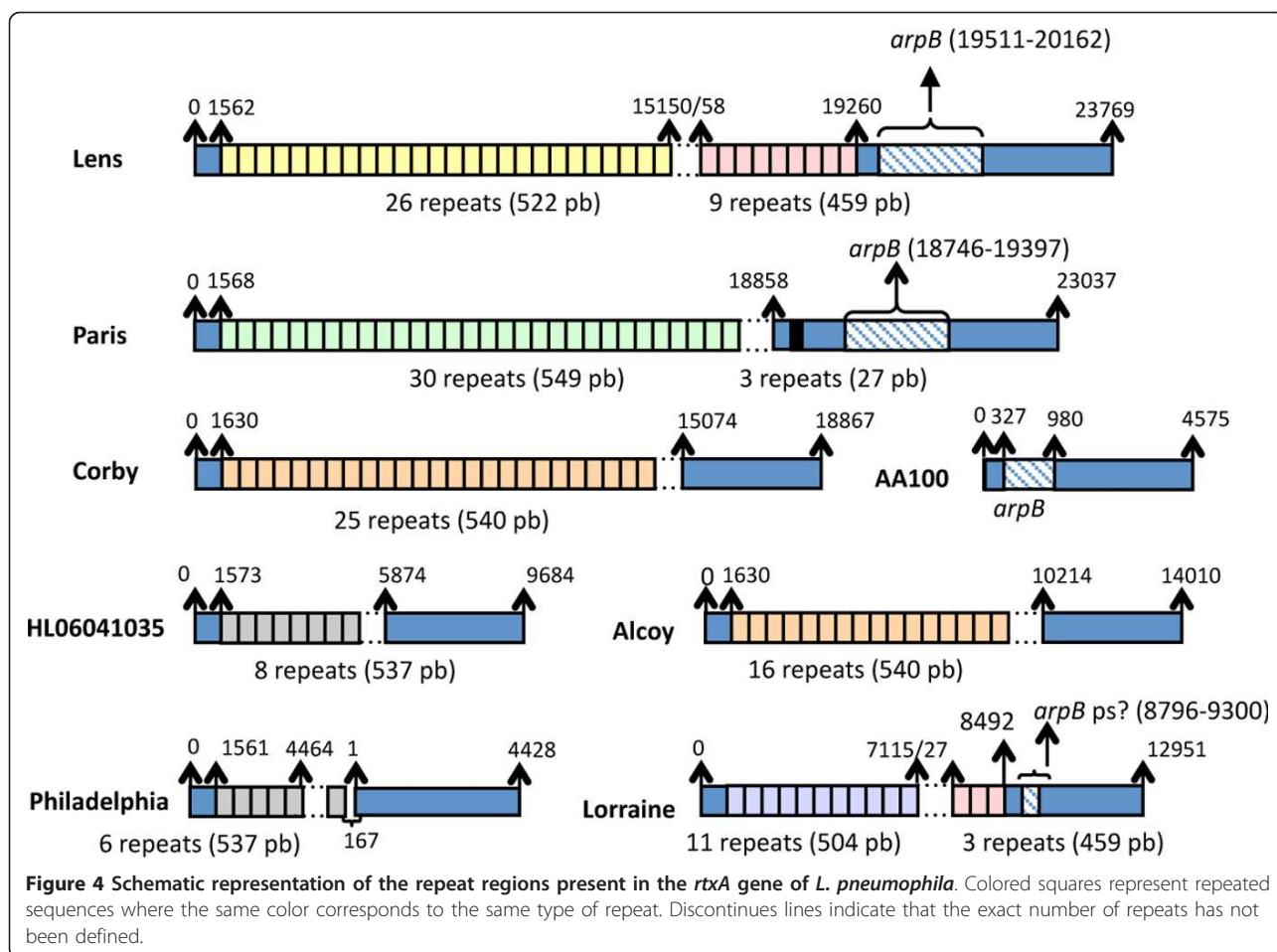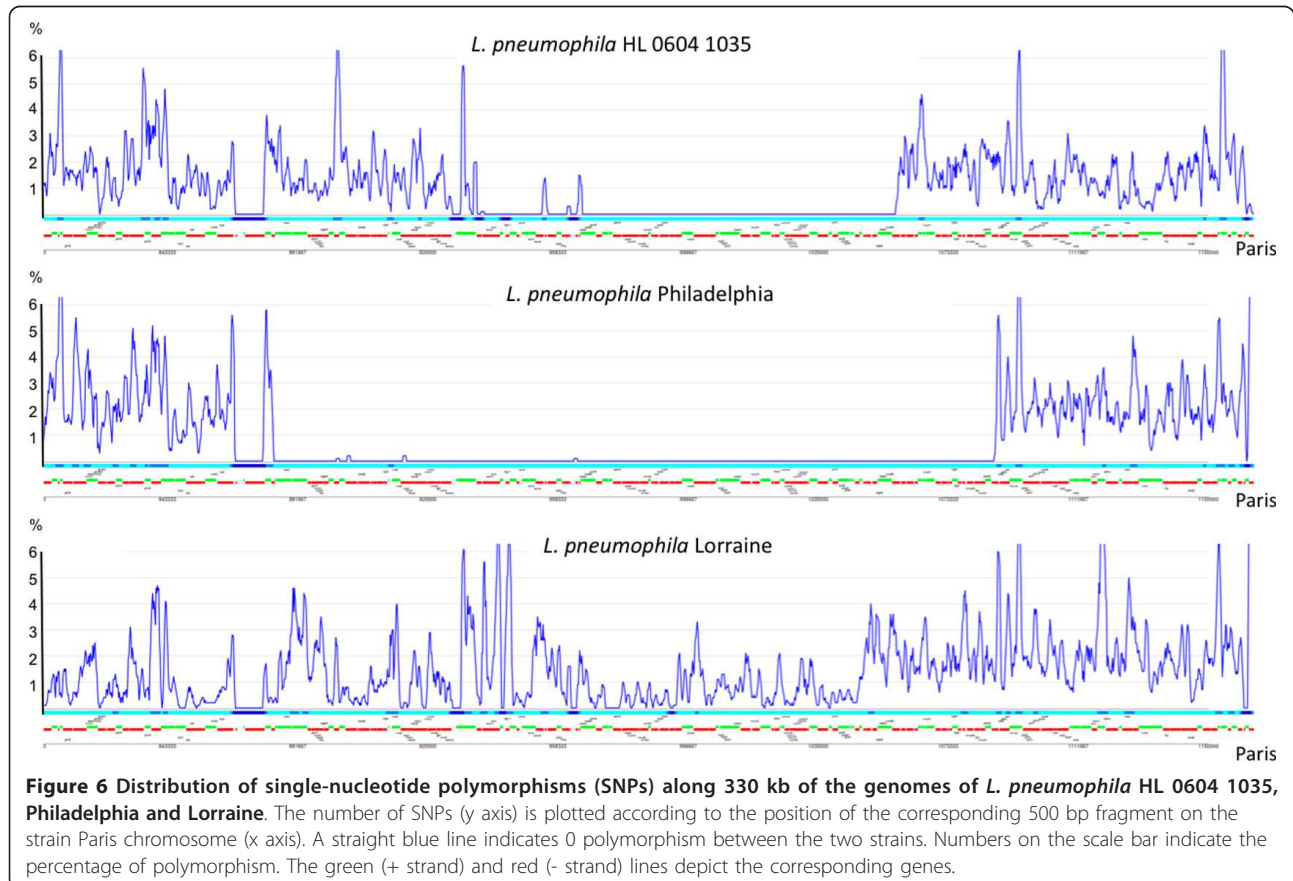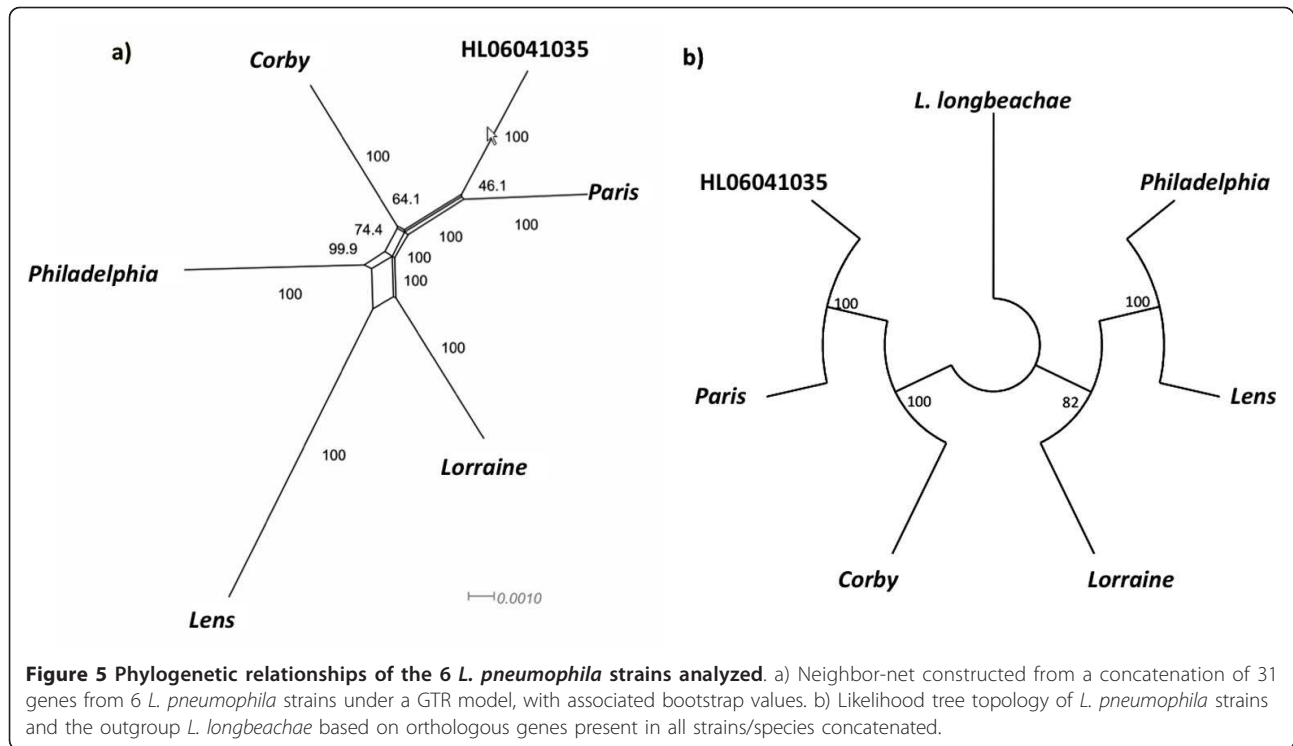
Figure 6 shows the distribution of single-nucleotide polymorphisms (SNPs) along 330 kb of the genome of *L. pneumophila* HL 0604 1035, Philadelphia and Lorraine as compared to the same region in the genome of strain Paris. We identified a region of 213 kbs a SNP frequency of 0.005%. Except an indel of 158 bs that shows higher polymorphism, only 11 SNPs are present in this region. This fragment may have evolved by conjugative transfer and recombination between strains Philadelphia and Paris. Among others, this region carries the genes necessary for lipopolysaccharide biosynthesis, that are also part of the smaller fragment that has been exchanged with strain HL 0604 1035. Our analyses suggest, that in addition to frequent intragenic recombination also recombination and horizontal transfer of large chromosomal fragments is taking place and shapes the chromosomes of *L. pneumophila*.

Conclusion

Analysis of the genome sequences of six *L. pneumophila* strains shows that the genomes of this environmental pathogen evolve by frequent HGT and high recombination rates. Most interestingly, these events take place between eukaryotes and prokaryotes and among different strains and species of *Legionella*. A genome-wide map analysis of nucleotide polymorphisms among these six strains demonstrated that each chromosome is a mosaic of large chromosomal fragments from different origins suggesting that exchanges of large DNA regions of over 200 kb have contributed to the genome dynamics in the natural population. The many T4SS might be implicated in exchange of these fragments by conjugal transfer. Plasmids also play a role in genome diversification and are exchanged among strains and circulate even between different species of *Legionella*. Importantly, plasmids seem to excise and integrate into the genome probably depending on environmental cues. However, *L. pneumophila* encodes also several toxin anti-toxin that might help to stabilize certain mobile genetic elements. In the near future, the analyses of 100 s of genomes thanks to new generation sequencing combined with molecular studies should provide further clues about the genetic mechanisms and the evolutionary forces that shape the *Legionella* genomes.

Figure 5 Phylogenetic relationships of the 6 *L. pneumophila* strains analyzed. a) Neighbor-net constructed from a concatenation of 31 genes from 6 *L. pneumophila* strains under a GTR model, with associated bootstrap values. b) Likelihood tree topology of *L. pneumophila* strains and the outgroup *L. longbeachae* based on orthologous genes present in all strains/species concatenated.

Figure 6 Distribution of single-nucleotide polymorphisms (SNPs) along 330 kb of the genomes of *L. pneumophila* HL 0604 1035, Philadelphia and Lorraine. The number of SNPs (y axis) is plotted according to the position of the corresponding 500 bp fragment on the strain Paris chromosome (x axis). A straight blue line indicates 0 polymorphism between the two strains. Numbers on the scale bar indicate the percentage of polymorphism. The green (+ strand) and red (- strand) lines depict the corresponding genes.

Methods

Bacterial strains and sequence accession numbers

The strains sequenced in this study are *L. pneumophila* strain Lorraine [EMBL: FQ958210, EMBL:FQ958212] and *L. pneumophila* HL 0604 1035 [EMBL:FQ958211]. Strain Lorraine was isolated in 2004 from a patient and was recently described as a newly emerging endemic clone [26]. *L. pneumophila* strain HL 0604 1035 (ST 734, Bellingham subgroup of the Dresden panel) was isolated in 2006 from a water supply system in a French hospital that it is colonizing since more than 10 years.

Sequencing and assembly

The complete genome sequence of *L. pneumophila subsp. pneumophila* strain HL06041035 (A) and strain Lorraine (B) were determined using a Sanger/pyrosequencing hybrid approach. A shotgun library was constructed with 10kb size fragments, obtained after mechanical shearing of the total genomic DNA, and cloned into vector pCNS (pSU derived). Sequencing with vector-based primers was carried out using the ABI 3730 Applera Sequencer. A total of 20736 (A) and 21888 (B) reads (~4 fold-coverage) were analyzed and assembled with 502731 (A) and 555541 (B) reads (~15 fold-coverage) obtained with Genome Sequencer GS20 (Roche Applied Science). For the assembly, we used the Arachne "HybridAssemble" version (Broad Institute, http://www.broad.mit.edu) that combines the contigs obtained with 454 sequencing with Sanger reads. To validate the assembly, the Mekano interface (Genoscope), based on visualization of clone links inside and between contigs, was used to check the clone coverage and misassemblies. In addition, the consensus was confirmed using Consed functionalities http://www.phrap.org: the consensus quality and the high quality discrepancies. The finishing step was achieved by PCR, primer walking and *in vitro* transposition technology (Template Generation System™ II Kit; Finnzyme, Espoo, Finland), and a total of 930 (A) and 999 (B) sequences (109, 165 and 656 respectively for *L. pneumophila subsp. pneumophila* strain HL06041035 and 62, 204 and 733 respectively for *L pneumophila subsp. pneumophila* str. Lorraine) were needed for gap closure and quality assessment.

Sequence analysis and annotation

The two newly sequenced *L. pneumophila* genomes were integrated into the MicroScope platform [77] to perform automatic and expert annotation of the genes, and comparative analysis with the other *L. pneumophila* strains already published. In addition the annotations of the previously published genomes were updated. The system integrates, for each predicted gene, the results of multiple bioinformatics methods (Blast result on UniProt and specialized genomic data, InterPro, COG, PRIAM, synteny group computation using the complete bacterial genomes available at NCBI RefSeq, etc; more information on the syntaxic and functional annotation process is given in [78]). In addition, many genomic and metabolic comparative tools are also available [77]. For details see https://www.genoscope.cns.fr/agc/microscope/home/index.php.

Definition of orthologous genes

To define orthologous chromosomal genes among the different *L. pneumophila* strains, pseudogenes and mobile elements were not taken into account due to the difficulty of ortholog assignment for these genes. Putative orthologous relations were defined as gene couples fulfilling two criteria: (i) having a bidirectional best hit (BBH) with an alignment threshold of 55% identity over at least 60% of the query sequence and target size (ii) and being in synteny. Subsequently, putative genes without any orthologous relation due to reduced identity percentage were integrated in a pre-existing orthologue group if they were flanked by orthologous genes showing gene order conservation (microsynteny). A final step of manual curation was carried out for each doubtful case.

Sequence alignments

For each gene of the selected data set, the nucleotide sequence was aligned based on the amino acid sequence using *tranalign*/EMBOSS package http://emboss.sourceforge.net/. Subsequently genes were concatenated in different data sets.

Identification of eukaryotic like proteins and eukaryotic domain carrying proteins

Eukaryotic domains were identified by analyzing the results obtained for all genes using the Interpro database that is integrated in MAGE. For the identification of eukaryotic like proteins we developed a new method. First we constructed two databases, one containing all and only eukaryotic sequences retrieved from public databases and a second one containing all and only prokaryotic sequences. From the second database we excluded the proteins of bacterial genera for which eukaryotic like protein-domains have been found in high proportions (e.g. parasites of protozoa) or bacterial genera that are reported to establish a symbiotic relationship with amoeba (for a detailed list see Additional file 8, Table S7). Those proteins, that showed a better, normalized blast score against eukaryotic proteins than to those present in the prokaryotic database were retrieved as eukaryotic like proteins. Parameters established for blast were: minimum identity: 25%; minimum

ratio avec query: 60%; minimum ratio avec target: 50%. The final results were manually checked.

Phylogenetic Analysis

For phylogentic reconstruction of the *L. pneumophila* strains analyzed in this work several data sets were used: (i) 31 housekeeping genes described to be essential for all prokaryotes were selected based on the study of Zeigler [67] (Table 7 and Additional file 9, Figure S2) for a multi locus sequence analysis (MLSA) approach for which gene each was analyzed individually and as a concatenated alignment, (ii) a concatenated alignment of 2434 orthologous genes present in all analyzed *L. pneumophila* strains (iii) a concatenated alignment of 1867 orthologous genes present in all analyzed *L. pneumophila* strains and in the selected out group, *Legionella longbeachae* strain NSW150. An analysis of genetic divergence was performed using DNAsp vs 5.00.07 [79] using the 31 selected housekeeping genes. For phylogenetic reconstruction maximum likelihood (ML) methods were used to infer phylogenetic relationships for all data sets. Prior to ML analyses, a DNA substitution model for each gene or data set was selected using Modeltest v3.06 [80] and the Akaike information criterion. ML heuristic searches were performed using 500 random taxon-addition replicates with tree bisection and reconnection (TBR) and branch swapping. ML bootstrap support was determined using 1000 bootstrap replicates. The ML best trees were rooted on *L. longbeachae* when added. A network reconstruction was done for the same data set (i) using SplitsTree4 (version 4.10) [81]. The NeighborNet method and the GTR distance model were used to create the network.

Congruence test

The 31 genes selected for a MLST approach were tested for the significance of topological differences in the obtained phylogenetic trees using several methods. The first approach was based on the consensus of individual gene trees. The consensus tree was inferred using the CONSENSE program in the PHYLIP package http://evolution.genetics.washington.edu/phylip.html applying the extended majority rule. Secondly we tested the significance of topological differences in phylogenetic trees using the Shimodaira-Hasegawa (SH) test. The SH test compares the likelihood score (-lnL) of a given data set across its ML tree *versus* the -lnL of that data set across alternative topologies, which in this case are the ML phylogenies for other data sets. The differences in the -lnL values are evaluated for statistical significance using 1000 replicates based on resampling estimated with the log-likelihood (RELL) method (PAUP version 4.0b10; http://paup.csit.fsu.edu/. We applied the test using all

the trees obtained with individual genes, with the concatenated alignment against the alignment of each individual gene and with the alignment of all the 31 genes concatenated.

Recombination analysis

The 31 genes selected for a MLST approach and its corresponding concatenated alignment, were screened for the presence of putative recombination events by using RDP 2.0b08 [82]. This program identifies recombinant sequences and recombination breakpoints applying several methods. We selected six of them; two phylogenetic methods (which infer recombination when different parts of the genome result in discordant topologies): RDP [68], 2000) and Bootscanning [83]; and four nucleotide substitution methods (which examine the sequences either for a significant clustering of substitutions or for a fit to an expected statistical distribution): Maxchi and Chimaera [84], GeneConv [85] and Sis-scan [86]. We considered only those recombination events in our analysis that were identified by at least two methods. The common settings for all methods were (i) to consider sequences as circular, (ii) a statistical significance of P < 0.05, and (iii) a Bonferroni correction for multiple comparisons implemented in RDP.

Additional material

Additional file 1: Table S1: Nucleotide identity of 140 selected Dot/Icm substrates of strain Philadelphia and of their orthologs in the *L. pneumophila* strains analyzed in this study.

Additional file 2: Table S2: Genes specific of strain HL 0604 1035 with respect to strains Paris, Lens, Philadelphia, Corby and Lorraine.

Additional file 3: Table S3: Genes specific of strain Lorraine with respect to strains Paris, Lens, Philadelphia, Corby and HL0604 1035.

Additional file 4: Table S4: Summary of genetic diversity parameters for the 31 selected *L. pneumophila* genes used to establish the phylogeny.

Additional file 5: Table S5: Results for the SH Test of alternative topologies for the 6 analyzed *L. pneumophila* strains.

Additional file 6: Table S6: Conserved domains and repeats of the *rtxA* gene in 8 *L. pneumophila* strains.

Additional file 7: Figure S1 - Distribution of single-nucleotide polymorphisms (SNPs) along the genome of *L. pneumophila* HL 0604 1035 as compared to strains Lens, Philadelphia, Corby and Lorraine. The number of SNPs (y axis) is plotted according to the position of the corresponding 500 bp fragment on the strain Paris chromosome (x axis). A straight blue line indicates 0 polymorphism between the two strains. Numbers on the scale bar indicate the percentage of polymorphism. Yellow blocks indicate chromosomal regions with a SNP number lower than 0,005%.

Additional file 8: Tables S7 - List of bacterial genera removed from our prokaryotic database.

Additional file 9: Figure S2: Distribution of the 31 genes selected for establishing the phylogeny of *L. pneumophila* species. The coordinates are given with respect to the chromosome of *L. pneumophila* strain Paris. Numbers next to gene names indicate the first

position of the corresponding gene starting from the origin of replication.

Abbreviations
ANK: ankyrin motif; CRISPR: Clustered regularly interspaced short palindromic repeats; HGT: horizontal gene transfer; ML: maximum likelihood; nt: nucleotide; Sg1: serogroup 1; T4SS: Type IV secretion system; T2SS: Type II secretion system;

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Authors' contributions
LGV and CB designed the study. SJ and JE supplied material and expertise; VB and BV performed genome sequencing; LGV and CR performed the genome annotation and analysis work, CM and RZ set up the LegioScope database. LGV and CB drafted and wrote the manuscript. All authors contributed to and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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