Genome Dynamics in *Legionella*: The Basis of Versatility and Adaptation to Intracellular Replication

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Legionella pneumophila is a bacterial pathogen present in aquatic environments that can cause a severe pneumonia called Legionnaires' disease. Soon after its recognition, it was shown that *Legionella* replicates inside amoeba, suggesting that bacteria replicating in environmental protozoa are able to exploit conserved signaling pathways in human phagocytic cells. Comparative, evolutionary, and functional genomics suggests that the *Legionella*–amoeba interaction has shaped this pathogen more than previously thought. A complex evolutionary scenario involving mobile genetic elements, type IV secretion systems, and horizontal gene transfer among *Legionella*, amoeba, and other organisms seems to take place. This long-lasting coevolution led to the development of very sophisticated virulence strategies and a high level of temporal and spatial fine-tuning of bacteria host–cell interactions. We will discuss current knowledge of the evolution of virulence of *Legionella* from a genomics perspective and propose our vision of the emergence of this human pathogen from the environment.

Legionellosis or Legionnaires' disease is a severe pneumonia caused by bacteria belonging to the genus *Legionella*. The first member of this genus, *Legionella pneumophila*, was recognized as a human pathogen during an outbreak of severe pneumonia in 1976 in Philadelphia, PA (Fraser et al. 1977; McDade et al. 1977). Since then, outbreaks occur every year, in particular in the industrialized world, and legionellosis remains an up-to-date health problem. Thirty-five years after the discovery of *Legionella pneumophila*, the genus *Legionella* has grown considerably as it now contains 59 species (http://old.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Legionella), all of which

are ubiquitous, environmental bacteria. Although 24 different species of *Legionella* have been isolated at least once from humans, *L. pneumophila* is the major cause of Legionnaires' disease worldwide as it causes more than 90% of the diagnosed cases. *Legionella longbeachae* is the second cause of legionellosis, responsible for 3.9% of the cases (Yu et al. 2002; Newton et al. 2010). Interestingly, the epidemiology is different in Australia and New Zealand, where *L. pneumophila* accounts for only 45.7% but *L. longbeachae* for 30.4% of the cases. Thus, research focuses mainly on *L. pneumophila* and *L. longbeachae*. In contrast, little or nothing is known about the other *Legionella* species with respect to

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their environmental distribution, ecology, or the capacity to trigger human disease. All *Legionella* species, except *L. longbeachae*, which is present mainly in compost and potting mix, are found in freshwater environments (Whiley and Bentham 2011).

Legionella are intracellular bacteria whose natural hosts are aquatic protozoa, in which these bacteria replicate and are protected from harsh environmental conditions (Rowbotham 1980). *Legionella* are able to parasitize at least 20 different species of amoeba, two species of ciliated protozoa, and one slime mold (Lau and Ashbolt 2009), but are associated most frequently with amoeba belonging to the genera *Acanthamoeba*, *Hartmannella*, and *Naegleria* (Fields 1996). Interestingly, not all *Legionella* will grow in the same amoebal host (Rowbotham 1986) and certain amoeba seem also to be resistant to *Legionella* infection (Atlan et al. 2012). Thus, *Legionella* are broad host-range protozoan parasites but some species, in particular *L. pneumophila*, are also pathogens of humans. The vehicle of transmission to humans is aerosols loaded with *Legionella*. Changes in the human lifestyle in the last decade led to an increased number of artificial water systems such as air conditioning units, cooling towers, showers, and fountains, where water is given off in a fine spray providing the bacterium with the possibility to reach humans as an accidental host. However, although *Legionella* is able to infect humans, human infection is a dead end for *Legionella* replication, as person-to-person transmission has never been reported. Therefore, the evolution of virulence traits in *L. pneumophila* and *L. longbeachae* has resulted from the interaction with environmental protozoa.

The capacity of *Legionella* to infect its natural hosts, amoeba or ciliated protozoa but also human macrophages, indicates that the mechanisms developed by this bacterium to infect low eukaryotes can also be used for infection of cells of high eukaryotes like humans as was proposed the first time by Rowbotham in 1980 (Rowbotham 1980). This also suggests that virulence factors of *Legionella* probably target conserved eukaryotic pathways, allowing *Legionella* to infect many phagocytic eukaryotic cells like differ-

ent aquatic protozoa, ciliates, and human cells. Therefore, the identification of what are these factors, how they have been acquired, and what are their functions, is fundamental to decipher host–pathogen interactions and the virulence strategies used by intracellular pathogens.

In recent years, it has been shown that several intracellular bacterial pathogens are able to enter and replicate in environmental amoebae, similar to what is known for *Legionella* (Molmeret et al. 2005). This observation has led to the idea that free-living amoebae are the “Trojan horses” of the microbial world, as they have a role in survival, replication, and transmission of bacteria (Winiecka-Krusnell and Linder 1999). The interaction between both organisms provides bacteria with the capacity to infect human cells and to become human pathogens. Furthermore, genome analyses and comparative genomics of amoeba-associated bacteria revealed that common virulence factors are present in bacteria living with amoebae (Schmitz-Esser et al. 2010). This finding suggested that the genes coding for these virulence factors may be interchanged and that they may thus be part of mobile genetic elements. The complete set of mobile genetic elements of a genome is commonly called “mobilome” (Siefert 2009), a term we will use hereafter. Particularly in bacteria living with amoebae, this mobilome seems to be highly dynamic. Because *L. pneumophila* is probably the best-studied amoeba-associated bacterium, it has become a model to study the role of amoebal hosts in bacterial pathogenesis and the acquisition of virulence factors from this host–pathogen relationship.

In this review, we will present the state-of-the-art of the evolution of virulence in *Legionella* from a genomics perspective and how current data increased our knowledge about *Legionella* virulence evolution and host–pathogen interaction and acquisition of virulence factors in general.

THE *Legionella* GENOMES ARE GENETICALLY DIVERSE

L. pneumophila strains Philadelphia, Paris, and Lens were the first *Legionella* genomes se-

quenced (Cazalet et al. 2004; Chien et al. 2004). Each strain contains one circular chromosome that is characterized by an average G + C content of about 38% and by a size between 3.3 and 3.5 Mb. In each strain ~3000 protein-coding genes are predicted, representing a coding capacity of 88% (Table 1A). Comparative genomics revealed that ~300 genes (~10%) are specific for each strain. Taking into account that these strains belong to the same species and serogroup, the genomic diversity among them is relatively high. Further analysis of the gene content of 217 *L. pneumophila* strains using a *Legionella* DNA array confirmed that the gene content of the *L. pneumophila* genome is highly diverse (Cazalet et al. 2008). Both studies suggested that the main source of diversity among the *Legionella* genomes is mobile genetic elements and horizontal gene transfer among *L.*

pneumophila strains, but also among strains belonging to different *Legionella* species, other bacterial species, and most surprisingly, probably also between *Legionella* and their eukaryotic hosts (Cazalet et al. 2004, 2008).

Recent years have seen five new *L. pneumophila* genomes sequenced providing the possibility to study their diversity and evolution in depth (Table 1A) (Steinert et al. 2007; D'Auria et al. 2010; Schroeder et al. 2010; Gomez-Valero et al. 2011b). Comparative analyses of the eight available genomes showed that the core genome contains 2405 orthologous groups of genes, and each genome when compared with the seven others contains between 154 and 271 strain-specific genes (Fig. 1). Furthermore, more than 1000 genes are not present in all eight genomes and thus also belong to the flexible gene content or accessory genome. In contrast, the *L. long-*

Table 1. General features of the *Legionella* genome sequences published

	(A) <i>L. pneumophila</i>							
	Paris	Lens	Philadelphia	Corby	Alcoy	130b	Lorraine	HL06041035
Chromosome size (Mb)	3.5	3.3	3.4	3.6	3.5	3.5	3.5	3.5
G + C content (%)	38	38	38	38	38	38	38	38
Number of genes	3178	3034	3083	3290	3197	3293	3170	3184
Number of protein-coding genes	3079	2921	2999	3193	3191	3288	3080	3079
Pseudogenes	45	59	32	44	?	?	37	53
tRNA	43	43	43	44	?	42	44	43
16S/23S/5S	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3
Coding density (%)	87	87	88	87	86		87	87
Plasmids	1	1	0	0	0	0	1	0

	(B) <i>L. longbeachae</i>				
	NSW 150#	D-4968+	ATCC39462+	98072+	C-4E7+
Chromosome size (Kb)	4.0	4.0	4.1	4.0	3.9
G + C content (%)	37.1	37.0	37.0	37.0	37
Number of genes	3660	3557	—	—	—
16S/23S/5S	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4
No. of contigs > 0.5–300 kb	Complete	13	64	65	63
N50 contig size ^a	Complete	—	138 kb	129 kb	134 kb
Percentage of coverage ^b	100%	96.3	96.3	93.4	93.1
Number of SNP with NSW150	—	1900	1611	16 853	16 820
Plasmids	1	1	0	1	1

^aN50 contig size, calculated by ordering all contig sizes and adding the lengths (starting from the longest contig) until the summed length exceeds 50% of the total length of all contigs.

^bFor SNP detection: #, completed sequence; +, draft sequence, not completed; SNP, single nucleotide polymorphism.

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Figure 1. Shared and specific gene content of eight *L. pneumophila* genomes. Each petal represents a genome with an associated color. The number in the center of the diagram represents the orthologous groups of genes shared by all the genomes. The number inside of each individual petal corresponds to the specific genes of each genome with nonorthologous genes in any of the other genomes. The orthologous groups were defined using the program PanOCT (Fouts et al. 2012) (the parameters used were e -value $\leq 1 \times 10^{-5}$, percent identity ≥ 35 , and length of the match ≥ 75).

beachae genomes, five of which have been sequenced to date (Cazalet et al. 2010; Kozak et al. 2010), have a slightly larger genome size of 3.9–4.1 Mb and show much lower diversity among them (Table 1B). When comparing two strains of *L. longbeachae* belonging to different serogroups (Sg), the overall DNA polymorphism is less than 0.4%, whereas this polymorphism is $\sim 2\%$ between *L. pneumophila* strains of the same Sg (Cazalet et al. 2010). Interestingly, despite the fact that *L. pneumophila* and *L. longbeachae* cause the same human disease, high divergence of these two species was observed. Only 2290 (65.2%) *L. longbeachae* genes are orthologous to *L. pneumophila* genes, whereas 1222 (34.8%) are *L. longbeachae* specific with respect to *L. pneumophila* Paris, Lens, Philadelphia, and Corby (Cazalet et al. 2010; Gomez-Valero et al. 2011b). The different genome analyses, when combined, revealed that the accessory genome of *Legionella*, containing approximately 3500 genes when eight *L. pneumophila* and five *L. longbeachae* genomes are analyzed, is large and, most interestingly, the main part seems to

be transferable, thus constituting the mobilome of the genus.

THE MOBILOME OF *Legionella* HAS A DIVERSE ORIGIN

The *Legionella* mobilome comprises plasmids, integrative conjugative elements, insertion sequences, and genomic-island-like regions like most of the prokaryotic genomes. However, it also has a particular feature, which is a large array of so-called eukaryotic-like genes that were identified during the *L. pneumophila* genome analyses of strains Paris and Lens in 2004 (Cazalet et al. 2004). These genes code for proteins that show the strongest similarity, not to prokaryotic but to eukaryotic proteins, or encode motifs known to be implicated in protein–protein interactions, which are present only or primarily in eukaryotes. The eukaryotic-like proteins were predicted to mimic host proteins to allow intracellular replication of *Legionella* and are thus good candidates for being implicated in host–pathogen interactions (Cazalet et al. 2004). Interestingly, many of these potential virulence factors never had been identified in a prokaryotic genome before the discovery in *L. pneumophila*, suggesting that *L. pneumophila* may have developed specific mechanisms to cross talk with its eukaryotic hosts (Bruggemann et al. 2006). The presence of a high number of eukaryotic-like proteins in the genus *Legionella* was further confirmed when analyzing different *L. pneumophila* and *L. longbeachae* genomes (de Felipe et al. 2005; Cazalet et al. 2010; Kozak et al. 2010; Schroeder et al. 2010; Gomez-Valero et al. 2011b). Thus, the *Legionella* genomes reflect the interaction of this bacterium with amoeba, which is probably the driving force in the evolution of *Legionella* pathogenicity.

Although few eukaryotic-like genes were then also identified in other bacteria like *Rickettsia belli* or *Wolbachia* (Ogata et al. 2006; Walker et al. 2007), *Legionella* is the bacterium with the widest variety of eukaryotic-like proteins and eukaryotic domain-carrying proteins; a finding that still holds true today, despite the hundreds of different bacterial genome sequences that have been sequenced and analyzed

since then. In 2004, more than 60 of these proteins were described in the genomes of strains Paris and Lens (Cazalet et al. 2004) and in 2005, in strain Philadelphia (de Felipe et al. 2005). Later, the analysis of the distribution of these genes among more than 200 *L. pneumophila* strains using DNA arrays showed that the conservation of certain eukaryotic-like genes within the species *L. pneumophila* was high, indicating strong environmental selection pressures for their preservation (Cazalet et al. 2008).

Recently, the sequencing and analyses of five additional *L. pneumophila* genomes revealed that over half of the eukaryotic-like proteins are present in all eight *L. pneumophila* strains (Gomez-Valero et al. 2011a,b). We thus propose that these conserved eukaryotic-like proteins have probably allowed the ancestor of *L. pneumophila* to better adapt to the intracellular environment, but now most of them are evolving as part of the core genome. Interestingly, approximately 30% of the proteins containing eukaryotic motifs are also present in the species *L. longbeachae* (Cazalet et al. 2010; Gomez-Valero et al. 2011a). These proteins might be part of the core of eukaryotic-like proteins essential for the genus *Legionella* for its intracellular replication. However, simultaneously, a flexible pool of eukaryotic-like proteins is present in the *Legionella* genomes. It represents more than half of the eukaryotic-like proteins identified to date in different *L. pneumophila* strains. The fact that even phylogenetically very closely related strains like the *L. pneumophila* strains HL06041035 and Paris present some differences in their repertoire of eukaryotic-like proteins suggests that the acquisition and loss of these genes is an ongoing process that has taken place multiple times and further underlines the high dynamics and plasticity of the *Legionella* genomes. In addition to horizontal gene transfer a process of convergent evolution might be important in the evolution of *Legionella* as, although many of the proteins containing eukaryotic motifs are not conserved between the species *L. pneumophila* and *L. longbeachae*, the same eukaryotic motifs like F-box, U-box, ankyrin, or serine threonine protein kinase (STPK) motifs are found in both species (Cazalet et al. 2010). A similar observa-

tion has been made when the genome sequence of the amoeba symbiont “*Candidatus Amoebophilus asiaticus*” has been analyzed. Interestingly, many of the eukaryotic motifs identified in *Legionella* are also present in this bacterium (Schmitz-Esser et al. 2010). Most interestingly, an enrichment analysis comparing the fraction of all functional protein domains among 514 bacterial proteomes revealed that all genomes of bacteria replicating in amoeba were enriched in protein domains that are predominantly found in eukaryotic proteins (Schmitz-Esser et al. 2010). The most highly enriched domains were ANK repeats, LRR, SEL1 repeats, and F- and U-box domains, all of which are also present in *L. pneumophila* and *L. longbeachae*. Taken together, genome analyses of different bacteria associated with amoeba undertaken since the *L. pneumophila* genome sequence had been published in 2004, showed that bacteria that can exploit amoebae as hosts share a set of eukaryotic domain proteins that are most probably important for bacteria–host–cell interactions, despite their different lifestyles and their large phylogenetic diversity. Thus, a global, convergent adaptation mechanism to intracellular parasitism seems to exist in amoeba-associated bacteria.

EUKARYOTIC-LIKE PROTEINS OF *Legionella* ARE VIRULENCE FACTORS THAT MIMIC HOST PROTEINS

One important prediction from the genome analysis of strains Paris and Lens was that the eukaryotic-like proteins of *Legionella* are secreted virulence factors able to interfere with many different host-signaling pathways by mimicking host proteins (Cazalet et al. 2004; Bruggemann et al. 2006). Therefore, many groups started functional studies focusing on these proteins and within a short period of time the importance of the eukaryotic-like proteins for the virulence of *Legionella* was shown (de Felipe et al. 2008; Nora et al. 2009; Hubber and Roy 2010). The main secretion system of *Legionella*, essential for intracellular growth, is the type IVB Dot/Icm secretion system (Marra and Shuman 1992; Berger and Isberg 1993). Using many dif-

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ferent methods it has been shown that at least 275 *L. pneumophila* proteins are secreted by this system (Campodonico et al. 2005; de Felipe et al. 2005; Shohdy et al. 2005; de Felipe et al. 2008; Burstein et al. 2009; Heidtman et al. 2009; Zhu et al. 2011), which represents nearly 10% of the protein-coding genes predicted in the *L. pneumophila* genomes. At least 75 of these are eukaryotic-like proteins or carry eukaryotic domains and, for certain of these, it has also been shown experimentally that they interfere with host–cell signaling pathways (Table 2).

The diverse motifs identified, like ankyrin repeats, F-box and U-box domains, STPK domains, coil-coiled domains, SET, and Sell or Sec-7 domains indicate also very diverse functional roles of these proteins. This fact is, for example, reflected in the very high number of different posttranslational modifications *Legionella* is able to induce in its hosts. To date, it has been shown that *L. pneumophila* proteins are able to ubiquitinate, phosphorylate, lipidate, glycosylate, AMPylate, DeAMPylate, phosphocholate, and dephosphoryl-cholate specific host proteins to modulate multiple host pathways to the pathogen's advantage (Rolando and Buchrieser 2012). Recently, a new posttranslational modification induced by *L. pneumophila* was reported. The *Legionella*-secreted effector, RomA, was shown to be a SET domain-containing methyltransferase that uniquely modifies host chromatin to repress gene expression and promote efficient intracellular bacterial replication (Rolando et al. 2013). Interestingly, a combination of eukaryotic domains was predicted in several of the eukaryotic-like proteins, leading to a modular protein structure. One example is AnkB/LegAU13/Ceg27/Lpg2144/Lpp2082 that contains three different eukaryotic protein domains: an F-box domain, an ankyrin domain, and a CaaX motif (Table 2). The F-box domain interacts with the SKP1 component of the SCF1 ubiquitin ligase complex and allows *Legionella* to exploit the host ubiquitination machinery, whereas the ankyrin motif, a widespread protein–protein interaction motif, probably interacts with the cellular target like Parvin B described for strain Paris (Price et al. 2009; Lomma et al. 2010). The F-box domain, as well as the two

ankyrin protein–protein interaction domains, is essential for the biological function of AnkB. The CaaX motif exploits the host prenyltransferase machinery to facilitate membrane localization of *L. pneumophila* effector proteins (Ivanov et al. 2010; Price et al. 2010). Another modular protein is SdhA/Lpg0376, a Dot/Icm-secreted virulence factor that contains a GRIP and a coiled-coil domain (Table 2). SdhA is required for growth within macrophages and protection from host–cell death (Laguna et al. 2006). Furthermore, the *Legionella*-containing vacuole is actively stabilized by the SdhA protein during intracellular replication, which affords the bacterium protection from cytosolic host factors that degrade bacteria and initiate immune responses (Creasey and Isberg 2012). AnkY/LegA9/Lpg0402 is also a modular protein as it contains an STPK domain and an ankyrin motif. However, to date, its functional role and putative implication in virulence has not been shown.

A specific example for the amazing temporal and spatial fine-tuning *Legionella* has evolved in its interactions with the host cell is the Dot/Icm-secreted effector LubX/Lpg2830. LubX (for *Legionella* U-box) is a protein containing two U-box domains (U-box1 and U-box2) similar to eukaryotic E3 ubiquitin ligases that function as a ubiquitin ligase in conjunction with host UbC5a or UbC5c E2 enzymes and mediates polyubiquitination of cellular Clk1 (Cdc2-like kinase) (Kubori et al. 2008). U-box1 seems to be critical for ubiquitin ligation, whereas the U-box2 domain interacts with the substrate. Recently, it was shown that the secreted *L. pneumophila* effector protein SidH is a second target of LubX within host cells. LubX directly binds and polyubiquitinates SidH in vitro and mediates its proteasomal degradation in infected cells. LubX is the first example of a bacterial metaeffector that regulates spatially and temporally the expression level of another effector (Kubori et al. 2010). The identification of this mechanism points to the fact that there might be many different levels of regulation among the large number of *Legionella* effectors as well as between the effectors and their host targets.

Table 2. List of selected substrates of the Dot/Icm secretion system in *L. pneumophila* and *L. longbeachae* strains

		<i>L. pneumophila</i> strains							<i>L. longbeachae</i> strains					Name	Product or associated motif	Function
	Phila	130b	Paris	Lens	Corby	Alcoy	Lorraine	HL06041035	NSW150	D-4968	ATCC39462	98072	C-4E7			
<i>Legionella</i> core genome	<i>lpg0103</i>								<i>llo3312</i>					<i>vipF</i>	Amino-terminal acetyltransferase, GNAT	Putative modulation of host protein trafficking
	<i>lpg0257</i>								<i>llo2362</i>					<i>sdeA</i>	Multidrug resistance protein	Adhesion, invasion
	<i>lpg0376</i>								<i>llo0548</i>					<i>sdhA</i>	GRIP, coiled-coil	Putative antiapoptotic
	<i>lpg0405</i>								<i>llo2845</i>					-	Spectrin	Unknown
	<i>lpg0422</i>								<i>llo2801</i>					<i>legY</i>	Putative glucan 1,4- α -glucosidase	Unknown
	<i>lpg0483</i>								<i>llo2705</i>					<i>ankC/legA12</i>	Ankyrin	Unknown
	<i>lpg0515</i>								<i>llo3224</i>					<i>legD2</i>	Phytanoyl-CoA dioxygenase	Unknown
	<i>lpg1426</i>								<i>llo1791</i>					<i>vpdC</i>	Patatin	Unknown
	<i>lpg1483</i>								<i>llo1682</i>					<i>legK1</i>	STPK	Modulation of host immune signaling
	<i>lpg1661</i>								<i>llo1691</i>					-	Putative N-acetyl transferase	Unknown
	<i>lpg1950</i>								<i>llo1397</i>					<i>ralF</i>	Sec7 domain	Protein recruitment to the LCV
	<i>lpg2222</i>								<i>llo1443</i>					<i>lpnE</i>	Putative β -lactamase (sel1 and TTR domains)	Host cell entry
	<i>lpg2298</i>								<i>llo1707</i>					<i>ylfA/legC7</i>	Coiled-coil	Putative modulation of host protein trafficking
	<i>lpg2300</i>								<i>llo0584</i>					<i>ankH/legA3/ankW</i>	Ankyrin, NF- κ B inhibitor	Intracellular proliferation
	<i>lpg2322</i>								<i>llo0570</i>					<i>ankK/legA5</i>	Ankyrin	Unknown
	<i>lpg2456</i>								<i>llo0365</i>					<i>ankD/legA15</i>	Ankyrin	Unknown
	<i>lpg2556</i>								<i>llo2218</i>					<i>legK3</i>	STPK	Unknown
	<i>lpg2832</i>								<i>llo0214</i>					-	Hypothetical protein	Unknown
	<i>lpg2936</i>								<i>llo0081</i>					-	rRNA methyltransferase E	Unknown
<i>L. pneumophila</i> core genome	<i>lpg0038</i>													<i>ankQ/legA10</i>	Ankyrin	Unknown
	<i>lpg0403</i>													<i>ankG/ankZ/ legA7</i>	Ankyrin	Unknown
	<i>lpg0436</i>													<i>ankJ/legA11</i>	Ankyrin	Intracellular proliferation
	<i>lpg0695</i>													<i>ankN/ankX/legA8</i>	Ankyrin	Modulation of vesicular transport
	<i>lpg1488</i>													<i>lgt3/legC5</i>	Coiled-coil	Cytotoxicity
	<i>lpg1701</i>													<i>ppeA/legC3</i>	Coiled-coil	Putative modulation of host protein trafficking
	<i>lpg1718</i>													<i>ankI/legA54</i>	Ankyrin methyltransferase	Unknown
	<i>lpg1884</i>													<i>ylfB/legC2</i>	Coiled-coil	Putative modulation of host protein trafficking
	<i>lpg1962</i>													<i>lirB/ppiB/rotA</i>	Rotamase	Unknown
	<i>lpg1976</i>													<i>pieG/legG1</i>	Regulator of chromosome condensation	Unknown
	<i>lpg1978</i>													<i>setA</i>	Putative glucosyltransferase	Putative modulation of host protein trafficking
	<i>lpg2137</i>													<i>legK2</i>	Ca-depPK	ER recruitment to the LCV
	<i>lpg2144</i>													<i>ankB/legAU13/ ceg27</i>	Ankyrin, F-box, CaaX	Recruitment of polyubiquitinated proteins to the LCV
	<i>lpg2176</i>													<i>legS2</i>	Sphingosine-1-phosphate lyase	Autophagy
	<i>lpg2215</i>													<i>legA2</i>	Ankyrin	Unknown
	<i>lpg2410</i>													<i>vpdA</i>	Patatin domain	Unknown
	<i>lpg2452</i>													<i>ankF/legA14/ ceg31</i>	Ankyrin	Unknown
	<i>lpg2793</i>													<i>lepA</i>	Effector protein A	Release of bacteria
	<i>lpg2999</i>													<i>legP</i>	Astacin protease	Unknown

Continued

Table 2. Continued

L. pneumophila strains									L. longbeachae strains								
		Phila	130b	Paris	Lens	Corby	Alcoy	Lorraine	HL06041035	NSW150	D-4968	ATCC39462	98072	C-4E7	Name	Product or associated motif	Function
L. longbeachae core genome										llo0037					-	Ankyrin	Unknown
										llo1168					-	Ankyrin	Unknown
										llo1371					-	Ankyrin, coiled-coil	Unknown
										llo2668					-	Ankyrin	Unknown
										llo3081					-	Ankyrin, patatin-like phospholipase	Unknown
										llo3093					-	Ankyrin, STPK	Unknown
										llo0114					-	LRR	Unknown
										llo1681					-	STPK	Unknown
										llo1427					-	F-Box	Unknown
										llo0448					-	U-Box	Unknown
										llo2643					-	PPR, Coiled-coil	Unknown
										llo2200					-	TTL	Unknown
										llo2327					-	SH2	Unknown
										llo2352					-	PAM2	Unknown
										llo1196					-	Snare	Unknown
										llo0793					-	Phosphatidylinositol-4-phosphate 5-kinase	Unknown
										llo2329					-	Ras-related small GTPase, Miro-like domain	Unknown
										llo2249					-	Miro-like domains	Unknown
										llo1892					-	Putative immunoglobulin I-set domain	Unknown
Legionella accessory genome										llo0990					-	Ankyrin	Unknown
										llo3116					-	LRR	Unknown
										llo3118					-	LRR	Unknown
	lpg0041														-	Putative metalloprotease	Unknown
	lpg0171														legU1	F-box motif	Interaction with host ubiquitination machinery
	lpg0402														ankY/legA9	Ankyrin, STPK	Unknown
	lpg1328														legT	Thaumatoin domain	Unknown
	lpg1355														sidG	Coiled-coil	Interaction with dot/icms system—translocation
	lpg1684														-	Unknown	Unknown
	lpg1890														legLC8	LRR, coiled-coil	Unknown
	lpg1947														lem16	Spectrin	Unknown
	lpg1948														legLC4	LRR, coiled-coil	Unknown
	lpg2224														ppgA	Regulator of chromosome condensation	Unknown
	lpg2400														legL7	LRR	Unknown
	lpg2830														lubX/legU2	U box motif	Polyubiquitination of host proteins
	lpg2862														Lgt2/legC8	Glucosyltransferase	Cytotoxicity

Filled squares represent presence, white squares absence of a gene. ER, endoplasmic reticulum; LCV, *Legionella*-containing vacuole; STPK, serine/threonine protein kinase; LRR, leucine rich repeat; TTL, tubulin-tyrosine ligase; SH2, Src homology 2; PAM, PCI/PINT-associated module; Rab1 GAP, GTPase-activating protein; TTR, tetratricopeptide repeats; Ca-depPK, calmodulin-dependent protein kinase.

EUKARYOTIC-LIKE PROTEINS WITNESS THE LONG-LASTING COEVOLUTION OF *Legionella* WITH EUKARYOTIC CELLS

The presence of these many eukaryotic-like proteins in the genome of amoeba-associated bacteria raises the question of the origin of this part of the genome. Are these eukaryotic-like genes laterally acquired or are they the result of adaptive changes generating motifs similar to the eukaryotic ones? The latter is known as “convergent evolution” in contrast to horizontal gene transfer (HGT). Although both can lead to similar proteins, similarity resulting from HGT is detectable at the primary sequence level, whereas convergent evolution more frequently leads to similar tridimensional structures but not necessarily to similar amino acid sequences. *Legionella* possesses all necessary factors for acquiring genes laterally as these bacteria are naturally competent and a complete recombination machinery is present, thus it is conceivable that eukaryotic-like proteins have been acquired by HGT. To analyze this question, different phylogenetic analyses of some of these eukaryotic-like proteins have been performed revealing a clustering of the *Legionella* eukaryotic-like proteins with eukaryotic sequences, further supporting the hypothesis that these were acquired by HGT from eukaryotic organisms like amoeba (de Felipe et al. 2005; Nora et al. 2009; Lurie-Weinberger et al. 2010; Gomez-Valero et al. 2011a). An example is the enzyme arylamine *N*-acetyltransferase (NAT) encoded by *lpp2516* in strain Paris. The *L. pneumophila* NAT is an atypical member of the arylamine *N*-acetyltransferase family that allows *L. pneumophila* to detoxify aromatic amine chemicals and to grow in their presence (Kubiak et al. 2012). This enzyme probably allows *L. pneumophila* that is exposed to many chemicals in its natural and man-made environments, to better adapt and survive in these environments (Kubiak et al. 2012). Phylogenetic reconstruction shown in Figure 2A indicates that horizontal acquisition of this gene from ciliates is the most probable origin of this enzyme in *Legionella* (Kubiak et al. 2012).

Another convincing example is the eukaryote-like sphingosine-1-phosphate lyase of *L.*

pneumophila that is encoded by *lpp2128* in strain Paris. It has been shown that the *L. pneumophila* Spl is a Dot/Icm-secreted effector that is able to complement the sphingosine-sensitive phenotype of *Saccharomyces cerevisiae* and that colocalizes with host-cell mitochondria (Degtyar et al. 2009). Figure 2B shows a phylogenetic reconstruction of Spl sequences that we have undertaken. The *L. pneumophila* Spl protein sequence clearly falls into the eukaryotic clade of SPL sequences and those of aquatic protozoa are the closest phylogenetic relatives, suggesting acquisition of this gene by HGT from an amoeba host. Most interestingly, *Legionella* also encodes proteins not present in any prokaryote sequenced to date, as amoebae are the only organisms in which we identified sequences with significant similarity. An example is Lpg1684 that codes a protein of unknown function and belongs to the eukaryotic D123 protein family. Eukaryotic-like proteins may have been acquired also from other eukaryotic organisms like viruses. For example, the eukaryotic-like protein Lpg2416 is an ankyrin-containing protein whose best homolog is present in the *Acanthamoeba polyphaga* mimivirus, a giant virus infecting *Acanthamoeba* that are also the hosts of *Legionella* (Lurie-Weinberger et al. 2010). Furthermore, recent studies have shown that these eukaryotic-like proteins can also be exchanged among different amoeba-related bacteria, adding complexity to the possible evolutionary scenarios. For example, the sequencing and analysis of the genome of *L. drancourtii* have identified numerous cases of exchange between the intracellular bacteria of the order Legionellales and Chlamydiales (Gimenez et al. 2011). However, for several eukaryotic-like proteins, a clear description of their phylogenetic history remains difficult, as the acquisition events might have been too ancient for the phylogenetic signal in the sequences to be preserved. Moreover, in some of these proteins, the eukaryotic sequence is restricted to a motif and therefore to a very short region, for which phylogenetic analyses are less evident. Furthermore, only few genome sequences of aquatic amoeba and other protozoa that are most probably the main donors are currently present in the databases. Only

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Figure 2. Phylogenetic tree of selected eukaryotic-like proteins of *Legionella* spp. (A) Phylogeny of the enzyme arylamine *N*-acetyltransferase (NAT) (Lpp2516) of *L. pneumophila*. (B) Phylogeny of the sphingosine-1 phosphate lyase Lpp2128 of *L. pneumophila*. Homolog sequences were recruited from a database containing only completed genome sequences. Selected representatives of all eukaryotic groups and one representative of each bacterial species are included in the analyses. After blastp only significant hits were recruited (e -value $< 10 \times 10^{-4}$), and only one hit for each species was retained. The alignments were performed with T-coffee (expresso) for Lpp2616 and Muscle for Lpp2128, and followed by manual curation. Phylogenies were reconstructed using a distance method (NJ) with 1000 bootstrap replicates. The corresponding support values are shown in each node (values lower than 50 are not represented). Bars represent 20% and 10% of estimated phylogenetic divergence, respectively. (Legend continues on following page.)

Figure 2. (Continued). The clustering of *Legionella* and eukaryotes was confirmed in both cases by in-parallel likelihood reconstructions.

once the gap of genome sequences of aquatic protozoa in the databases is filled will we be able to fully understand the origin and history of these eukaryotic-like proteins.

Independently of the origin of these eukaryotic-like genes, their mechanism of integration remains unknown. How are these eukaryotic genes without any similarity to prokaryotic sequences integrated in the *Legionella* genome?

Many eukaryotic genes have introns, but their prokaryotic counterparts do not. How can these introns be processed or how did they “disappear?” It is tempting to propose that this happened not via HGT but via “horizontal RNA transfer.” Indeed, the *L. pneumophila* genomes encode a reverse transcriptase that could be implicated in this process. However, the integration mechanism remains to be discovered.

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Moreover, there is evidence for the presence of a secretion signal in the carboxy terminal of the Dot/Icm substrates (Nagai et al. 2005; Kubori et al. 2008; Huang et al. 2010); therefore, acquisition of this signal is also a challenge for these proteins to be secreted. Thus, although we start to discern how these eukaryotic proteins have appeared in the *Legionella* genomes, many questions with respect to the detailed mechanism of acquisition and integration remain unanswered.

THE *Legionella* ACCESSORY GENES ARE PART OF THE MOBILOME

The most evident component of the *Legionella* accessory genome is the plasmids. The strains Paris, Lens, and Lorraine each contain a plasmid of 132, 60, and 150 kb, respectively (Cazalet et al. 2004; Chien et al. 2004). Likewise, *L. longbeachae* strain NSW150 possesses a plasmid of 72 kb, and also strain D-4968 seems to contain a plasmid (Kozak et al. 2010). These replicons show a heterogeneous distribution among the different genomes, as the same plasmids or partially similar plasmids are present in different strains (Cazalet et al. 2008). Likewise, a comparative analysis of these plasmids revealed that the 30.4 kb Tra region present in the plasmid of *L. pneumophila* strain Paris shows much higher identity with the Tra region located on the *L. longbeachae* plasmid, than with those of the other *L. pneumophila* strains sequenced (Gomez-Valero et al. 2011b). Interestingly, regions coding proteins homologous to Tra proteins probably coding for a conjugative machinery are present in all *Legionella* plasmids. They are similar to F-type T4SS that allow the synthesis of a long and flexible pilus for conjugation in liquid and solid media (Lawley et al. 2003). However, such a system is also found in a chromosomal localization in the *L. pneumophila* strain Philadelphia and in *L. longbeachae* strain NSW150. In both strains, this region is inserted in a tRNA gene next to an integrase and it is bordered by flanking repeats. The presence of these elements suggests that these T4SSs are mobile and that their heterogeneous distribution is the result of the lateral movement of these

plasmids. Furthermore, these conjugative elements can be found in different plasmids or can be completely or partially present in the chromosome, indicating that they might have the capacity to integrate and excise from the *Legionella* genomes. Indeed, this has been shown for the region carrying the Lvh genes. Lvh is a genomic-island-like region that encodes a T4ASS implicated in conjugation and in virulence-related phenotypes under laboratory conditions mimicking the spread of Legionnaires' disease from environmental niches (Ridenour et al. 2003; Bandyopadhyay et al. 2007). This region can be integrated in the chromosome but can also excise in a site-specific manner to exist as a low copy number plasmid that is undoubtedly transferable among strains (Doleans-Jordheim et al. 2006). Indeed, it is present in five of the eight sequenced *L. pneumophila* strains, in *L. longbeachae* strain D-4968 (Kozak et al. 2010), and according to hybridization results also partly in *Legionella rubrilucens* (Cazalet et al. 2008). Interestingly, in the *Legionella* strains where the Lvh T4SS is not present, another Tra system, a P-type T4SS that codes for short and rigid pili that allow surface mating for conjugation, is present in the same chromosomal position (Gomez-Valero et al. 2011b). This remarkable diversity and mobility of conjugative elements probably coding T4SSs in *Legionella* and their presence in all strains, suggests a role in the bacterial adaptation to the different environments and hosts.

An intriguing feature of these probably mobile regions is their association with genes coding for homologs of a putative phage repressor protein (PrpA) and for homologs of LvrA, LvrB, and LvrC, first described for the Lvh region of *L. pneumophila*. LvrC is a homolog of CsrA, an RNA-binding protein crucial for the regulation of the switch between the replicative and transmissive phases of *L. pneumophila* (Molofsky and Swanson 2004). It is tempting to assume that the CsrA homolog encoded on each of these regions is implicated in the regulation of the mobility and/or the integration or excision of these islands. Perhaps under certain conditions it is advantageous for *Legionella* to have multiple copies of these plasmids to achieve a higher expression of these genes or for promoting high

frequencies of DNA interchange to rapidly adapt to new conditions. This idea is further underlined by the observation that the presence of homologous Lvr loci is not only limited to the Lvh or Tra regions, but they are also present in other genomic locations that seem to be mobile. Thus, large parts of the accessory genome of *Legionella* are mobile, and it seems that their mobility is regulated by a particular mechanism that still remains elusive.

CONCLUSIONS: THE *Legionella* ACCESSORY GENOME IS PART OF A GLOBAL MOBILOME CONFERRING VERSATILITY

One of the best-known characteristics of obligate, intracellular bacteria is a reduction of the genome size as many genes become dispensable given the stability of the intracellular environment they inhabit. In addition, there is a minor probability of DNA exchange as they live in a

closed niche (Moya et al. 2008). Extreme examples are the symbiotic bacteria *Buchnera aphidicola*, an insect endosymbiont found in the aphid *Cinaria cedri* with a genome size of 416 Kb coding solely for 357 protein-coding genes (Pérez-Brocal et al. 2006) or the obligate intracellular parasite *Rickettsia prowazekii*, the causative agent of epidemic typhus with a 1.1 Mb genome (Andersson et al. 1998). However, this does not hold true for amoeba-associated bacteria like *Legionella*, as no signs that they follow a reductive evolutionary path are present in their genomes, although *Legionella* are probably replicating exclusively intracellularly. Furthermore, *Legionella* have highly dynamic genomes and a large flexible gene pool is present in the genus comprising plasmids, mobile genetic elements, and genes probably transferred from their hosts. This large mobilome confers to these bacteria their high versatility and capacity to adapt to many different environmental conditions and

Figure 3. Model of the genetic flow inside amoeba. The accessory genome of *Legionella*, other intracellular bacteria, mimivirus and amoeba are contributing to a “global mobilome” that is accessible to the different organisms living inside amoeba to adapt to different conditions and allows versatility in the host–pathogen interactions.

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to infect diverse hosts like amoeba, protists, probably algae, and humans. Thus, the intracellular environment provided by amoeba is different from the environment in which symbionts like (e.g., *Buchneria* or *Rickettsia* live), probably because amoeba may host different bacteria and viruses at the same time, allowing exchange of genetic material among a variety of organisms. We thus propose that amoeba and aquatic protozoa form communities with a large pool of exchangeable genetic material, and have a large common accessory genome, derived from the host and from the diverse intracellular organisms that inhabit them as parasites or symbionts (Fig. 3). This big “global mobilome” allows the constant acquisition of DNA and provides a large repertoire of diverse functions for adapting to changing environmental conditions. This large mobile gene pool combined with the high genome dynamics provide the basis of versatility and adaptability in *Legionella* and allow successful intracellular parasitism in many different hosts.

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Genome Dynamics in *Legionella*: The Basis of Versatility and Adaptation to Intracellular Replication

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