More than 18,000 effectors in the *Legionella* genus genome provide multiple, independent combinations for replication in human cells

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The genus *Legionella* comprises 65 species among, which *Legionella pneumophila* is a human pathogen. To understand the evolution of an environmental to an accidental human pathogen, we have functionally analyzed 80 Legionella genomes spanning 58 species. Uniquely, an immense repository of 18,000 secreted proteins encoding 137 different eukaryotic-like domains and over 200 eukaryotic-like proteins is paired with a highly conserved T4SS. Specifically, we show that eukaryotic Rho and Rab-GTPase domains are found nearly exclusively in eukaryotes and *Legionella*. Translocation assays for selected Rab-GTPase proteins revealed that they are indeed T4SS secreted substrates. Furthermore, F/U-box and SET domains were present in >70% of all species suggesting that manipulation of host signal transduction, protein turnover and chromatin modification pathways are fundamental intracellular replication strategies for Legionellae. In contrast, the Sec-7 domain was restricted to *L. pneumophila* and seven other species, indicating effector repertoire tailoring within different amoebae. Functional screening of 47 species revealed 60% were competent for intracellular replication in THP-1 cells, but interestingly this phenotype was associated with diverse effector assemblages. These data, combined with evolutionary analysis indicate that the capacity to infect eukaryotic cells has been acquired independently many times within the genus and that a highly conserved T4SS secretes an exceptional number of different proteins shaped by inter-domain gene transfer. Furthermore we revealed the surprising extent to which Legionellae have co-opted genes and thus cellular functions from their eukaryotic hosts and provide a new understanding of how dynamic reshuffling and gene-acquisition has led to the emergence of human pathogens.

Legionella | co-evolution | horizontal gene transfer | protozoa

Introduction

Legionnaires' disease or legionellosis is an atypical pneumonia caused by bacteria of the genus *Legionella*. Shortly after the discovery of *L. pneumophila* (1) it was reported that this bacterium is pathogenic for freshwater and soil amoebae of the genera *Acanthamoeba* and *Naegleria* (2). This finding led to a new perception in microbiology, whereby bacteria that parasitize protozoa can utilize similar processes to infect human cells. Sequencing and analyses of the *L. pneumophila* genome substantiated this idea, when it revealed the presence of a large number and variety of eukaryotic-like domains within the predicted proteome (3). Many of these proteins, termed effector proteins, were shown to be secreted into the host cell where they facilitate *Legionella* intracellular replication within a specialized compartment termed the *Legionella* containing vacuole (LCV) (3, 4). Overall, the type IV secretion system (T4SS), Dot/Icm, secretes more than 300 different effector proteins into the host cell and is indispensable for virulence of *L. pneumophila* (5-8). The presence of the Dot/Icm T4SS in other *L. pneumophila* strains and in selected *Legionella* species had also been reported (9-12) but recent genome scale studies of *Legionella* (13-15) indicated that the T4SS system is present in every *Legionella* strain analyzed.

Despite high conservation of the Dot/Icm system among different *Legionella* species, effector repertoires appear to vary greatly. An analysis of putative T4SS effectors of *L. longbeachae*, the second most frequent cause of Legionnaires' disease, revealed that only about 50% of the virulence factors described in *L. pneumophila* were also present in the genome of *L. longbeachae* (16). Recently, Burstein *et al.* (14) analyzed 38 *Legionella* species using a machine learning approach to predict T4SS effectors and Joseph *et al.* (15) examined *Legionella* genome dynamics, both concluding that DNA interchange between different species is rare. However, still little is known about the potential of the different species to cause human disease and about the impact and the specific characteristics of the T4SS effectors on the evolution of new human pathogens within this environmental bacterial genus.

Here we present a comprehensive analysis of the *Legionella* genus genome, covering 80 *Legionella* strains belonging to 58 *Legionella* species and subspecies. We establish a pan-genus pool of putative T4SS effectors and show that this comprises over 18,000 proteins and identify more than 200 new eukaryotic-like proteins and 137 eukaryotic domains, including a unique class

Significance

***Legionella* comprises 65 species for which aquatic amoebae are the natural reservoirs. Using functional and comparative genomics to deconstruct the entire bacterial genus we reveal the surprising parallel evolutionary trajectories that have led to the emergence of human pathogenic *Legionella*. An unexpectedly large and unique repository of secreted proteins (>18,000) containing eukaryotic-like proteins acquired from all domains of life (plant, animal, fungal, archaea) is contrasting with a highly conserved type 4 secretion system. This study reveals an unprecedented environmental reservoir of bacterial virulence factors, and provides a new understanding of how reshuffling and gene-acquisition from environmental eukaryotic hosts, may allow for the emergence of human pathogens.**

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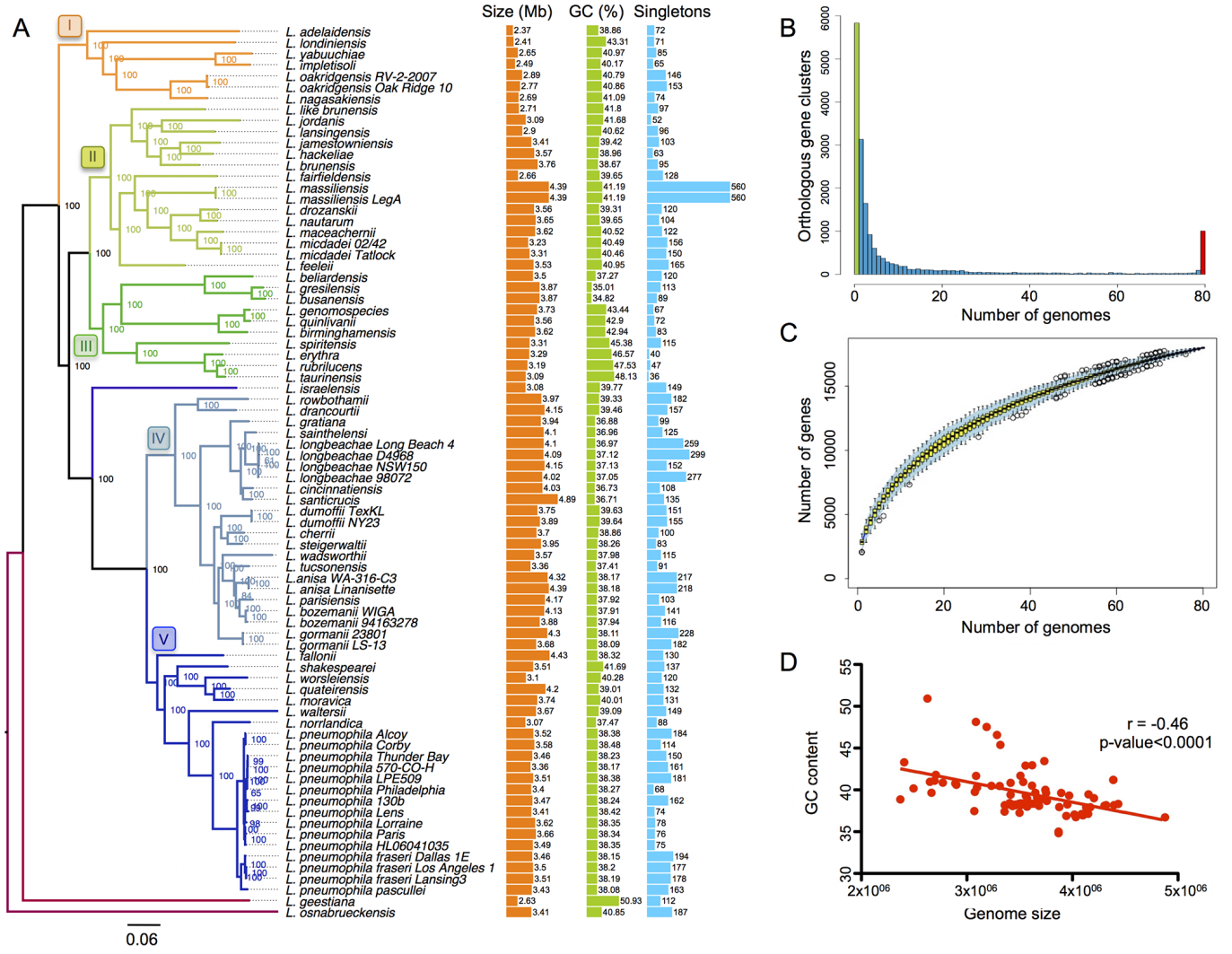

Fig. 1. The Legionella genomes are diverse in size and gene content. A) Phylogeny of the genus based on the core genome, genome size, GC content and number of singletons of each species are depicted. Numbers represent bootstrap values. Branches are coloured according to the clade they belong to. Genome size and GC content include plasmids if present in the corresponding species. The number of singletons is based on the results of OrhtoMCL (takes into account orthologs and paralogs). Each species has been compared to the others without taking into account strains from the same species to avoid bias due to the number of strains sequenced within a species. B) Occurrence of genes within the 80 analysed Legionella genomes. Left end of the x-axis, genes present in a single genome (strain specific genes; 5832 ≈32% of the pangenome); right end of the x-axis, genes present in all 80 genomes (core-genome; 1008 genes ≈6% of the pan-genome) C) Gene accumulation curve for the total number of proteins of the 80 genomes. D) Negative correlation between genome size and GC content indicating high acquisition of foreign genes (Pearson's correlation coefficient equal to -0.46 with a p-value<0.0001)

of putative bacterial Rab GTPases. We confirmed experimentally that a subset of these proteins translocate into the host cell upon infection. We conclude that the T4SS is highly conserved at the sequence level, but the effector proteins secreted are highly diverse.

Results and discussion

The *Legionella* genus genome is dynamic and characterized by frequent genetic exchange. We sequenced 58 *Legionella* species of which 16 were newly sequenced, and analyzed them in combination with all publicly available genomes (80 genomes in total) (**SI Appendix, Table S1**). The *Legionella* genomes were extremely diverse, as the genome size varied from 2.37Mb (*L. adelaidensis*) to 4.88Mb (*L. santicrucis*), the GC content from 34.82% (*L. busanensis)* to 50.93% (*L. geestiana)* and the number of clusters of orthologous genes as defined with OrthoMCL was 17,992 of which 5,832 (32%) were strain specific (singletons) (**Fig. 1A**). Only 1,008 genes (6%) constituted the core genome (**Fig. 1B**), compared to an earlier analysis of 38 *Legionella* species, which found 16,416 clusters of orthologous and 1,054 core genes (14). The addition of 40 new genomes comprising 16 newly sequenced *Legionella* species in our study increased the number of orthologous gene clusters by over 1,576 and decreased the core genome by 46 genes, underlining the high diversity of the *Legionella* genus. This difference suggested that the *Legionella* genus pan-genome is far from fully described and that sequencing of additional *Legionella* species will increase the genus gene repertoire significantly. This was supported by the rarefaction curve that does not reach a plateau (**Fig. 1C**).

The highly dynamic nature of these genomes is also seen in the analysis of the strain specific genes and the accessory genome as it highlights the presence of several mobile genetic elements; often associated with genes encoding for transfer regions/conjugative elements such as the type IVA secretion systems (T4ASS). These T4ASSs (classified as T4SSF, G, I and T (17) are present in each strain to varying degrees indicating that they circulate among the different *Legionella* strains (**SI Appendix, Table S2**) and therefore drive genome dynamics and

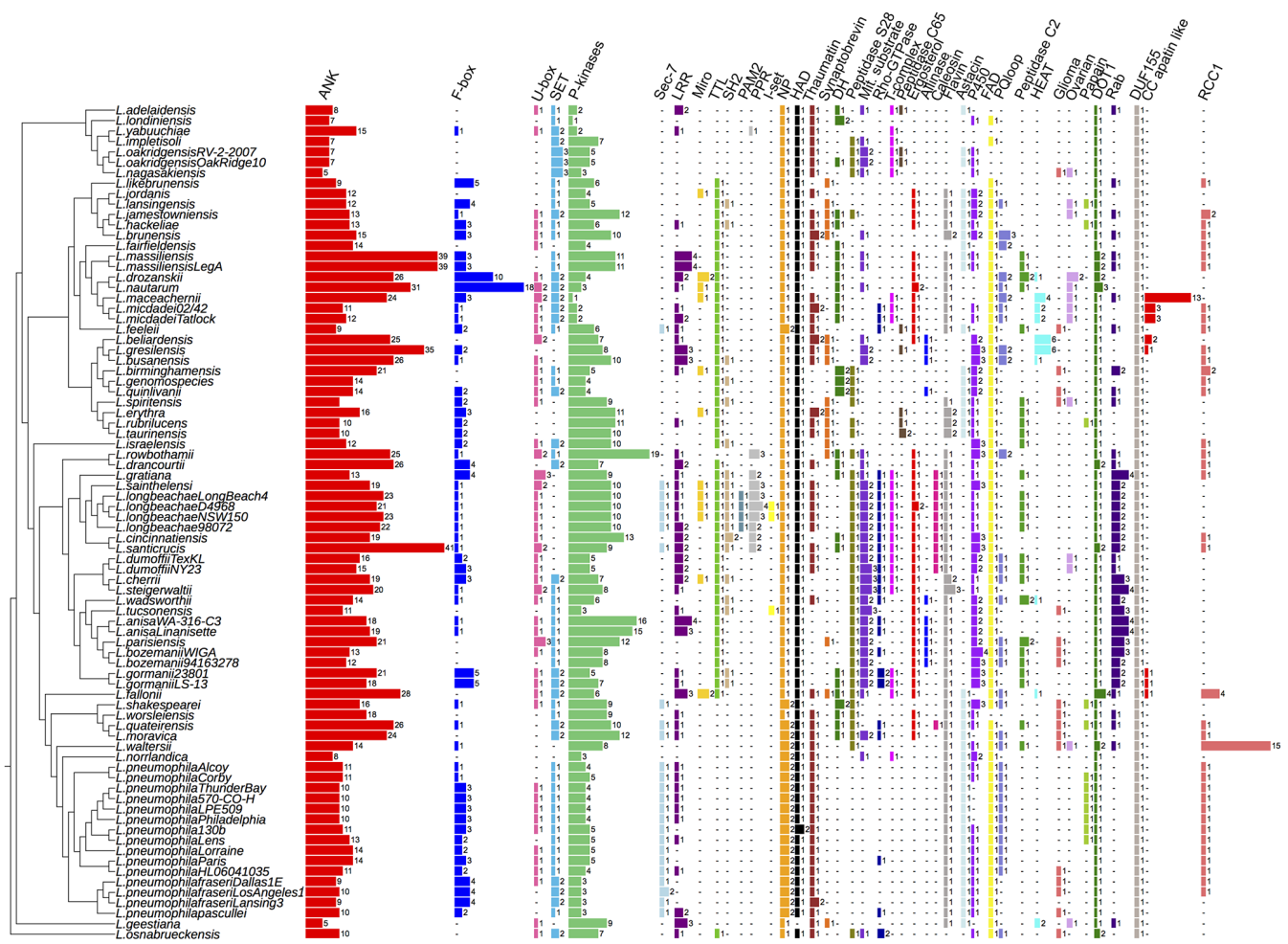

Fig. 2. Eukaryotic domains have a diverse distribution within the genus Legionella suggesting multiple acquisition events. The number and distribution of the 41 most frequently identified eukaryotic motifs within the genus Legionella are shown. Numbers represent the number of proteins containing this eukaryotic motif. Abbreviations used: ANK (ankyrin), F-box, U-box), SET domain, Pkinases (protein kinases), Sec-7 domain, LLR (leucine rich repeats), Miro (Mitochondrial Rho domain), TTL (tubulin-tyrosine ligase), SH2 (The Src homology 2), PAM2 (ataxin-2, C-terminal), PPR (pentatricopeptide repeat), I-set (immunoglobulin I-set), NP (nucleoside phosphatase gda1/cd39), HAD (HAD-superfamily hydrolase), DH (Dbl homology domain), Mit. Substrate (mitochondrial substrate/solute carrier), Rho GTPases-activating protein domain, T-complex (T-complex10/11), PC65 (Peptidase C65 otubain), Ergosterol (Ergosterol biosynthesis), Flavin (flavin monooxygenase-like), Astacin (Peptidase M12A, astacin), Cyt:P450 (Cytochrome P450), Cytokine FAD (Cytokinin dehydrogenase 1, FAD/cytokinin binding domain), PQ loop repeat, Peptidase C2 (calpain, catalytic domain), LR glioma (Leucine-rich glioma-inactivated, EPTP repeat), Ovarian (Ovarian tumour, otubain), Papain (Peptidase C1A, papain C-terminal, DOT1 (Histone methylation DOT1) , Rab small GTPases, DUF155, C/C (Clathrin/coatomer adaptor, adaptinlike), RCC1 (Regulator of chromosome condensation).

diversification. It has been suggested that the incorporation of foreign DNA via horizontal gene transfer (HGT) is responsible for an increase in the AT content and the increase in genome size (18). Indeed, we found a negative correlation between the genome size and the GC content for the *Legionella* genomes, which also suggests frequent HGT (**Fig. 1D**) (19). Despite the importance of flagella for transmission to new hosts as shown for *L. pneumophila*, flagella encoding genes were not conserved in all species, but showed a patchy distribution, as 23 of the 80 strains analyzed lacked flagella genes (**SI Appendix, Fig. S1**). The analyses showed that the *Legionella* genus genome is highly diverse, dynamic and shaped by HGT.

The genus *Legionella* encodes proteins with 137 different eukaryotic domains. Interpro scan analysis of all 58 *Legionella* species revealed the presence of 137 different eukaryotic motifs/domains in the genus *Legionella* (**SI Appendix, Table S3**) according to the definition that an eukaryotic domain is one that is found in >75% of eukaryotic genomes and <25% in prokaryotic genomes. The most abundant eukaryotic domains identified were ankyrin repeats. Interestingly, *L. santicrucis and L. massiliensis* encoded 41 and 39 ankyrin domains, respectively (**Fig. 2**). Ankyrin motifs were found frequently associated with other eukaryotic motifs and thus constituted modular proteins associated with eukaryotic F-box, U-box, Rab or SET domains. Notably, F-box and U-box domains were present in more than two thirds of the species analyzed (**Fig. 2**) suggesting manipulation of the host ubiquitin-system is a fundamental virulence strategy of *Legionella* species. Generally, the genomes contained one to three F-box containing proteins with the exception of *L. nautarum* and *L. dronzanskii*, which contained 18 and 10, respectively. The SET domain containing protein RomA of *L. pneumophila* that induces a unique host chromatin modification (20) is present in 46 of the 58 *Legionella* species suggesting the ability of many *Legionella* species to manipulate host chromatin (**Fig. 2**). Interestingly, the Sec-7 domain present in the effector RalF, a bacterial ARF guanine exchange factor and the first described Dot/Icm effector

Table 1. Homology of *Legionella* Rab domains-containing proteins against protozoan Rab proteins

Domain	Protein	First blast hit	Identity	Coverage	e-value
Rab	Lade0491	*Entamoeba histolytica*	35%	52%	4.E-17
Rab	LgoA0634	*Paramecium tetraurelia*	33%	51%	2.E-19
Rab	Llo3288	*Ichthyophthirius multifiliis*	42%	53%	4.E-31
Rab	Lstei0814	*Tetrahymena thermophila*	34%	86%	3.E-26
Rab	Lstei2185	*Stentor coeruleus*	38%	55%	6.E-29
Rab	Lbir2252	*Entamoeba invadens*	32%	55%	5.E-15
Rab	Lges1860	*Entamoeba histolytica*	34%	55%	7.E-25
Rab+ Fbox	Lwad3214	*Paramecium tetraurelia*	34%	35%	7.E-14
Rab	Lgra2891	*Guillarda theta*	36%	56%	2.E-19
Rab	Lgra3435	*Entamoeba histolytica*	35%	59%	2.E-27
Rab	Lma1540	*Paramecium tetraurelia*	34%	55%	1.E-17
Rab + ank	LmasA3690	*Oxytricha trifallax*	34%	19%	2.E-19
Rab	Lqua0234	*Dictyostelium fasciculatum*	38%	34%	1.E-25
Rab	Lquin3026	*Tetrahymena thermophila*	34%	57%	1.E-19
Rab	Lspi0161	*Naegleria gruberi*	34%	24%	7.E-24
Rab	Lwal3261	*Paramecium tetraurelia*	33%	85%	7.E-18

Each Rab protein listed in the table represents a different orthologous group. Results are based on blastp searches using the non-redundant NCBI database.

of *L. pneumophila* (21) was present in only eight (*L. pneumophila*, *L. longbeachae*, *L. feelei*, *L. sainthelensis*, *L. santicrucis*, *L. shakespeari*, *L. quateirensis L. moravica*) of the 58 *Legionella* species analyzed, suggesting that, different effectors may compensate for RalF activity or that LCV biogenesis varies among different species (**Fig. 2**).

One newly identified motif in *Legionella* was the ergosterol reductase ERG4/ERG24 (IPR001171) domain. Ergosterol is the primary sterol in the cell membranes of filamentous fungi, present in membranes of yeast and mitochondria (22). Importantly, it is also the major sterol of amoebae such as *A. castellanii* and *A. polyphaga,* the natural hosts of *Legionella* (23, 24). We found that 31 *Legionella* species encoded one or two proteins with the ERG4/ERG24 domain (**Fig. 2**). The *L. longbeachae* protein (L1o1320) containing this domain showed 56% aa identity to that encoded by the amoeba *Naegleria gruberi* and 30% aa identity to that encoded by *A. castellani* strain Neff. This domain was also present in other amoebae related bacteria such as *Parachlamydia acanthamoebae* and *Protochlamydia naegleriophila*, as well as *Coxiella burnetii*. Phylogenetic analyses suggested that *L. longbeachae* acquired this domain from amoeba (**SI Appendix, Fig. S2A**).

Phylogenetic analyses of the here identified C-terminal alliinase and Caleosin domains present in *L. beliardensis* and *L. anisa* or the *L. longbeachae* clade (**Fig. 2**), respectively further supported acquisition of these domains from plants, amoeba or fungi **(SI Appendix, Fig. S2B-C).** They probably help *Legionella* to fight competitor bacteria or fungi in amoebae or in the environment. Taken together, our analyses highlight key domains preferentially present in protozoa, fungi, plants or animals that have been acquired by different *Legionella* species.

A unique case in the prokaryotic world: *Legionella* encode small GTPase-like domains The Ras-related small GTPase superfamily comprises more than 150 members in humans, which function as key regulators of signal transduction in almost all cellular processes(25). These enzymes bind and hydrolyse GTP to GDP and activate downstream effectors when bound to GTP. The first identified member was the p21-Ras protein, an evolutionary conserved small GTPase that controls cell proliferation, survival and migration through its effector binding at RAF/MAPK and PI3K (26). The Ras protein superfamily is subdivided into at least five distinct branches: Ras, Rho, Rab, Arf and Ran (27). Evolutionarily conserved orthologs are found in Drosophila, C. elegans, S. cerevisiae, S. pombe, Dictyostelium and plants (28).

The only Rab-like protein in a prokaryotic genome was reported in the *L. longbeachae* genome sequence (16). However, upon analysis of our 80 *Legionella* strains, we identified 184 small GTPases of which 104 could be classified with a very high confidence as Rab, Ras or Rho like proteins (34 Ras, 71 Rab and one Rho domain) (**SI Appendix, Table S4** and **Fig. S3**). Blastp analysis of these proteins in the NCBI database revealed that 149 of the 184 small GTPases of *Legionella* were exclusively present in *Legionella* and eukaryotic organisms (**Table 1**). The Rab domain was localized to different parts of the effector proteins, and a subset of Rab proteins carried additional domains such as U-box domains, ankyrin motifs or F-box domains (**Fig. 3A**). Alignment of the different Rab domains identified in the *Legionella* genomes revealed that the structural features of eukaryotic Rab domains were conserved among the *Legionella* proteins (**SI Appendix, Fig. S4**).

To analyze further the evolutionary history of the Ras-related domains in *Legionella* we undertook phylogenetic analyses of these proteins. For example, the two *L. longbeachae* Rab proteins, Llo1716 and Llo3288, were present in all strains closely related to *L. longbeachae*, suggesting that they and their orthologous share a common origin and evolved from a gene acquired by the ancestor of all these species (**SI Appendix, Fig. S5**). Further phylogenetic analysis of 16 Rab proteins present in eight different *Legionella* species showed that these Rab domains were acquired by HGT, mainly from protozoa (**Fig. 3B and SI Appendix, Fig. S6A-P**). Recently a novel isoform of Rab5D was identified in the *Acanthamoeba polyphaga mimivirus* (APMV) and all group I members of the *Mimiviridae* (29). Phylogenetic analyses suggested that the Rab GTPase was acquired by an ancestor of the *Mimiviridae* family and Rabs from *Mimiviridae*, *Plasmodium* and few lower eukaryotes form a separate clade (29). Thus, *Legionella* and APMV that both infect the protozoa *Acanthamoeba* encode Rab proteins most likely to mimic and subvert host cell function. To substantiate that these proteins act in the host cell, we determined whether the Rab containing proteins were bona fide substrates of the Dot/Icm T4SS by creating fusion proteins between the 16 different Rab proteins and the catalytic domain of the TEM-1 beta-lactamase (indicated by a star in **SI Appendix, Fig. S5**). Translocation assays were performed using wild type *L.*

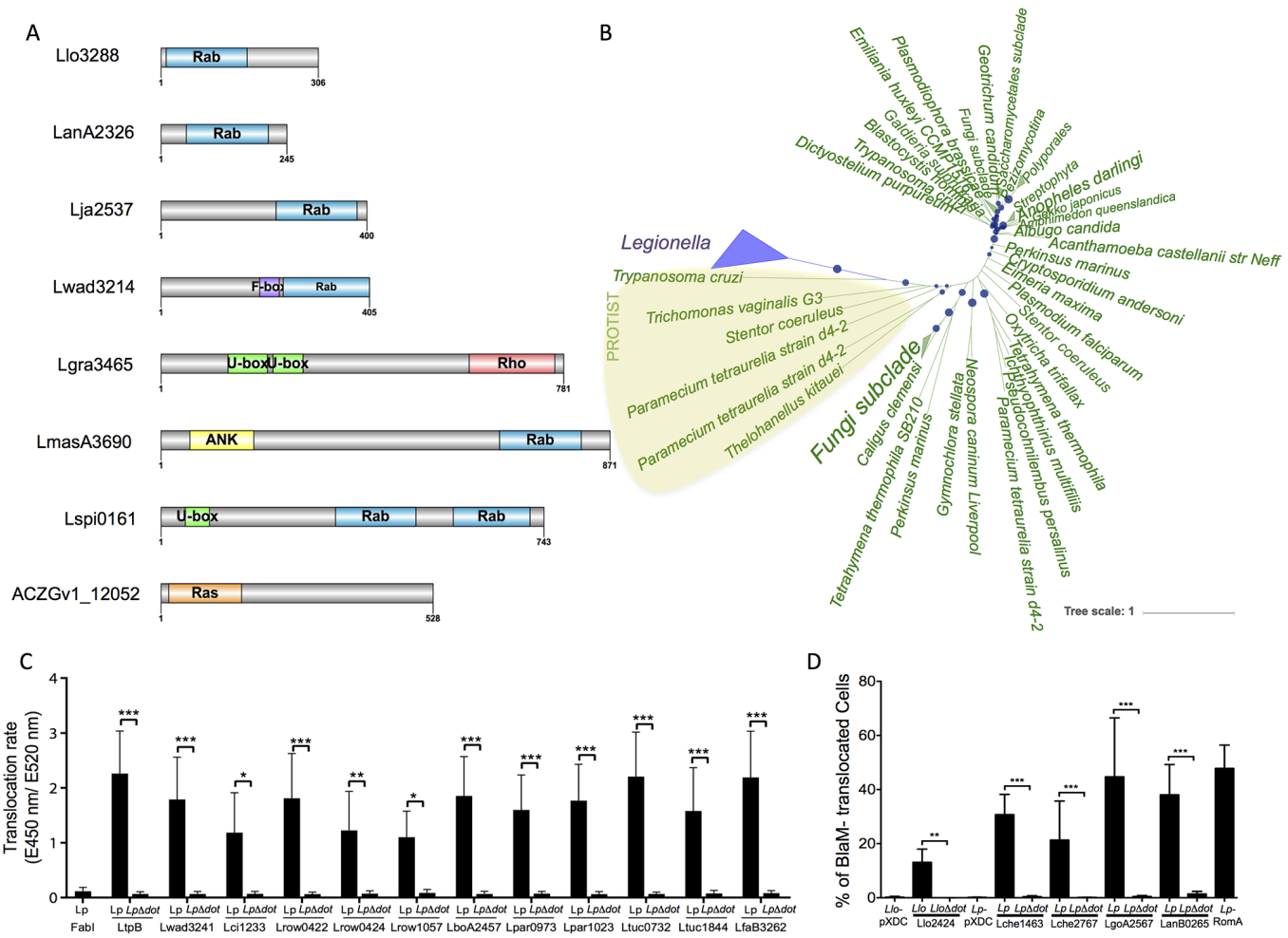

Fig. 3. Domain organization of small GTPases in Legionella and phylogenetic analyses of the Llo3288 Rab proteins suggests eukaryotic origin. A) Domain organization of the different small GTPases proteins identified. B) Unrooted tree of Llo3288 and homologues recruited by blastp constructed using likelihood. Local support values are represented with circles on the corresponding branches and size of circles is proportional to the values (only local support of at least 0.7 are shown). C) Translocataion of selected proteins using the beta-lactamase translocataion assay and infection of Raw264.7 cells for 1h with Lp wild typeor LpΔdotA expressing BlaM-effector fusions analysed with a microplate reader. Three independent experiments (n=9) were done. Statistical significance was determined by 2-way Anova with multiple comparisons test (*, P<0.05; **, P<0.01; ***, P<0.001. D) Transloctaion of selected proteins using the beta-lactamase translocataion assay and infectionof THP-1 cells at an MOI of 50 during 1h 30min with Lp and Llo strains in before addition of CCF4-AM and analyses by flow cytometry. Histograms show the frequency of BlaM- translocated, blue fluorescence-emitting cells as means ± SD of three independent experiments (n=12). Statistical significance was determined by Wilcoxon matched pairs test (**, P<0.01; ***, P<0.001).). Lp, L. pneumophila wild type; Llo, L. longbeachae wild type; Lp ΔdotA, L. pneumophila ΔdotA; LloΔ, L. longbeachae ΔdotA.

pneumophila as a surrogate host and compared with an isogenic Dot/Icm mutant (Δ*dotA*). All 16 Rab motif-containing proteins were translocated by *L. pneumophila* but not by the Δ*dotA* mutant (**Fig. 3C-D**).

More than 250 different eukaryotic like proteins are encoded in *Legionella* genomes. In addition to modular effectors with eukaryotic domains, the *Legionella* genome encodes proteins that are similar to eukaryotic proteins, many of which are proven effectors of the Dot/Icm T4SS. A wider search for eukaryotic like proteins in the *Legionella* genus identified 2196 eukaryotic like proteins representing more than 400 different orthologous groups that matched better to eukaryotes than to prokaryotes from a total of 6809 different orthologous proteins that matched with eukaryotic proteins. Among these, we identified 156 proteins with a eukaryotic domain, and 210 new eukaryotic-like proteins (**SI Appendix, Table S5**). Furthermore, 152 eukaryotic like proteins detected possess a higher GC content (40%-62%) than the rest of the genome indicating recent HGT. Phylogenetic analysis of selected, newly identified proteins suggested that these were acquired from eukaryotes. As an example, **SI Appendix, Fig. S7** shows the protein LanA0735 from *Legionella anisa*, a species frequently found in artificial water systems. This protein belongs to the pyridine nucleotide-disulfide oxidoreductase family, a subfamily of the FAD dependent oxidoreductase family. LanA0735 showed some similarity to thioredoxin reductase that exists as two major ubiquitous isoenzymes in higher eukaryotic cells, one cytosolic and the other one mitochondrial. The cytosolic form has been implicated in interference with the acidification of the lysosomal compartment in *C. elegans* (30), and thus LanA0735 may help *Legionella* avoid vacuole acidification during infection.

Among the proteins defined as eukaryotic like, two previously described phospholipases of *L. pneumophila*, PlcB (Lpp1411/Lpg1455) and PlcA (Lpp0565/Lpg0502) were identified in our analysis as eukaryotic proteins. The only other bacteria encoding these two enzymes are *Pseudomonas* and amoebae-associated bacteria. The two enzymes have phospholipase activity

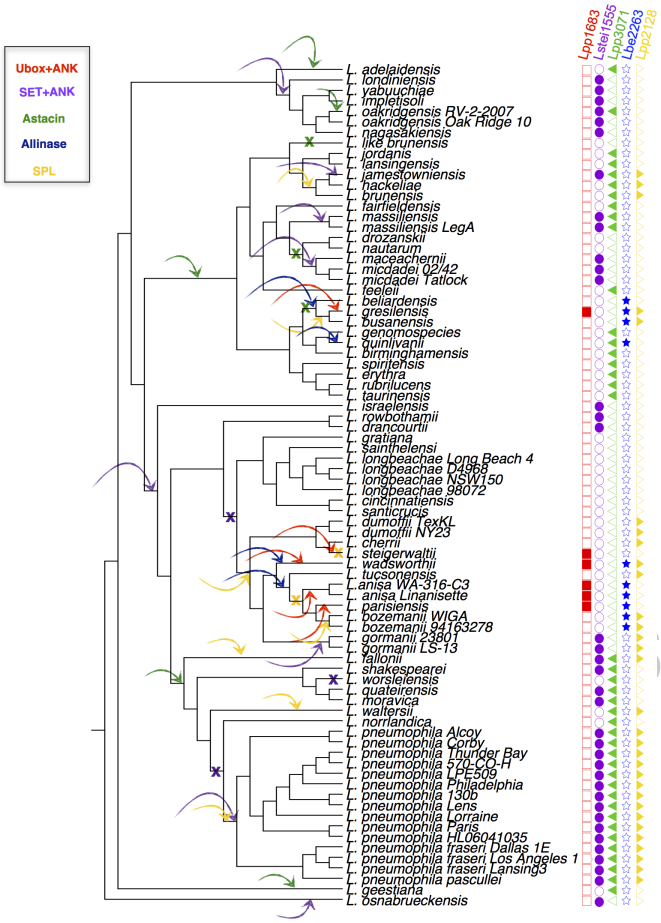

Fig. 4. Gain/loss prediction for selected eukaryotic proteins and domain containing proteins. Circles on the branches represent gain events, crosses loss events.The full squares, circles, triangles or stars indicate the presence of the respective protein; the emptysquares, circles, triangles or stars indicate that the protein is absent in this species.

(31), but their role in infection is unknown. Here they were predicted as phosphatidylcholine-hydrolyzing phospholipase C. Phosphatidylcholine is a eukaryotic membrane phospholipid that is present in only about 15% of prokaryotic species, in particular bacteria interacting with eukaryotes (32). *L. pneumophila* belongs to the phosphatidylcholine-containing group of bacteria, which includes *Francisella tulurensis* or *Brucella abortus* (33). These pathogens use the phosphatidylcholine synthase pathway exclusively for phosphatidylcholine formation and are thought to depend on choline supplied from the host cell (34). Indeed, it has been shown that phosphatidylcholin synthesis is required for *L. pneumophila* virulence (35). Thus, it is tempting to infer that the role of these enzymes may be to help acquire choline from the host cell.

Evolutionary history of eukaryotic domains and eukaryotic proteins. It is intriguing that *Legionella* species encode such a diverse repertoire of eukaryotic domains and eukaryotic-like proteins. To understand better this unique feature of the genus we analyzed the evolutionary history of these proteins. After phylogenetic reconstruction of the genus *Legionella* based on the core genome (at least 50% identical) (**Fig. 1A**), we analyzed the distribution of the eukaryotic motifs and the eukaryotic proteins with respect to the evolution of the genus. For most we found patchy distribution, as the repertoire of these proteins is variable among the different *Legionella* species (**Fig. 2**). Such a distribution is indicative of gain and loss events during the evolution of the genus. To analyze further how these proteins may have evolved in *Legionella* we selected 25 eukaryotic motifs representing 2,837 different proteins in over 800 orthologous groups and used the program Gloome to analyze the gain and loss events for these proteins. We found that the number of gain events (1,197/69%) considerably exceeded the number of loss events (549/31%), a bias that was even stronger when using parsimony (1,628 gain events *versus* 89 loss events) (**SI Appendix, Fig. S8**). These results were confirmed also when using a more conservative approach by taking a probability cut-off for the stochastic model of 0.8 instead of 0.5, and when analyzing each motif separately.

An exemplary view of this result is shown for four proteins encoding different motifs (U-box and ankyrin repeat, SET domain and ankyrin repeat, astacin domain and allinase domain; **Fig. 4).** Loss events are indicated by a star and gain events by a dot. The number of gain events exceeds the number of loss events, indicating that in the *Legionella* genus gene acquisition is dominant. Moreover, gene acquisition seems to be an ongoing and frequent process in the genus *Legionella* given the high number of events we observed and the fact that most of them are localized in the terminal branches of the tree (**SI Appendix, Fig. S8**). To analyse if eukaryotic-like proteins have the same evolutionary history, we took the sphingosine1-phosphate lyse (*L*pSpl) (36, 37) as an example. Indeed, when running the same analyses this gene also appeared to have been gained multiple times during the evolution of the genus (**Fig. 4**).

Thus, in comparison to most prokaryotic species analysed to date, more gene gain events are evident than loss events during evolution of the *Legionella* genus, which is also corroborated by the fact that the ancestral genomes were probably smaller (**Fig. 1A**, cluster I). Indeed, as seen in **Fig. 1A,** in each of the defined phylogenetic clusters only few genomes have a larger size *e.g.* in cluster II *L. massiliensis* is the only species with a big genome, thus the most parsimonious explanation is that the ancestor of this clade had a small genome and in the branch leading to *L. massiliensis* gene gain occurred. This finding is similar to what was described for the adaptation of louse-borne intracellular pathogens and amoeba associated bacteria. It is well known that the specialization of intracellular bacteria is associated with genome reduction, and extreme genome reduction cane be seen in louse-borne human specialists. In contrast, nonspecialized intra-amoebal microorganisms exhibit a genome larger than their relatives due to gene conservation and acquisition (38).

The Dot/Icm secretion system is a highly conserved machinery secreting thousands of different proteins. The Dot/Icm T4SS is indispensable for intracellular replication of *L. pneumophila* in both amoeba and macrophages (39). In stark contrast to the high genetic diversity observed in the *Legionella* genomes, the Dot/Icm T4SS is part of the core genome as it is present in all species analyzed and the organization of the constituent proteins is highly conserved, even at the amino acid level. The proteins comprising the secretion machinery show an average amino acid identity of more than 50% and some even more than 90% when compared to the *L. pneumophila* Dot/Icm components (**SI Appendix Fig. S9A and Table S6**). The most conserved proteins are DotB, a secretion ATPase (86-100% aa identity) and IcmS, a small acidic cytoplasmic protein (74-98% aa identity). This high conservation is even seen with one of the few non-*Legionella* species that encode a Dot/Icm system, *Coxiella burnetti*.

The only gene of the Dot/Icm system that is not present in all *Legionella* species is *icmR*. IcmR interacts with IcmQ as a chaperone preventing IcmQ self-dimerization (40). Although IcmQ is highly conserved, the gene encoding IcmR is frequently replaced by one or two non-homologous genes encoding for proteins that are called FIR because they can functionally replace IcmR (41). When overlapping the occurrence of the different FIR genes with the phylogeny of the species, most phylogenetically closely related

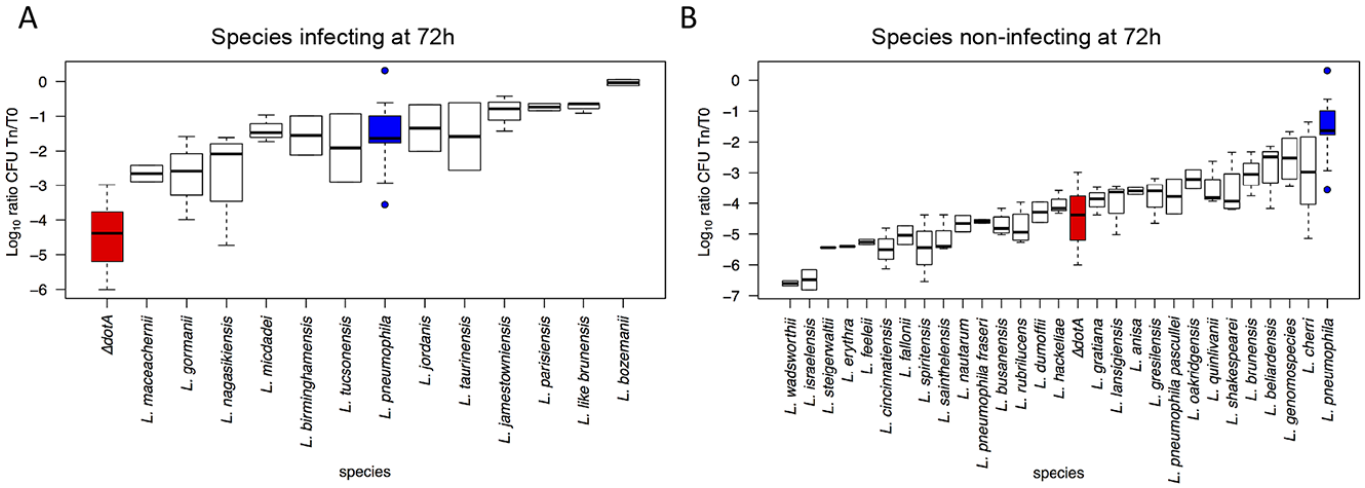

Fig. 5. The replicative capacity of the different Legionella species in THP-1 cells correlates with their epidemiological features. Replication of each strain at the time point 72h after infection of THP-1 cells is shown (24h and 48h of infection are shown in SI Appendix,Fig. S14. Intracellular replication was determined by recording the number of colony-forming units (CFU) after plating on BCYE agar. L. pneumophila Paris, representative of a replicating strain (blue box); L. pneumophila ΔdotA, representative of non-replicating strain (red box). The strains are ordered according to the mean replication values. A) Legionella species replicating like or significantly better than L. pneumophila Paris. B) Species with no or significantly lower replication capacities than L. pneumophila Paris.

species share homologous FIR genes (**SI Appendix, Fig. S10**). Apart from two conserved regions (**SI Appendix, Fig. S11**), the absence of sequence homology among FIR proteins indicates that *icm*R is an extremely fast evolving gene and therefore probably under positive selection. The reason why this gene is extremely divergent is still unknown but could be also linked to the high variety of Dot/Icm effectors described in this genus. Thus, except for the FIR genes, the Dot/Icm T4SS is highly conserved and encoded in a very dynamic genetic context.

It has been shown previously, that the more than 300 substrates of the *L. pneumophila* Dot/Icm system are not universally present within the genus *Legionella* as among 38 *Legionella* species only seven core effectors had been described (14). Surprisingly, when adding the 40 additional genomes and 16 new *Legionella* species sequenced in this study, we identified 8 core effectors instead of seven. A comparison of the two studies confirmed Lpg0103 (VipF), Lpg0107 (RavC), Lpg2300 (LegA3/AnkH/AnkW), and Lpg2815 (IroT/MavN) as core substrates (14) (**SI Appendix, Fig. S9B and Table S7**). Three of the previously defined core substrates (Lpg0140, Lpg2832, Lpg3000) were present in two genomes as two consecutive genes instead of one, however, this fragmentation might be a sequencing error, and thus we considered these substrates also as core substrates (**SI Appendix Table S7**). In our study we identified one additional core effector gene, *lpg1356/lpp1310.* This protein has been reported by Lifshitz and colleagues (42) as secreted protein, but had not been included in the Burstein effector search, which explains the different result (**SI Appendix, Fig. S9B and Table S7).** Similarly, to most of the other core substrates, their functions are not known, but Lpg1356 encodes eight eukaryotic Sel-1 motifs similar to LpnE, a L. pneumophila virulence determinant that influences vacuolar trafficking (43). Furthermore, seven other genes are present in all but one, two or four genomes, thus they might have important functions in host pathogen interactions (**SI Appendix Table S7**). Interestingly, when the effector repertoire of several strains of one species is compared the conservation of the effectors is very high (between 82 and 97%) (**SI Appendix Table S8**). However, if more strains than two are available for a species as it is the case for *L. pneumophila* where 11 strains could be compared, the conservation of the effector pool is only 65% (264 of the 408 different effectors identified in the 11 strains) (**SI Appendix Table S8**). Thus the *L. pneumophila* core effector set is also smaller than previously thought. Taken together, the genus *Legionella* has 8 core substrates present in all genomes and seven additional ones that are present in nearly all genomes.

Interestingly, whereas the number of core Dot/Icm substrates is extremely small, the number and the diversity of predicted Dot/Icm substrates is extremely high. Indeed, through a machine learning approach, Burstein *et al* predicted that the *Legionella* genus would encode 5,885 effectors (14). Here we extended these analyses and identified 4,767 proteins with eukaryotic motifs that have a high probability to be secreted effectors as shown for the Rab-like proteins. If we consider that the orthologous of these proteins in each species are also effectors then the number raises to 7103 (representing 1145 different orthologous proteins) (**SI Appendix Fig. S9C**). Moreover, we identified 2,196 eukaryotic like proteins representing 414 different orthologous genes, which form together with the above-mentioned eukaryotic motif carrying proteins 1,400 different putative orthologous substrates of the Dot/Icm T4SS. Finally, when adding to the effectors predicted in this study (based on their similarity to eukaryotic domains and proteins), the effectors previously described in *L. pneumophila* and their orthologues (more than 7000 proteins representing about 300 different orthologous), as well as the effectors predicted by the machine learning approach and their orthologous (more than 10 000 proteins representing about 900 different orthologous) (14) the total number of different effectors rises to almost 18,000 proteins (more than 1,600 orthologous groups) (**SI Appendix, Table S9 and Fig. S9C**). Therefore, the *Legionella* genus has by far the highest number and widest variety of effectors described for an intracellular bacterium. Furthermore, when calculating the growth accumulation curve for Dot/Icm predicted effectors, this number should still increase with the sequencing of new *Legionella* genomes, as the plateau is not reached yet (**SI Appendix, Fig. S9D**).

The ability to infect human cells has been acquired independently several times during the evolution of the genus *Legionella*. Among the 65 *Legionella* species known, *L. pneumophila* is responsible for over 90% of human disease, followed by *L. longbeachae* (2-7% of cases, except Australia and New Zealand with 30% (44)). Certain *Legionella* species such as *L. micdadei*, *L. dumoffii* or *L. bozemanii* have once or sporadically been associated with human disease (44), and all other species seem to be environmental bacteria only. The reasons for these differences are not known. To explore whether all species are able to replicate in human cells we chose the human macrophage like cell line

THP-1 as model and tested the replication capacity of 47 different *Legionella* species. Infections were carried out in duplicates or triplicates and colony-forming units were recorded at 24h, 48h and 72h post infection. Levels of intracellular replication were compared to wild type *L. pneumophila* strain Paris and an isogenic non-replicating Δ*dotA* mutant as reference strains (**Fig. 5 and SI Appendix, Fig. S12 and S13**). Results were also compared to data previously reported for different *Legionella* species in THP-1, U937 and A549 cells, Mono Mac 6, mouse and guinea pig derived macrophages, or in guinea pigs (**SI Appendix, Table S10**). When results at 72 h after infection were analyzed, 28 of the 47 species tested were impaired for intracellular replication whereas nine species replicated similarly to *L. pneumophila* Paris or better (**Fig. 5**). These nine species were *L. gormanii*, *L. jamestowniensis*, *L. jordanis*, *L. like brunensis*, *L. maceachernii*, *L. micdadei*, *L. nagasikiensis L. parisiensis,* and *L. tucsonensis*. Interestingly, *L. jamestowniensis*, for which one human case has been reported (45), replicated better than *L. pneumophila* Paris. Indeed, *L. jamestowniensis* productively infects human U937-derived phagocytes. The remaining eight species showed variable replication patterns being significantly different from *L. pneumophila* Paris only in one or two of the three analyzed time points (**SI Appendix, Fig. S12**). Broadly, the species most frequently reported from human disease (*L. pneumophila, L. longbeachae, L. micdadei, L. bozemanii* and *L. dumoffii*) are also those that replicated robustly in THP-1 cells. The only exception was the *L. dumoffii* strains that were impaired for replication in THP-1 cells but which have been shown to replicate in other cell types and guinea pigs. Taken together, there is a convincing correlation between the frequency of isolation from human disease and the ability to grow in macrophage-like cells.

To analyze this further, we overlapped the replication results with the phylogeny of the genus. Apart from the small cluster containing *L. beliardensis*, *L. gresilensis* and *L. busanensis,* which were all unable to grow in THP-1 cells, replicating and non-replicating strains were mixed in the phylogeny (**SI Appendix, Fig. S14**). This suggests that the capacity to replicate in human cells has been acquired independently several times during evolution of the *Legionella* genus, possibly as a result of recruiting effectors that allow adaptation to particular niches. To understand whether a specific set of effectors is necessary to infect human cells, we further analyzed the combination of effectors present in the strains isolated from human disease and effectors present in strains capable of replicating in THP-1 cells. Surprisingly, no specific set of effectors could be attributed to strains capable of replicating in human cells or isolated from human disease, although among these strains certain conserved motifs always present were identified, such as ankyrin motifs, F-box or SET-domains, suggesting that common pathways need to be subverted to cause human infection. Thus, the capacity to infect human cells has been acquired independently, several times during the evolution of the genus *Legionella*.

In conclusion, the analysis of 80 *Legionella* strains representing 58 different *Legionella* species has revealed a contrasting picture of the *Legionella* genus. It encodes a highly conserved T4SS predicted to secrete more than 18,000 proteins, of which only 8 are conserved throughout the genus. Together the genomes portray an extremely diverse genus shaped by massive inter-domain horizontal gene transfer, circulating mobile genetic elements and eukaryotic like proteins. Our in-depth analyses of eukaryotic features of the *Legionella* genomes identified 137 different eukaryotic domains of which Rab or Ras domain-containing proteins were quasi unique to the genus *Legionella*. The secretion assays undertaken for 16 of these Rab or Ras domain-containing proteins confirmed that these were translocated Dot/Icm effectors. In addition to the eukaryotic domains, we identified 210 orthologous groups of eukaryotic like proteins. If all these proteins in the different species and their orthologues are taken into account, we found more than 8,000 proteins that have been shaped by inter-domain horizontal gene transfer in the genus *Legionella*. Thus, to our knowledge the genus *Legionella* contains the widest variety and highest number of eukaryotic proteins and domains of any prokaryotic genus genome analyzed to date. Analyzing more strains per species will probably discover new unknown effectors increasing our knowledge of the set of tools used by *Legionella* to infect eukaryotic cells. Although eukaryotic proteins and domains were a universal feature of the genus *Legionella*, the repertoire of these proteins for each species was different. Surprisingly, even when the same motif was present in different species, these were often present in different proteins with no orthology. In accordance with this finding, our evolutionary analysis of the presence/absence of these domains and proteins suggested that these proteins were mostly acquired through gene gain events.

When exploring the replication capacity of 47 different *Legionella* species in human macrophage-like cell line THP-1, we found that the 23 species were capable of replicating in THP-1 cells. However, these did not cluster in the phylogeny, indicating that the capacity to replicate in macrophages can be achieved by different combinations of effectors, and this capacity has been acquired several times during the evolution of the *Legionella* genus. As humans are an accidental host for *Legionella*, the capacity to replicate in macrophages may also have been obtained by a coincidental acquisition of different virulence properties initially needed to adapt to a specific natural host, such as amoebae. Indeed, due to the high conservation of key signaling pathways in professional phagocytes such as amoebae and human macrophages, different combinations of effectors may allow *Legionella* species to infect higher eukaryotic cells by chance.

Here we show that all *Legionella* species have acquired eukaryotic proteins that likely modulate specific host functions to allow intracellular survival and replication in eukaryotic host cells. At a certain point, the evolution of a combination of effector proteins that allow replication in human cells may inadvertently lead to the emergence of new human pathogens from environmental bacteria.

Material and Methods

The materials and methods are described at length in *SI Appendix*. This includes: Sequencing and assembly, sequence processing and annotation, pan/core genome, ortholog and singleton definition, phylogenetic reconstruction and evolutionary analysis, phylogenetic analyses of Rab and eukaryotic-like proteins, infection assays, statistical analysis, and translocation assays. The raw sequence reads were deposited in the European Nucleotide Archive (study accession number PRJEB24896). The sequences and annotations can be accessed through: https://github.com/bbi-ip/Legionella_genus_proteins.git

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