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

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27 **Running title:** *Legionellae* and the emergence of human pathogens

28
29 **Key words:** *Legionella*, protozoa, co-evolution, horizontal gene transfer, virulence

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1 **Significance**

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

12

13 **Abstract**

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

1 \body

2 **Introduction**

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

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

28 Here we present a comprehensive analysis of the *Legionella* genus genome, covering
29 80 *Legionella* strains belonging to 58 *Legionella* species and subspecies. We establish a pan-
30 genus pool of putative T4SS effectors and show that this comprises over 18,000 proteins and
31 identify more than 200 new eukaryotic-like proteins and 137 eukaryotic domains, including a
32 unique class of putative bacterial Rab GTPases. We confirmed experimentally that a subset of
33 these proteins translocate into the host cell upon infection. We conclude that the T4SS is
34 highly conserved at the sequence level, but the effector proteins secreted are highly diverse.

35

1 **Results and discussion**

2 **The *Legionella* genus genome is dynamic and characterized by frequent genetic**
3 **exchange.** We sequenced 58 *Legionella* species of which 16 were newly sequenced, and
4 analyzed them in combination with all publicly available genomes (80 genomes in total) (**SI**
5 **Appendix, Table S1**). The *Legionella* genomes were extremely diverse, as the genome size
6 varied from 2.37Mb (*L. adelaidensis*) to 4.88Mb (*L. santicrucis*), the GC content from
7 34.82% (*L. busanensis*) to 50.93% (*L. geestiana*) and the number of clusters of orthologous
8 genes as defined with OrthoMCL was 17,992 of which 5,832 (32%) were strain specific
9 (singletons) (**Fig. 1A**). Only 1,008 genes (6%) constituted the core genome (**Fig. 1B**),
10 compared to an earlier analysis of 38 *Legionella* species, which found 16,416 clusters of
11 orthologous and 1,054 core genes (14). The addition of 40 new genomes comprising 16 newly
12 sequenced *Legionella* species in our study increased the number of orthologous gene clusters
13 by over 1,576 and decreased the core genome by 46 genes, underlining the high diversity of
14 the *Legionella* genus. This difference suggested that the *Legionella* genus pan-genome is far
15 from fully described and that sequencing of additional *Legionella* species will increase the
16 genus gene repertoire significantly. This was supported by the rarefaction curve that does not
17 reach a plateau (**Fig. 1C**).

18 The highly dynamic nature of these genomes is also seen in the analysis of the strain
19 specific genes and the accessory genome as it highlights the presence of several mobile
20 genetic elements; often associated with genes encoding for transfer regions/conjugative
21 elements such as the type IVA secretion systems (T4ASS). These T4ASSs (classified as
22 T4SSF, G, I and T (17) are present in each strain to varying degrees indicating that they
23 circulate among the different *Legionella* strains (**SI Appendix, Table S2**) and therefore drive
24 genome dynamics and diversification. It has been suggested that the incorporation of foreign
25 DNA via horizontal gene transfer (HGT) is responsible for an increase in the AT content and
26 the increase in genome size (18). Indeed, we found a negative correlation between the genome
27 size and the GC content for the *Legionella* genomes, which also suggests frequent HGT (**Fig.**
28 **1D**) (19). Despite the importance of flagella for transmission to new hosts as shown for *L.*
29 *pneumophila*, flagella encoding genes were not conserved in all species, but showed a patchy
30 distribution, as 23 of the 80 strains analyzed lacked flagella genes (**SI Appendix, Fig. S1**).
31 The analyses showed that the *Legionella* genus genome is highly diverse, dynamic and
32 shaped by HGT.

33

34 **The genus *Legionella* encodes proteins with 137 different eukaryotic domains.** Interpro
35 scan analysis of all 58 *Legionella* species revealed the presence of 137 different eukaryotic

1 motifs/domains in the genus *Legionella* (**SI Appendix, Table S3**) according to the definition
2 that an eukaryotic domain is one that is found in >75% of eukaryotic genomes and <25% in
3 prokaryotic genomes. The most abundant eukaryotic domains identified were ankyrin repeats.
4 Interestingly, *L. santicrucis* and *L. massiliensis* encoded 41 and 39 ankyrin domains,
5 respectively (**Fig. 2**). Ankyrin motifs were found frequently associated with other eukaryotic
6 motifs and thus constituted modular proteins associated with eukaryotic F-box, U-box, Rab or
7 SET domains. Notably, F-box and U-box domains were present in more than two thirds of the
8 species analyzed (**Fig. 2**) suggesting manipulation of the host ubiquitin-system is a
9 fundamental virulence strategy of *Legionella* species. Generally, the genomes contained one
10 to three F-box containing proteins with the exception of *L. nautarum* and *L. dronzanskii*,
11 which contained 18 and 10, respectively. The SET domain containing protein RomA of
12 *L. pneumophila* that induces a unique host chromatin modification (20) is present in 46 of the
13 58 *Legionella* species suggesting the ability of many *Legionella* species to manipulate host
14 chromatin (**Fig. 2**). Interestingly, the Sec-7 domain present in the effector RalF, a bacterial
15 ARF guanine exchange factor and the first described Dot/Icm effector of *L. pneumophila* (21)
16 was present in only eight (*L. pneumophila*, *L. longbeachae*, *L. feelei*, *L. sainthelensis*,
17 *L. santicrucis*, *L. shakespeari*, *L. quateirensis* *L. moravica*) of the 58 *Legionella* species
18 analyzed, suggesting that, different effectors may compensate for RalF activity or that LCV
19 biogenesis varies among different species (**Fig. 2**).

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

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

1 present in protozoa, fungi, plants or animals that have been acquired by different *Legionella*
2 species.

4 **A unique case in the prokaryotic world: *Legionella* encode small GTPase-like domains**

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

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

25 To analyze further the evolutionary history of the Ras-related domains in *Legionella*
26 we undertook phylogenetic analyses of these proteins. For example, the two *L. longbeachae*
27 Rab proteins, Llo1716 and Llo3288, were present in all strains closely related to
28 *L. longbeachae*, suggesting that they and their orthologous share a common origin and
29 evolved from a gene acquired by the ancestor of all these species (**SI Appendix, Fig. S5**).
30 Further phylogenetic analysis of 16 Rab proteins present in eight different *Legionella* species
31 showed that these Rab domains were acquired by HGT, mainly from protozoa (**Fig. 3B and**
32 **SI Appendix, Fig. S6A-P**). Recently a novel isoform of Rab5D was identified in the
33 *Acanthamoeba polyphaga mimivirus* (APMV) and all group I members of the *Mimiviridae*
34 (29). Phylogenetic analyses suggested that the Rab GTPase was acquired by an ancestor of
35 the *Mimiviridae* family and Rabs from *Mimiviridae*, *Plasmodium* and few lower eukaryotes

1 form a separate clade (29). Thus, *Legionella* and APMV that both infect the protozoa
2 *Acanthamoeba* encode Rab proteins most likely to mimic and subvert host cell function. To
3 substantiate that these proteins act in the host cell, we determined whether the Rab containing
4 proteins were bona fide substrates of the Dot/Icm T4SS by creating fusion proteins between
5 the 16 different Rab proteins and the catalytic domain of the TEM-1 beta-lactamase (indicated
6 by a star in **SI Appendix, Fig. S5**). Translocation assays were performed using wild type
7 *L. pneumophila* as a surrogate host and compared with an isogenic Dot/Icm mutant ($\Delta dotA$).
8 All 16 Rab motif-containing proteins were translocated by *L. pneumophila* but not by the
9 $\Delta dotA$ mutant (**Fig. 3C-D**).

10
11 **More than 250 different eukaryotic like proteins are encoded in *Legionella* genomes.** In
12 addition to modular effectors with eukaryotic domains, the *Legionella* genome encodes
13 proteins that are similar to eukaryotic proteins, many of which are proven effectors of the
14 Dot/Icm T4SS. A wider search for eukaryotic like proteins in the *Legionella* genus identified
15 2196 eukaryotic like proteins representing more than 400 different orthologous groups that
16 matched better to eukaryotes than to prokaryotes from a total of 6809 different orthologous
17 proteins that matched with eukaryotic proteins. Among these, we identified 156 proteins with
18 a eukaryotic domain, and 210 new eukaryotic-like proteins (**SI Appendix, Table S5**).
19 Furthermore, 152 eukaryotic like proteins detected possess a higher GC content (40%-62%)
20 than the rest of the genome indicating recent HGT. Phylogenetic analysis of selected, newly
21 identified proteins suggested that these were acquired from eukaryotes. As an example, **SI**
22 **Appendix, Fig. S7** shows the protein LanA0735 from *Legionella anisa*, a species frequently
23 found in artificial water systems. This protein belongs to the pyridine nucleotide-disulfide
24 oxidoreductase family, a subfamily of the FAD dependent oxidoreductase family. LanA0735
25 showed some similarity to thioredoxin reductase that exists as two major ubiquitous
26 isoenzymes in higher eukaryotic cells, one cytosolic and the other one mitochondrial. The
27 cytosolic form has been implicated in interference with the acidification of the lysosomal
28 compartment in *C. elegans* (30), and thus LanA0735 may help *Legionella* avoid vacuole
29 acidification during infection.

30 Among the proteins defined as eukaryotic like, two previously described
31 phospholipases of *L. pneumophila*, PlcB (Lpp1411/Lpg1455) and PlcA (Lpp0565/Lpg0502)
32 were identified in our analysis as eukaryotic proteins. The only other bacteria encoding these
33 two enzymes are *Pseudomonas* and amoebae-associated bacteria. The two enzymes have
34 phospholipase activity (31), but their role in infection is unknown. Here they were predicted
35 as phosphatidylcholine-hydrolyzing phospholipase C. Phosphatidylcholine is a eukaryotic

1 membrane phospholipid that is present in only about 15% of prokaryotic species, in particular
2 bacteria interacting with eukaryotes (32). *L. pneumophila* belongs to the phosphatidylcholine-
3 containing group of bacteria, which includes *Francisella tularensis* or *Brucella abortus* (33).
4 These pathogens use the phosphatidylcholine synthase pathway exclusively for
5 phosphatidylcholine formation and are thought to depend on choline supplied from the host
6 cell (34). Indeed, it has been shown that phosphatidylcholin synthesis is required for
7 *L. pneumophila* virulence (35). Thus, it is tempting to infer that the role of these enzymes may
8 be to help acquire choline from the host cell.

9
10 **Evolutionary history of eukaryotic domains and eukaryotic proteins.** It is intriguing that
11 *Legionella* species encode such a diverse repertoire of eukaryotic domains and eukaryotic-like
12 proteins. To understand better this unique feature of the genus we analyzed the evolutionary
13 history of these proteins. After phylogenetic reconstruction of the genus *Legionella* based on
14 the core genome (at least 50% identical) (**Fig. 1A**), we analyzed the distribution of the
15 eukaryotic motifs and the eukaryotic proteins with respect to the evolution of the genus. For
16 most we found patchy distribution, as the repertoire of these proteins is variable among the
17 different *Legionella* species (**Fig. 2**). Such a distribution is indicative of gain and loss events
18 during the evolution of the genus. To analyze further how these proteins may have evolved in
19 *Legionella* we selected 25 eukaryotic motifs representing 2,837 different proteins in over 800
20 orthologous groups and used the program Gloome to analyze the gain and loss events for
21 these proteins. We found that the number of gain events (1,197/69%) considerably exceeded
22 the number of loss events (549/31%), a bias that was even stronger when using parsimony
23 (1,628 gain events *versus* 89 loss events) (**SI Appendix, Fig. S8**). These results were
24 confirmed also when using a more conservative approach by taking a probability cut-off for
25 the stochastic model of 0.8 instead of 0.5, and when analyzing each motif separately.

26 An exemplary view of this result is shown for four proteins encoding different motifs
27 (U-box and ankyrin repeat, SET domain and ankyrin repeat, astacin domain and allinase
28 domain; **Fig. 4**). Loss events are indicated by a star and gain events by a dot. The number of
29 gain events exceeds the number of loss events, indicating that in the *Legionella* genus gene
30 acquisition is dominant. Moreover, gene acquisition seems to be an on-going and frequent
31 process in the genus *Legionella* given the high number of events we observed and the fact that
32 most of them are localized in the terminal branches of the tree (**SI Appendix, Fig. S8**). To
33 analyse if eukaryotic-like proteins have the same evolutionary history, we took the
34 sphingosine1-phosphate lyse (*LpSpl*) (36, 37) as an example. Indeed, when running the same

1 analyses this gene also appeared to have been gained multiple times during the evolution of
2 the genus (Fig. 4).

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

15
16 **The Dot/Icm secretion system is a highly conserved machinery secreting thousands of**
17 **different proteins.** The Dot/Icm T4SS is indispensable for intracellular replication of
18 *L. pneumophila* in both amoeba and macrophages (39). In stark contrast to the high genetic
19 diversity observed in the *Legionella* genomes, the Dot/Icm T4SS is part of the core genome as
20 it is present in all species analyzed and the organization of the constituent proteins is highly
21 conserved, even at the amino acid level. The proteins comprising the secretion machinery
22 show an average amino acid identity of more than 50% and some even more than 90% when
23 compared to the *L. pneumophila* Dot/Icm components (SI Appendix Fig. S9A and Table S6).
24 The most conserved proteins are DotB, a secretion ATPase (86-100% aa identity) and IcmS, a
25 small acidic cytoplasmic protein (74-98% aa identity). This high conservation is even seen
26 with one of the few non-*Legionella* species that encode a Dot/Icm system, *Coxiella burnetii*.

27 The only gene of the Dot/Icm system that is not present in all *Legionella* species is
28 *icmR*. IcmR interacts with IcmQ as a chaperone preventing IcmQ self-dimerization (40).
29 Although IcmQ is highly conserved, the gene encoding IcmR is frequently replaced by one or
30 two non-homologous genes encoding for proteins that are called FIR because they can
31 functionally replace IcmR (41). When overlapping the occurrence of the different FIR genes
32 with the phylogeny of the species, most phylogenetically closely related species share
33 homologous FIR genes (SI Appendix, Fig. S10). Apart from two conserved regions (SI
34 Appendix, Fig. S11), the absence of sequence homology among FIR proteins indicates that
35 *icmR* is an extremely fast evolving gene and therefore probably under positive selection. The

1 reason why this gene is extremely divergent is still unknown but could be also linked to the
2 high variety of Dot/Icm effectors described in this genus. Thus, except for the FIR genes, the
3 Dot/Icm T4SS is highly conserved and encoded in a very dynamic genetic context.

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

30 Interestingly, whereas the number of core Dot/Icm substrates is extremely small, the
31 number and the diversity of predicted Dot/Icm substrates is extremely high. Indeed, through a
32 machine learning approach, Burstein *et al* predicted that the *Legionella* genus would encode
33 5,885 effectors (14). Here we extended these analyses and identified 4,767 proteins with
34 eukaryotic motifs that have a high probability to be secreted effectors as shown for the Rab-
35 like proteins. If we consider that the orthologous of these proteins in each species are also

1 effectors then the number raises to 7103 (representing 1145 different orthologous proteins)
2 (SI Appendix Fig. S9C). Moreover, we identified 2,196 eukaryotic like proteins representing
3 414 different orthologous genes, which form together with the above-mentioned eukaryotic
4 motif carrying proteins 1,400 different putative orthologous substrates of the Dot/Icm T4SS.
5 Finally, when adding to the effectors predicted in this study (based on their similarity to
6 eukaryotic domains and proteins), the effectors previously described in *L. pneumophila* and
7 their orthologues (more than 7000 proteins representing about 300 different orthologous), as
8 well as the effectors predicted by the machine learning approach and their orthologous (more
9 than 10 000 proteins representing about 900 different orthologous) (14) the total number of
10 different effectors rises to almost 18,000 proteins (more than 1,600 orthologous groups) (SI
11 Appendix, Table S9 and Fig. S9C). Therefore, the *Legionella* genus has by far the highest
12 number and widest variety of effectors described for an intracellular bacterium. Furthermore,
13 when calculating the growth accumulation curve for Dot/Icm predicted effectors, this number
14 should still increase with the sequencing of new *Legionella* genomes, as the plateau is not
15 reached yet (SI Appendix, Fig. S9D).

16
17 **The ability to infect human cells has been acquired independently several times during**
18 **the evolution of the genus *Legionella*.** Among the 65 *Legionella* species known,
19 *L. pneumophila* is responsible for over 90% of human disease, followed by *L. longbeachae*
20 (2-7% of cases, except Australia and New Zealand with 30% (44)). Certain *Legionella* species
21 such as *L. micdadei*, *L. dumoffii* or *L. bozemanii* have once or sporadically been associated
22 with human disease (44), and all other species seem to be environmental bacteria only. The
23 reasons for these differences are not known. To explore whether all species are able to
24 replicate in human cells we chose the human macrophage like cell line THP-1 as model and
25 tested the replication capacity of 47 different *Legionella* species. Infections were carried out
26 in duplicates or triplicates and colony-forming units were recorded at 24h, 48h and 72h post
27 infection. Levels of intracellular replication were compared to wild type *L. pneumophila*
28 strain Paris and an isogenic non-replicating $\Delta dotA$ mutant as reference strains (Fig. 5 and SI
29 Appendix, Fig. S12 and S13). Results were also compared to data previously reported for
30 different *Legionella* species in THP-1, U937 and A549 cells, Mono Mac 6, mouse and guinea
31 pig derived macrophages, or in guinea pigs (SI Appendix, Table S10). When results at 72 h
32 after infection were analyzed, 28 of the 47 species tested were impaired for intracellular
33 replication whereas nine species replicated similarly to *L. pneumophila* Paris or better (Fig. 5).
34 These nine species were *L. gormanii*, *L. jamestowniensis*, *L. jordanis*, *L. like brunensis*,
35 *L. maceachernii*, *L. micdadei*, *L. nagasakiensis* *L. parisiensis*, and *L. tucsonensis*. Interestingly,

1 *L. jamestowniensis*, for which one human case has been reported (45), replicated better than
2 *L. pneumophila* Paris. Indeed, *L. jamestowniensis* productively infects human U937-derived
3 phagocytes. The remaining eight species showed variable replication patterns being
4 significantly different from *L. pneumophila* Paris only in one or two of the three analyzed
5 time points (**SI Appendix, Fig. S12**). Broadly, the species most frequently reported from
6 human disease (*L. pneumophila*, *L. longbeachae*, *L. micdadei*, *L. bozemanii* and *L. dumoffii*)
7 are also those that replicated robustly in THP-1 cells. The only exception was the *L. dumoffii*
8 strains that were impaired for replication in THP-1 cells but which have been shown to
9 replicate in other cell types and guinea pigs. Taken together, there is a convincing correlation
10 between the frequency of isolation from human disease and the ability to grow in
11 macrophage-like cells.

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

27 In conclusion, the analysis of 80 *Legionella* strains representing 58 different
28 *Legionella* species has revealed a contrasting picture of the *Legionella* genus. It encodes a
29 highly conserved T4SS predicted to secrete more than 18,000 proteins, of which only 8 are
30 conserved throughout the genus. Together the genomes portray an extremely diverse genus
31 shaped by massive inter-domain horizontal gene transfer, circulating mobile genetic elements
32 and eukaryotic like proteins. Our in-depth analyses of eukaryotic features of the *Legionella*
33 genomes identified 137 different eukaryotic domains of which Rab or Ras domain-containing
34 proteins were quasi unique to the genus *Legionella*. The secretion assays undertaken for 16 of
35 these Rab or Ras domain-containing proteins confirmed that these were translocated Dot/Icm

1 effectors. In addition to the eukaryotic domains, we identified 210 orthologous groups of
2 eukaryotic like proteins. If all these proteins in the different species and their orthologues are
3 taken into account, we found more than 8,000 proteins that have been shaped by inter-domain
4 horizontal gene transfer in the genus *Legionella*. Thus, to our knowledge the genus *Legionella*
5 contains the widest variety and highest number of eukaryotic proteins and domains of any
6 prokaryotic genus genome analyzed to date. Analyzing more strains per species will probably
7 discover new unknown effectors increasing our knowledge of the set of tools used by
8 *Legionella* to infect eukaryotic cells. Although eukaryotic proteins and domains were a
9 universal feature of the genus *Legionella*, the repertoire of these proteins for each species was
10 different. Surprisingly, even when the same motif was present in different species, these were
11 often present in different proteins with no orthology. In accordance with this finding, our
12 evolutionary analysis of the presence/absence of these domains and proteins suggested that
13 these proteins were mostly acquired through gene gain events.

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

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

30

31 **Material and Methods**

32 The materials and methods are described at length in *SI Appendix*. This includes: Sequencing
33 and assembly, sequence processing and annotation, pan/core genome, ortholog and singleton
34 definition, phylogenetic reconstruction and evolutionary analysis, phylogenetic analyses of
35 Rab and eukaryotic-like proteins, infection assays, statistical analysis, and translocation

1 assays. The raw sequence reads were deposited in the European Nucleotide Archive (study
2 accession number PRJEB24896). The sequences and annotations can be accessed through:
3 https://github.com/bbi-ip/Legionella_genus_proteins.git

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11 12 **Author contributions**

13 SJ and LGV, ELH contributed to sample collection and strain analyses DNA extraction and
14 sequencing; CR, DC, SM, AEPC, MR, SP, SR, JD, JC, SDD, GNS to functional experiments,
15 data analyses and interpretation. The manuscript was written by LGV and CB with input from
16 co-authors. The project was conceived, planned and supervised by LGV, GD, GF and CB.

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- 29
- 30
- 31

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

15

16 **Figure 2: Eukaryotic domains have a diverse distribution within the genus *Legionella***
17 **suggesting multiple acquisition events.** The number and distribution of the 41 most
18 frequently identified eukaryotic motifs within the genus *Legionella* are shown. Numbers
19 represent the number of proteins containing this eukaryotic motif. Abbreviations used: ANK
20 (ankyrin), F-box, U-box), SET domain, Pkinases (protein kinases), Sec-7 domain, LLR
21 (leucine rich repeats), Miro (Mitochondrial Rho domain), TTL (tubulin-tyrosine ligase), SH2
22 (The Src homology 2), PAM2 (ataxin-2, C-terminal), PPR (pentatricopeptide repeat), I-set
23 (immunoglobulin I-set), NP (nucleoside phosphatase gda1/cd39), HAD (HAD-superfamily
24 hydrolase), DH (Dbl homology domain), Mit. Substrate (mitochondrial substrate/solute
25 carrier), Rho GTPases-activating protein domain, T-complex (T-complex10/11),
26 PC65 (Peptidase C65 otubain), Ergosterol (Ergosterol biosynthesis), Flavin (flavin
27 monooxygenase-like), Astacin (Peptidase M12A, astacin), Cyt:P450 (Cytochrome_P450),
28 Cytokine FAD (Cytokinin dehydrogenase 1, FAD/cytokinin binding domain), PQ loop repeat,
29 Peptidase C2 (calpain, catalytic domain), LR glioma (Leucine-rich glioma-inactivated, EPTP
30 repeat), Ovarian (Ovarian tumour, otubain), Papain (Peptidase C1A, papain C-terminal,
31 DOT1 (Histone methylation DOT1) , Rab small GTPases, DUF155, C/C (Clathrin/coatomer
32 adaptor, adaptin-like), RCC1 (Regulator of chromosome condensation).

33

34 **Figure 3: Domain organization of small GTPases in *Legionella* and phylogenetic**
35 **analyses of the Llo3288 Rab proteins suggests eukaryotic origin.** A) Domain organization

1 of the different small GTPases proteins identified. **B)** Unrooted tree of Llo3288 and
2 homologues recruited by blastp constructed using likelihood. Local support values are
3 represented with circles on the corresponding branches and size of circles is proportional to
4 the values (only local support of at least 0.7 are shown). **C)** Transloctaion of selected proteins
5 using the beta-lactamase transloctaion assay and infection of Raw264.7 cells for 1h with *Lp*
6 wild type or *Lp* Δ *dotA* expressing BlaM-effector fusions analysed with a microplate reader.
7 Three independent experiments ($n=9$) were done. Statistical significance was determined by
8 2-way Anova with multiple comparisons test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). **D)**
9 Transloctaion of selected proteins using the beta-lactamase transloctaion assay and infection
10 of THP-1 cells at an MOI of 50 during 1h 30min with *Lp* and *Llo* strains in before addition of
11 CCF4-AM and analyses by flow cytometry. Histograms show the frequency of BlaM-
12 translocated, blue fluorescence-emitting cells as means \pm SD of three independent
13 experiments ($n=12$). Statistical significance was determined by Wilcoxon matched pairs test
14 (**, $P<0.01$; ***, $P<0.001$).). *Lp*, *L. pneumophila* wild type; *Llo*, *L. longbeachae* wild type;
15 *Lp* Δ *dotA*, *L. pneumophila* Δ *dotA*; *Llo* Δ , *L. longbeachae* Δ *dotA*.

16

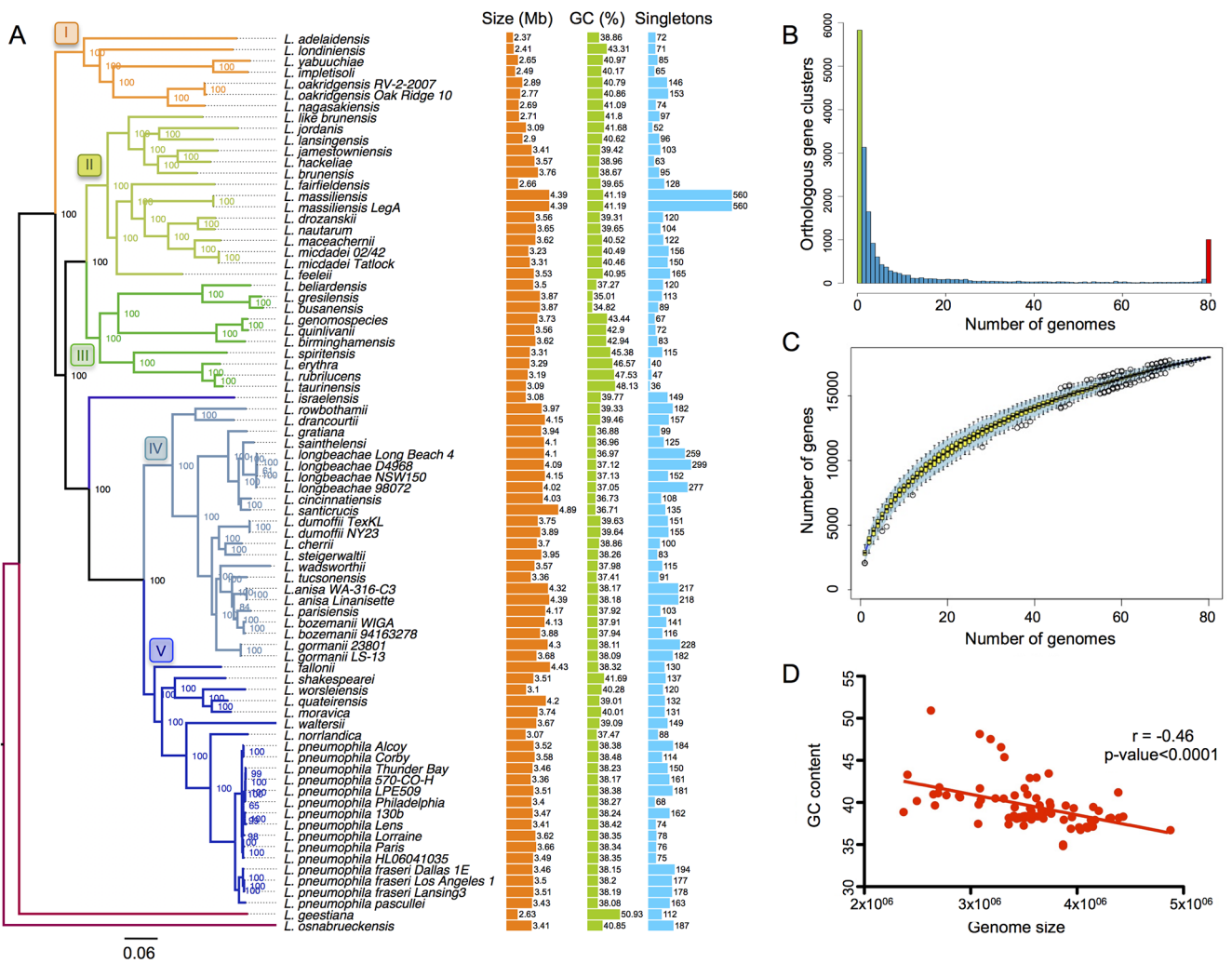
17 **Figure 4. Gain/loss prediction for selected eukaryotic proteins and domain containing**
18 **proteins.** Circles on the branches represent gain events, crosses loss events. The full squares,
19 circles, triangles or stars indicate the presence of the respective protein; the empty squares,
20 circles,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

21

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

31

32



Ubox+ANK
SET+ANK
 Astacin
 Allinase
 SPL

