Correction

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Correction for "Minimization of the *Legionella pneumophila* genome reveals chromosomal regions involved in host range expansion," by Tamara J. O'Connor, Yewande Adepoju, Dana Boyd, and Ralph R. Isberg, which appeared in issue 36, September 6, 2011, of *Proc Natl Acad Sci USA* (108:14733–14740; first published August 22, 2011; 10.1073/pnas.1111678108).

The authors note that the GenBank accession number for Hextuple 2q is CP003023, and the GenBank accession number for Hextuple 3a is CP003024.

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Minimization of the *Legionella pneumophila* genome reveals chromosomal regions involved in host range expansion

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Legionella pneumophila **is a bacterial pathogen of amoebae and humans. Intracellular growth requires a type IVB secretion system that translocates at least 200 different proteins into host cells. To distinguish between proteins necessary for growth in culture and those specifically required for intracellular replication, a screen was performed to identify genes necessary for optimal growth in nutrient-rich medium. Mapping of these genes revealed that the *L. pneumophila* chromosome has a modular architecture consisting of several large genomic islands that are dispensable for growth in bacteriological culture. Strains lacking six of these regions, and thus 18.5% of the genome, were viable but required secondary point mutations for optimal growth. The simultaneous deletion of five of these genomic loci had no adverse effect on growth of the bacterium in nutrient-rich media. Remarkably, this minimal genome strain, which lacked 31% of the known substrates of the type IVB system, caused only marginal defects in intracellular growth within mouse macrophages. In contrast, deletion of single regions reduced growth within amoebae. The importance of individual islands, however, differed among amoebal species. The host-specific requirements of these genomic islands support a model in which the acquisition of foreign DNA has broadened the *L. pneumophila* host range.**

bacterial genomes | transposon site hybridization | essential genes | genome organization | virulence factor

In its natural fresh water environment, *Legionella pneumophila* is an intracellular parasite of free-living amoebae (1). The host range of *L. pneumophila* is extensive because the bacterium is capable of replicating in at least 15 different species of amoebae. When contaminated water sources are aerosolized and inhaled by humans, *L. pneumophila* replicates in alveolar macrophages, causing a potentially fatal form of pneumonia (2, 3). This behavior makes *L. pneumophila* a generalist because it is able to grow in a diverse array of evolutionarily distinct host cell types. Although the majority of disease-causing organisms are specialists that exhibit a high degree of host tropism, jumping among hosts has been observed for a number of pathogens (4). It is thought that host jumping is followed by adaptation to the new host, which often selects for mutation, inactivation, or loss of virulence factors necessary for growth in the previous host (4). In some cases, this allows escape from immune sensing of the pathogen by the new host (5). This process of host specialization appears to be less prominent in *L. pneumophila* because it retains the ability to grow in a large assortment of amoebae. What determines the broad host range of this bacterium, how it is maintained, and how it contributes to the fitness of the bacterium in its natural environment are poorly understood.

The intracellular life cycle of *L. pneumophila* in all host cell types examined is very similar, despite these hosts being phylogenetically distant. The bacterium is internalized into a membrane-bound compartment that avoids delivery to the lysosome (3) and is remodeled through recruitment of host endoplasmic reticulum-derived vesicles into a ribosome-studded replication vacuole (6, 7). Intracellular growth of the bacterium requires a type IVB secretion system encoded by the *dot/icm* genes (8–10) that delivers bacterial proteins to the host cytoplasm (11), where they manipulate a variety of host cellular processes (12–15). To date, over 200 Dot/Icm translocated substrates (TS) have been identified (16–20). Although the host cell targets and mechanisms of action for a number of these substrates have been elucidated (21–28), the explicit roles of the majority of these proteins remain unclear. This is primarily attributable to the absence of any discernable phenotype of mutants lacking genes encoding these proteins (16, 21, 22, 29), a phenomenon that has been attributed to functional redundancy (16, 30–32).

To understand the nature of the intracellular environment of the host, how the bacterium handles the challenges encountered there, and the differential requirements for growth in different hosts, it is important to distinguish between genes that are essential for viability, basic metabolism, and proliferation and those that are specifically important for growth in a host. To address these issues, a genetic screen using a library of *L. pneumophila* transposon mutants was performed to identify genes that are essential for growth in nutrient-rich bacteriological media. Clustering of these essential genes to distinct loci defined several genomic islands that were dispensable for growth of the bacterium in rich media and macrophages but not in amoebae. Furthermore, the amoebae-specific requirements of these large genomic segments for intracellular growth demonstrated a modular organization of the *L. pneumophila* genome distinguishing between regions encoding genes devoted to fundamental biological processes and those dedicated to replication in a host.

Results

Genes Required for *L. pneumophila* Growth in Nutrient-Rich Bacteriological Media. A *mariner himar1*-based transposon consisting of a kanamycin resistance cassette flanked by divergently transcribed T_7 promoter elements (Fig. S1) was introduced into *L. pneumophila* Philadelphia 1 (33) to generate a library of transposon insertion mutants (*Materials and Methods*) for transposon site hybridization (TraSH) (34). A pool of 100,000 insertion mutants was generated, which, if distributed randomly, was pre-

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The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database.

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dicted to result in ~30 insertions per 1-kb gene (the average size of a *L. pneumophila* ORF). Based on the size of the library, and the fact that randomly chosen insertions showed no sequence specificity, it was judged to be exhaustive and sufficiently random for TraSH (34).

The TraSH library was used to identify genes that are important for growth of *L. pneumophila* in nutrient-rich bacteriological media (Fig. S1). To do this, an aliquot of the *L. pneumophila* transposon mutant library was grown to stationary phase in broth culture and plated for single colonies on solid charcoal buffered yeast extract thymidine (CYET) media. Those mutants containing transposon insertions in genes that are important for survival under these conditions will be underrepresented in this pool. To determine the abundance of each mutant in the pool, short regions of the chromosome directly adjacent to the transposon insertion sites were amplified and used to probe a custom gene array representing 94% of *L. pneumophila* Philadelphia 1 protein encoding genes (*Materials and Methods*). As a control, a genomic probe was generated by amplifying total DNA from the nonmutagenized parent strain Lp02 using random primers (*Materials and Methods*), and the relative intensities of each probe were determined at each site on the array. The average insertion/genomic probe ratio for each gene from 10 individual experiments was calculated.

The relative abundance of insertion mutations recovered in each gene was plotted as a histogram of the logarithm of the insertion/genomic probe ratio for each gene analyzed (Fig. 1). The majority of genes exhibited a ratio of ~1, indicating that these genes are not required for growth in nutrient-rich media. There is a prominent skewing of this plot toward genes in which there was a lower recovery of mutations relative to what was expected based on the distribution of the genomic probe intensities alone, which we interpret as representing insertions in loci resulting in a growth defect. Thus, genes for which the ratio was <0.266 (the point of skewing; Fig. 1, dotted line) and which were significantly depleted relative to the mean population ($P < 0.001$) were classified as being defective for growth in culture. Based on these criteria, 597 genes required for optimal growth in culture were identified (Dataset S1), including many associated with core metabolic pathways as well as 53 genes encoding proteins of unknown function. Viable mutants have been previously reported in 9 of the genes judged to be required for optimal growth in media (Dataset S1) (35–39); thus, these genes were excluded from further analysis. Of the 588 remaining genes, 98.0% are conserved in the other four sequenced strains of *L. pneumophila* (40–42) and 78.0% have been described as essential in at least one other bacterial species (Dataset S1).

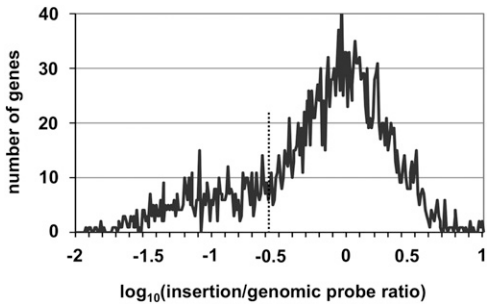

Fig. 1. Identification of genes essential for growth of *L. pneumophila* in nutrient-rich bacteriological media. Histogram plot of the $\log_{10}$(insertion/genomic probe intensity) for all *L. pneumophila* genes represented on the custom microarray. Skewing of the plot occurs at $10^{-0.575}$ (dotted line), corresponding to an insertion/genomic ratio of 0.266, or a 3.8-fold defect in growth. Genes with an insertion/genomic ratio <0.266 and whose value was statistically different from the mean population based on a Student *t* test *P* value <0.001 were classified as essential for growth in rich media. Data represent the mean of 10 independent experiments.

Large Contiguous Regions of the *L. pneumophila* Genome Are Dispensable for Growth in Culture. Seven distinct regions of the *L. pneumophila* chromosome were found to be devoid of genes required for optimal growth (Fig. 2*A* and Table 1). These clusters, ranging in size from 76 to 171 genes, make up 27.1% of all protein encoding genes. The grouping of these dispensable genes to distinct loci indicates that there is a structured, mosaic organization of the *L. pneumophila* genome that correlates with function.

Closer examination of the seven gene clusters revealed several features that distinguished them from the rest of the genome. First, these regions collectively encoded 49% of the known Dot/Icm TS (Fig. 2*A* and Table 1). In contrast, the genes encoding the Dot/Icm secretion system did not map to these clusters but, instead, were imbedded in regions required for optimum growth in culture (Fig. 2*A*). Second, all but one of the seven gene clusters exhibited a high degree of variability in terms of gene conservation and synteny compared with their respective counterparts in the other four sequenced strains of *L. pneumophila* (Fig. 2*B*). Third, six of the seven gene clusters had a significantly lower average %GC content relative to the rest of the genome (Fig. 2*C*), a common characteristic of DNA acquired via horizontal gene transfer (43). Indeed, of the 62 insertion sequence (IS) elements and phage-related genes annotated in the *L. pneumophila* Lp02 strain, which are characteristic of mobile genetic elements, 60% map to one of the seven gene clusters identified with a heavy concentration at the termini of these loci (Fig. S2).

Because individual genes from the TraSH screen were predicted to be dispensable for growth in bacteriological medium, we tested whether elimination of entire clusters of these genes resulted in viable bacterial strains. Of the seven gene clusters identified, deletion mutations for clusters 3 and 5 were successfully constructed, as were those for clusters 4, 6, and 7 after redefining their outer boundaries (Fig. 2*A* and Table 1). Of the remaining two clusters, only a partial deletion of cluster 2 consisting of two smaller internal segments could be isolated (Fig. 2*A* and Table 1), allowing further analysis of strains missing genes in this region. Cluster 1, however, could not be deleted under any circumstance, perhaps because of the lethality of deleting multiple genes simultaneously or an essential role for one or more of the genes from this region that were missing from the custom *L. pneumophila* arrays (Dataset S2). The correct end points of each deletion were confirmed by microarray analysis (Fig. S3) or by whole-genome sequencing (see below). The isolation of strains lacking large segments of consecutive genes validated the data obtained from the TraSH screen and showed that large contiguous regions of the chromosome are dispensable for growth in culture.

In addition to the five genetic loci deleted here, three other regions of the *L. pneumophila* Philadelphia 1 genome (Fig. 2*A*) have been identified, either directly or indirectly, as dispensable for growth in vitro: the *lvh* gene locus (*lpg1228–lpg1271*), which is absent from the WT strain used in this work (8); the *tra* locus (*lpg2057–lpg2115*) (44); and the metal efflux island (*mei*) (*lpg1006–lpg1096*) (45). TraSH data presented here support expendable roles for the *tra* and *mei* loci for growth in nutrient-rich media, because none of the genes encoded in these regions passed the essential gene criteria (Dataset S1). Collectively, these loci define an additional 6.6% of protein encoding genes experimentally demonstrated to be dispensable for viability and growth of *L. pneumophila* in rich media.

Five of the Single Cluster Deletion Mutants Show No Growth Defects During Culture in Nutrient-Rich Media. All six cluster mutants constructed were capable of growth on solid medium; however, the cluster 5 mutant produced markedly smaller colonies compared

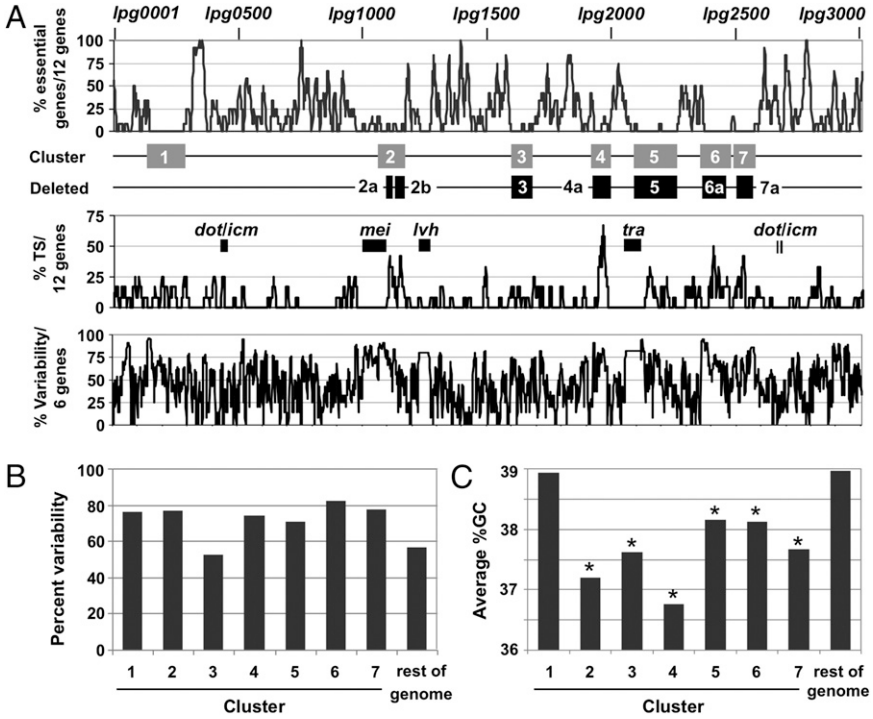

Fig. 2. Mosaic architecture of the *L. pneumophila* genome. (*A*) Seven genomic islands are devoid of genes essential for growth in rich media but exhibit a high concentration of Dot/Icm TS. (*Top*) Running average plot of the percentage of essential genes per 12 genes. The seven gene clusters identified (gray boxes) and the six genomic loci deleted (black boxes) are indicated. (*Middle*) Running average plot of the percentage of Dot/Icm TS per 12 genes. The locations of the *dot/icm*, *tra*, *lvh*, and *mei* loci (black boxes) are individually labeled. (*Bottom*) Running average plot of the percent variability per 6 genes. Pairwise comparison of the *L. pneumophila* Philadelphia 1 genome to Paris, Lens, Corby, and Alcoy strains was used to identify genes that are conserved or absent across all five species. Percent variability is the number of genes inserted or deleted relative to the total number of genes in that region. (*B*) Six of the genomic islands have a higher percent variability than the rest of the genome, as defined in *A*, compared with other sequenced strains of *L. pneumophila*. (*C*) Six genomic islands exhibit a lower average %GC for all genes in the cluster compared with the rest of the genome. Clusters exhibiting a statistically significant difference based on a two-tailed Student *t* test *P* value <0.05 relative to the rest of the genome are indicated by an asterisk. %GC, percent of guanine and cytosine bases.

with the WT strain (data not shown). Consistent with these observations, growth of the cluster 2ab, 3, 4a, 6a, and 7a mutants in liquid media was similar to that of the WT strain (Fig. 3*A*), demonstrating that their absence imparts no adverse effect on the growth of *L. pneumophila* in culture. In contrast, the cluster 5 mutant exhibited an initial decrease in turbidity at early time points; however, on recovery, it grew at a slower rate than the WT strain (doubling times of 4.4 ± 0.1 h and 3.3 ± 0.1 h, respectively) (Fig. 3*A*). Despite these defects, upon reaching postexponential phase, the mutant exhibited properties of the WT strain grown to this density, including synthesis of the secreted pigment pyomelanin (46, 47) and enhanced motility relative to exponentially growing bacteria (48).

Minigenome Strains of *L. pneumophila* Are Competent for Growth in Culture. To determine if a highly minimized genome of *L. pneumophila* was sufficient for growth in culture, the simultaneous deletion of all six clusters was attempted in a single strain (Table 1). The Δ2abΔ3Δ6aΔ7aΔ4a, or pentuple mutant, lacking 12.7% of protein encoding genes in the *L. pneumophila* genome (Fig. S3 and Table 1), showed similar growth kinetics compared with the WT strain in nutrient-rich liquid media (Fig. 3*B*). These results demonstrated that a significant portion of the *L. pneumophila* genome can be deleted without affecting viability or growth of the bacterium in a nutrient-rich environment, indicating that these regions are designated for purposes other than fundamental biological processes.

Deletion of cluster 5 in the pentuple mutant required generating a marked deletion using an antibiotic resistance cassette for selection. By this approach, viable Δ2abΔ3Δ6aΔ7aΔ4aΔ5:*cat*, or hextuple deletion mutants were obtained, which reduced the genome size by 18.5% compared with the WT strain. Similar to the Cluster 5 mutant, the majority of hextuple mutants exhibited smaller colony sizes than the WT strain when grown on solid media. However, a subset of these mutants generated significantly larger colonies (data not shown). Growth of one of these faster growing variants, 2q, in nutrient-rich liquid media was compared with that of a slower growing isolate, 3a (Fig. 3*C*). Both hextuple mutants showed a defect in growth at early time points similar to the Cluster 5 mutant, but the larger colony variant recovered in a shorter period, consistent with the acquisition of a suppressor mutation in the larger colony variant.

The entire genomes of both hextuple isolates were sequenced and compared with that of the parental strain, Lp02 (*SI Materials and Methods*). Each strain had the expected six cluster deletions as well as an unexpected point mutation in *tufB* that was also present in the Cluster 3 mutant, the progenitor for constructing the hextuple deletion strain. In addition, the slower growing 3a mutant coded for a substitution in RpoC at G195, a residue known to affect RNA polymerase β′ subunit stability (49). In contrast, the faster growing 2q isolate contained three point mutations: one in the intragenic region separating *lpg1464* and *lpg1465*, which encode proteins of unknown function; a mutation in the lysine biosynthesis gene *lysCA* (*lpg1811*) that results in substitution of an active site residue of the LysA domain (50); and an insertion mutation in *cpxR* (*lpg1438*) that causes premature translational termination of the encoded protein. CpxR is a master two-component response regulator that responds to

Table 1. Cluster deletion mutants

Cluster	Region	Total kilobases	No. genes	Percent total genes*	No. TS	Percent total TS†	Deletion mutant constructed	Growth in vitro‡	Intracellular growth			
									AJ	Dd	Hv	Ac
TraSH-identified clusters												
1	*lpg0140–lpg0286*	172	147	5.0	15	6.6	N					
2	*lpg1067–lpg1173*	133	107	3.6	18	8.0	N					
3	*lpg1603–lpg1686*	109	84	2.9	10	4.4	Y					
4	*lpg1924–lpg1999*	92	76	2.5	20	8.8	N					
5	*lpg2097–lpg2267*	224	171	5.8	17	7.5	Y					
6	*lpg2362–lpg2485*	132	124	4.2	18	8.0	N					
7	*lpg2489–lpg2579*	104	91	3.1	13	5.8	N					
Total		966	800	27.1	111	49.1						
Single-cluster deletion mutants												
2ab	*lpg1104–lpg1128, lpg1136—lpg1169*	77	59	2.0	15	6.6	Y	+	+	+	+	+
3	*lpg1603–lpg1686*	109	84	2.9	10	4.4	Y	+	+	+	–	–
4a	*lpg1933–lpg1999*	81	67	2.3	19	8.4	Y	+	+	+	+	–
5	*lpg2097–lpg2267*	224	171	5.8	17	7.5	Y	–	–	–	–	–
6a	*lpg2369–lpg2465*	107	97	3.3	18	8.0	Y	+	+	+	+	+
7a	*lpg2508–lpg2573*	77	66	2.2	9	4.0	Y	+	+	–	+	–
Combination-cluster deletion mutants												
3, 2ab		186	143	4.9	25	11.1	Y	+	+	+	–	–
3, 2ab, 6a		293	240	8.2	43	19.0	Y	+	+	+	–	–
3, 2ab, 6a, 7a		370	306	10.4	52	23.0	Y	+	+	–	–	–
3, 2ab, 6a, 7a, 4a	(pentuple)	451	373	12.7	71	31.4	Y	+	+	–	–	–
3, 2ab, 6a, 7a, 4a, 5	(hextuple)	675	545	18.5	88	38.9	Y	–	–	ND	ND	ND

Ac, *Acanthamoeba castellanii*; AJ, murine bone marrow-derived A/J macrophages; Dd, *Dictyostelium discoideum*; Hv, *Hartmanella vermiformis*; N, no; ND, not determined; Y, yes; +, growth comparable to WT; –, growth defect.

*Total number of *L. pneumophila* protein encoding genes = 2,942.

†Total number of *L. pneumophila* TS encoding genes = 226 (14–18) [including only TS with translocation efficiency ≥40% in (18)].

‡In vitro indicates growth in AYE nutrient-rich media.

bacterial envelope stress in *Escherichia coli* (51) and controls expression of genes encoding components of the Dot/Icm secretion system and its substrates in *L. pneumophila* (52). The appearance of suppressor mutations in the hextuple strains is consistent with this particular combination of gene deletions approaching the minimal gene set still capable of supporting *L. pneumophila* growth.

Consistent with the prediction that mutations in the hextuple 2q isolate suppress the effects of the Cluster 5 mutation, introduction of the *cpxR* insertion mutation into the cluster 5 mutant shortened its early growth defect by roughly 2 h and restored its rate of growth in exponential phase to that of the WT strain (doubling time of 3.2 ± 0.1 h compared with 4.3 ± 0.1 h for the Cluster 5 mutant) (Fig. 3*D*). Thus, forcing the deletion of cluster 5 in the pentuple mutant through positive selection resulted in the accumulation of mutations that allowed the bacterium to tolerate the loss of cluster 5 in combination with clusters 2ab, 3, 4a, 6a, and 7a.

***L. pneumophila* Minigenome Strains Are Competent for Growth in Macrophages.** The retarded growth of the hextuple mutants in broth culture, and the ready isolation of fast-growing derivatives that affect Dot/Icm expression, made it difficult to analyze intracellular growth of strains harboring this particular combination of deletions. Therefore, we analyzed the ability of the pentuple mutant (Δ2ab, 3, 4a, 6a, 7a) to grow within bone marrow-derived mouse macrophages. Remarkably, intracellular growth of this mutant was almost indistinguishable from that of the WT strain (Fig. 4*A*), despite the fact that this mutant was missing 31% of all known Dot/Icm TS (Table 1). As expected, any subcombination of cluster deletions also had no effect on growth within cultured macrophages (Fig. S4, dark bars). Therefore, almost one-third of the proteins targeted for host cells are completely dispensable for intracellular growth in this host cell type.

***L. pneumophila* Minigenome Strains Show Amoebae-Specific Defects in Intracellular Growth.** Because a large number of Dot/Icm TS encoding genes could be removed from the *L. pneumophila* genome without any clear effect on growth in macrophages, the behavior of the pentuple mutant was analyzed in the amoebal species *Acanthamoeba castellanii*, *Dictyostelium discoideum*, and *Hartmanella vermiformis* (Fig. 4*A* and Fig. S4), which mimic natural hosts of the bacterium in the environment.

In contrast to its behavior within bone marrow macrophages, the pentuple mutant was severely defective for growth in each of the amoebal species tested, with the most extreme defect observed in *A. castellanii* (Fig. 4*A*). To determine if these growth defects were attributable to the simultaneous deletion of all five gene clusters or could be attributed to a single cluster deletion, a variety of cluster deletion derivatives were analyzed for growth in these species (Fig. 4*B* and Fig. S4). The Cluster 2ab and Cluster 6a mutants grew as well as the WT strain in all three amoeba hosts tested (Fig. S4). In contrast, gene clusters 3 and 7a were differentially important for growth in *H. vermiformis* and *D. discoideum*, respectively (Fig. 4*B*). In *A. castellanii*, not only were both clusters 3 and 7a required for robust intracellular growth but, unlike in *H. vermiformis* and *D. discoideum*, growth of the Cluster 4a mutant was severely impaired in this host (Fig. 4*B*). Thus, the importance of a cluster in one host does not mean it is critical for growth in all hosts, demonstrating a host-specific requirement for genes in these regions.

In addition to the host-specific requirements observed for a subset of the cluster deletion mutants, three other behavioral trends stood out. First, both the cluster 4 and Δ2abΔ3Δ6a mutants were almost as defective as the pentuple mutant for growth in *A. castellanii* (Fig. 4*C*), demonstrating that growth defects of comparable magnitude could be generated in more than one fashion. The simultaneous deletion of multiple func-

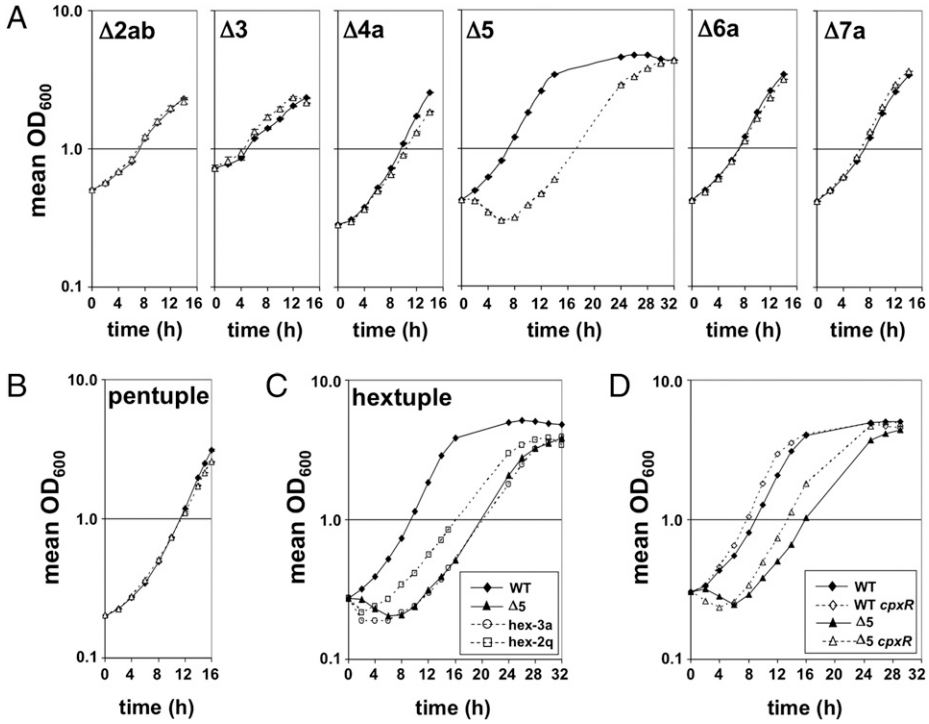

Fig. 3. *L. pneumophila* minimal genome strains exhibit robust growth in vitro. (*A*) Growth of all but the Cluster 5 single deletion mutants (△) is comparable to that of the WT strain Lp02 (◆) in nutrient-rich media. (*B*) Growth of the pentuple mutant (△) in rich media is similar to that of the WT strain (◆). (*C*) Differential growth of the hextuple mutant variants 2q and 3a relative to WT and the Cluster 5 mutant strains. (*D*) Partial rescue of the cluster 5 mutant in vitro growth defect by the hextuple 2q *cpxR* point mutation. Data represent the average of two independent experiments of two technical replicates each; error bars indicate ± SD.

tionally redundant virulence genes in different combinations likely accounts for this phenomenon. Second, each deletion mutant generally exhibited a growth defect in *A. castellanii* that was as great as or greater than that in *D. discoideum* or *H. vermiformis* (Fig. S4), consistent with *A. castellanii* being more restrictive for *L. pneumophila* growth than these other two species. Third, the growth defect of the pentuple mutant in *H. vermiformis* could not be explained simply by the absence of critical Dot/Icm TS, because the total yield of bacteria for this mutant was lower than that observed for a *dot*⁻ translocation deficient strain (Fig. 4*A* and Fig. S4).

The behavior of the pentuple mutant indicates that a large cohort of genes in the *L. pneumophila* genome are dispensable for growth within macrophages but are necessary for propagation in the bacterium's natural environmental host. The importance of some gene clusters for growth in only a subset of amoebal species and their apparent acquisition from an exogenous source indicate that expansion of the bacterial genome through the accumulation of foreign genetic material has broadened the host range of *L. pneumophila*.

Discussion

We have shown that the *L. pneumophila* genome is organized in a mosaic fashion, with conserved chromosomal segments encoding genes required for growth in culture. This modular arrangement of the genome allowed its targeted reduction by almost 13%, resulting in a mutant that grew as well as the WT strain in nutrient-rich medium. This series of essential regions defines a core genome that is also sufficient for growth in macrophages, demonstrating that the collection of virulence factors encoded by this core genome can support bacterial replication in a subset of hosts. Dispersed among these essential genomic regions are chromosomal islands of high plasticity that allow replication in a diverse set of amoebal hosts. The impaired growth of mutants lacking individual genomic islands demonstrates a role for these loci in expanding the host range of *L. pneumophila*.

The requirements of different genomic regions for *L. pneumophila* growth in specific hosts indicate that considerable variation exists in the intracellular niches occupied by the bacterium within these hosts, even though the membrane trafficking events that lead to formation of the replication vacuole appear to be similar. Possible sources of this host variation could include allelic differences in, or the absence of, host proteins targeted by the bacterium that render certain Dot/Icm TS ineffective, alterations in the availability of host nutrients, or differences in host antimicrobial defenses. The need to establish a replicative niche in the face of host variation is likely to be the selective pressure driving genome expansion. Therefore, we propose that expansion of the *L. pneumophila* genome through its acquisition of foreign genetic material has allowed the bacterium to replicate in hosts that are evolutionarily distant from one another. In clinical isolates of *L. pneumophila*, the large amount of genetic diversity in the regions of the chromosome that are dispensable for growth in vitro likely reflects variation in the amoeba population encountered by these strains in their respective environmental niches. Interaction of the bacterium with any one of multiple hosts in its fresh water habitat could provide the selective pressure for maintaining its diverse repertoire of virulence determinants and contribute to functional redundancy among Dot/Icm TS.

L. pneumophila strains are naturally competent for DNA uptake, allowing acquisition of genetic material from a variety of sources, including eukaryotes (17, 33, 40). Furthermore, the Legionellaceae appear to be capable of actively donating their own DNA to one another, because all sequenced strains encode at least one conjugative DNA apparatus (33, 40–42). The ability to acquire genetic material from multiple sources and reassort DNA among related species likely contributes to increased fitness of the bacterium in a diverse set of hosts.

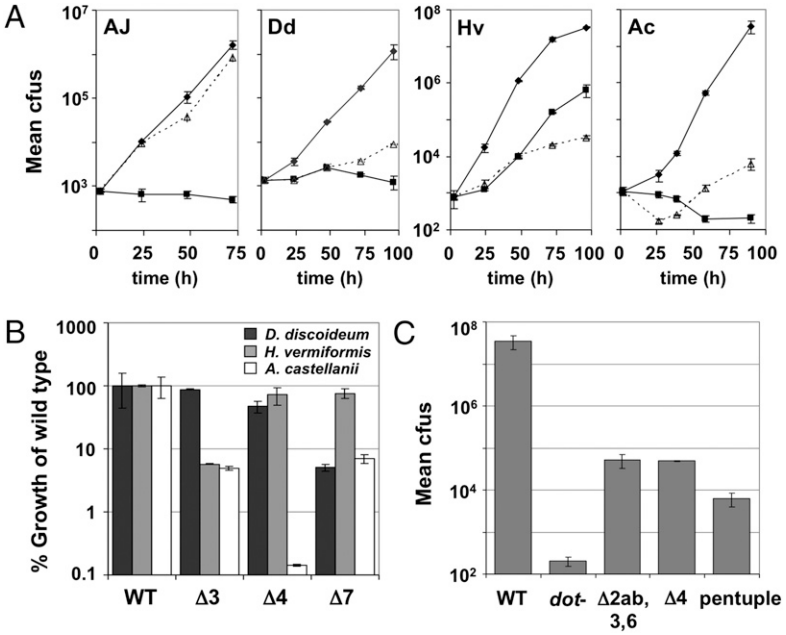

Fig. 4. Intracellular growth of the pentuple mutant is severely impaired in amoebae but not macrophage hosts. (*A*) Growth of WT (◆), *dot*⁻ (■), and pentuple mutant (△) strains in bone marrow-derived A/J macrophages (AJ) and the amoebal species *D. discoideum* (Dd), *H. vermiformis* (Hv), and *A. castellanii* (Ac). (*B*) Growth of the Cluster 3, 4, and 7 deletion strains in *D. discoideum*, *H. vermiformis*, and *A. castellanii* as a percentage of the WT strain. (*C*) Growth of the Δ2ab,3,6a, and 4a mutants in *A. castellanii* is comparable to that of the pentuple mutant. In each case, bacterial growth based on recovered number of cfus on solid media from lysed host cells was monitored over 3–4 d (encompassing 3 consecutive rounds of infection). Plotted is the total bacterial yield (*A* and C) over the course of the infection normalized relative to the WT strain by percent uptake at the initial time point tested or the percentage of growth (*B*) of each mutant relative to the WT strain. Data represent the average of two independent experiments of three technical replicates each; error bars indicate ±SD.

Continual interaction of *L. pneumophila* and amoebae over time may have promoted their coevolution in a manner reminiscent of type III effectors and the immune surveillance systems of plants (5). As *L. pneumophila* evolved effective strategies for promoting intracellular growth, amoebae may have also developed appropriate countermeasures for limiting bacterial replication. This may explain why one amoebal species, such as *A. castellanii*, is generally more restrictive than others. Unlike plant pathogens, however, which have constrained host ranges and have shed effectors that provide little advantage in their preferred hosts (4, 53), *L. pneumophila* appears to have retained a broad effector set to facilitate growth in a diverse group of aquatic species. Accumulation of foreign genetic material may enhance the *L. pneumophila* host range as well as facilitate its adaptation to antimicrobial strategies newly acquired by its hosts.

L. pneumophila is often referred to as an accidental pathogen of humans because its interaction with amoebae provides the selective pressure that allows replication within mammalian macrophages. The pentuple mutant showed robust growth in macrophages despite being severely defective for replication in all three amoebal hosts tested. This behavior supports the existence of an ancestral *L. pneumophila* strain that encodes a core set of virulence factors sufficient to promote its replication in an unidentified natural host. Further supporting this idea of a reduced genome with a collection of Dot/Icm TS that enables growth in a subset of hosts are the following observations. First, the pentuple mutant has intact copies of the only two Dot/Icm TS whose absence severely impairs intracellular growth, SdhA (13) and DimB/MavN (19, 54). Second, the *dot/icm* genes do not map to any of the seven gene clusters analyzed here but, instead, are located within the minimized genome (Fig. 2*A*). Identification of genes in the pentuple mutant required for replication in macrophages should facilitate the discovery of virulence factors specific to this host.

Work on other pathogens has posited the existence of pathogenicity islands, contiguous regions of DNA that provide the bacterium with the potential to colonize a host (55–57). Despite the identification of over 200 Dot/Icm TS, bioinformatic-based approaches to identify such islands in *L. pneumophila* did not predict the organized clustering of genes required for intracellular growth. The fact that the single most important determinants of disease, the *dot/icm* genes, are absent from these dispensable regions distinguishes these loci from classically defined pathogenicity islands (58). Instead, the genomic islands identified here by essential gene mapping are regions of host range expansion.

The ordered assembly of the chromosome, in which there are modules of DNA neither associated with a minimal gene set necessary for growth in bacterial culture nor characterized as pathogenicity islands, can be found in other organisms. For example, when we positionally mapped the essential genes of *Mycobacterium tuberculosis* for growth in rich media, as identified by Rubin and colleagues (59), 11 large gene clusters were devoid of essential genes (Fig. S5). These segments ranged in size from 42 to 112 genes, encompassing 22% of the *M. tuberculosis* genome. Four of these clusters included genes that encode proteins involved in intracellular survival of the bacterium, demonstrating a correlation between genes in these islands and virulence. Similar information about genome architecture based on experimentally identified regions necessary for optimal growth in bacterial culture should facilitate a more directed approach for genome minimization in other bacteria and provide a strategy for identifying novel virulence genes, including those involved in host range expansion.

This work demonstrates that the *L. pneumophila* genome was assembled in a modular fashion, building on a core genome that encodes determinants sufficient for growth in vitro as well as replication in selected hosts. Propagation in the environment, however, presumably selected for the ability to grow in multiple hosts and adapt to fluctuations in the host population, rendering

the core genome insufficient for survival of the organism in aquatic habitats. Sequestering genes responsible for host range expansion into contiguous regions ensures that the integrity of the core genome is preserved by allowing genetic exchanges resulting in host range alteration being isolated from chromosomal regions encoding functions involved in fundamental cellular processes. Host diversification of *L. pneumophila* would require that a large fraction of the newly acquired genetic material be maintained to allow growth in multiple hosts. Retention of additional virulence genes would depend on whether they provide an advantage to the bacterium in at least one of its natural hosts without impairing its growth in another. Presumably, the lack of selection against such proteins as Dot/Icm TS would allow *L. pneumophila* to maintain these proteins, thus accounting for the broader host range of this microorganism. Future studies will focus on distinguishing between factors involved in expanding the host range of *L. pneumophila* and those encoded by the core genome that play central roles in virulence.

Materials and Methods

Bacterial Strains, Cultures, Cells, and Growth Media. *L. pneumophila* strains were grown in liquid *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffered yeast extract (AYE) media (60) or on solid charcoal buffered yeast extract (CYE) media (61) containing 0.1 mg/mL CYET (Sigma), 40 μg/mL kanamycin, 5% (wt/vol) sucrose, or 5 μg/mL chloramphenicol when appropriate. *E. coli* strains were grown in liquid LB or on solid LB plates supplemented with 50 μg/mL ampicillin, 50 μg/mL kanamycin, 50 μg/mL streptomycin, 10 μg/mL tetracycline, or 17 μg/mL chloramphenicol when appropriate. The *E. coli* strain DH5α λpir (62) was used for all plasmid cloning with the exception of pTO100, which was cloned using a DH5α λpir F^+ strain (*SI Materials and Methods*). Strains are summarized in Table S1.

A/J primary bone marrow-derived mouse macrophages were prepared and cultured as described previously (8). *D. discoideum*, *A. castellanii* (ATCC30234; American Type Culture Collection), and *H. vermiformis* (ATCC50237; American Type Culture Collection), respectively, were cultured as described elsewhere (28, 63, 64). For infections in amoebae, the chromosomal thy^- allele in each bacterial strain analyzed was replaced with the thy^+ allele by allelic exchange as described (28).

Construction of *L. pneumophila* Transposon Mutant Library. Twenty-five individual aliquots of 3×10^9 electrocompetent *L. pneumophila* Philadelphia 1 strain Lp02 were electroporated with 75 ng of pTO100, recovered at 37 °C for 4 h in AYE, and then plated on CYET containing kanamycin and sucrose. Bacteria were incubated at 37 °C for 4 d. Roughly, 100,000 cfus were harvested into AYE and 20% (vol/vol) glycerol (final concentration of $\sim 2 \times 10^{10}$ bacteria/mL), aliquoted into 500-μL volumes, and stored at −80 °C.

TraSH Screen: Growth of *L. pneumophila* Transposon Library in Vitro. An aliquot of the *L. pneumophila* transposon mutant library ($\sim 10^{10}$ bacteria) was diluted in AYE media and then cultured overnight at 37 °C to stationary phase. Bacteria were plated for single colonies on solid CYET media containing kanamycin and grown at 37 °C for 4 d. An estimated 100,000–150,000 bacterial colonies were harvested, resuspended in AYE (~2,500 cfus/mL) and mixed to homogeneity. From this suspension, triplicate aliquots of 2×10^9 bacteria were used to isolate genomic DNA using a Qiagen DNeasy kit, including proteinase K and RNase digestion steps, and then pooled. This procedure was performed with 10 separate aliquots of the library for a total of 10 individual experiments. Array construction, TraSH probe generation, array hybridization, and analysis are detailed in *SI Materials and Methods*.

Construction of *L. pneumophila* Deletion Mutants. Gene clusters were deleted in *L. pneumophila* Philadelphia 1 strain Lp02 using a double-recombination strategy with the suicide vector pSR47s as described (65) with the following exception: 3,000-bp segments directly upstream and downstream of each cluster were used for homologous recombination. Primer pairs for plasmid construction are listed in Table S1. All plasmids were sequenced before use. For deletion of cluster 5 in the pentuple mutant, a BamHI chloramphenicol resistance cassette was cloned from pM_Cm (pM-Gm was a kind gift from Zhao-Qing Luo, Perdue University, West Lafayette, IN) into the BamHI site of the cluster 5 deletion plasmid, pYA71. For each mutant, 100–600 individual isolates were screened by PCR. For those mutants used in subsequent analyses, their corresponding deletion mutations were verified by microarray analysis (*SI Materials and Methods* and Fig. S3).

In Vitro Growth Curves. *L. pneumophila* strains grown on solid media were diluted in AYE media containing thymidine to $2–3 \times 10^8$ bacteria/mL. Bacterial growth at 37 °C was monitored over 16–26 h by diluting culture aliquots 1:5–1:10 in 1× PBS and measuring the absorbance at 600 nm in a Molecular Devices SpectroMax M5 spectrophotometer at regular intervals. Bacterial doubling time, t_d, was calculated as $t_d = (t_2 - t_1)/(\log_{10}2/(\log_{10}(q_2/q_1))$, where t_1 is time 1, t_2 is time 2, and q_1 and q_2 are the number of bacteria at t_1 and t_2, respectively.

Intracellular Growth Assays. Growth of *L. pneumophila* in A/J macrophages, *A. castellanii*, and *D. discoideum*, respectively, was performed as described in elsewhere (8, 28, 66). For growth in *H. vermiformis*, *H. vermiformis* was plated at 1×10^5 cells per well in a 96-well tissue culture plate in *H. vermiformis* media (ATCC1034; American Type Culture Collection) and then immediately challenged with *L. pneumophila* strains grown to postexponential phase at a multiplicity of infection of 0.05. Cells were incubated for 2 h at 35 °C and then rinsed twice with *H. vermiformis* media. At 2, 24, 48, 72, and 96 h postinfection, *H. vermifomis* were lysed with 0.02% saponin (Sigma) and growth of *L. pneumophila* was determined by plating host cell lysates on solid CYE media and counting the number of bacterial cfus.

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