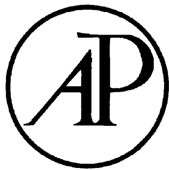

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The *Legionella pneumophila* GacA homolog (LetA) is involved in the regulation of *icm* virulence genes and is required for intracellular multiplication in *Acanthamoeba castellanii*

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Abstract

Legionella pneumophila, the causative agent of legionnaires' disease, is a broad-host-range facultative intracellular pathogen. Thus far, 24 genes (*icm*/*dot* genes) required for *L. pneumophila* intracellular growth, have been discovered. In this study, a deletion substitution was constructed in the *L. pneumophila* homolog of the *gacA* response regulator (*letA*) and its involvement in *L. pneumophila* pathogenicity and *icm*/*dot* gene expression was characterized. The *letA* mutant constructed had no intracellular growth defect when analyzed in HL-60 derived human macrophages, but it was found to be severely attenuated for intracellular growth in the protozoan host *Acanthamoeba castellanii*. The growth defect in amoebae was fully complemented by introducing the *L. pneumophila letA* gene on a plasmid. In addition, the LetA regulator was found to be involved in the expression of three *icm* genes (*icmT*, *icmP* and *icmR*). The level of expression of the *icmT::lacZ* and *icmR::lacZ* fusions was found to be higher, while the level of expression of the *icmP::lacZ* fusion was found to be lower when analyzed in the *letA* mutant strain, in comparison to the wild-type strain. We concluded that LetA has a moderate effect on *icm*/*dot* gene expression, but it probably plays a major role in the expression of *L. pneumophila* genes required for intracellular growth in protozoan hosts. A similar host specific phenotype was previously described for the RpoS sigma factor and the type II general secretion system of *L. pneumophila*.
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Keywords: Legionella; GacA; LetA; Intracellular growth; *icm*/*dot*

1. Introduction

Legionella pneumophila, the causative agent of legionnaires' disease, is a broad-host-range facultative intracellular pathogen. *L. pneumophila* is able to infect, multiply within, and kill human macrophages, as well as free living amoebae that serve as their environmental reservoir [1,2]. Two regions of *icm*/*dot* genes have been discovered in *L. pneumophila* [3,4], these genes were shown to be required for: upregulation of phagocytosis; inhibition of phagosome lysosome fusion at early times during infection; association of the phagosome with the rough endoplasmic reticulum and as a consequence of all that for intracellular multiplication in human macrophages and amoebae [5–12]. Fifteen out of the 24 Icm/Dot proteins were found to contain sequence homology to proteins involved in conjugation from IncI plasmids such as R64 [13,14]. In addition, the *icm*/*dot* genes were found to be required for *L. pneumophila* plasmid conjugation [15,16] and conjugal components were found to inhibit *L. pneumophila* intracellular growth [17]. The *icm*/*dot* system is believed to encode a type IV secretion system, that probably translocates effector molecule(s) to its host, and in this way modulate the properties of the phagosome [3,4,7].

Complementation and primer extension analysis indicated that the *icm*/*dot* genes are probably organized in nine transcriptional units (*icmTS*, *icmR*, *icmQ*, *icmPO*, *icmMLK-EGCD*, *icmJB*, *icmF-tphA*, *icmWX* and *icmV-dotA*) [15,16, 18–21]. Recently, 12 regulatory sites were identified in the upstream region of eight *icm*/*dot* genes. Seven of these sites were found to constitute the −10 promoter elements of the *icm* genes, where the other five, are expected to serve as binding sites for regulatory factors [19]. One of the transcription regulators known to be involved in controlling virulence genes of many gram-negative bacterial pathogens is the GacA (*g*lobal *ac*tivator) regulator. GacA and its

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orthologs were found in several species belongs to the gamma subdivision of *Proteobacteria* including, *Pseudomonas* species (*gacA*), *Erwinia carotovora* (*expA*), *Escherichia coli* (*uvrY*), *Vibrio cholera* (*varA*), and *Salmonella enterica* serovar typhimurium (*sirA*) (reviewed in [22]). The GacA/GacS two components system comprises a membrane associated sensor kinase (GacS) and its cognate response regulator (GacA) from the FixJ family (reviewed in [23]). Recently, it was found that the GacA regulator is probably involved in regulation of its target genes by a post-transcriptional mechanism, where it was shown to function together with other regulatory factors [24,25].

The phenotype of *gacA* mutant strains in different pathogens suggests that it play a key role in the regulation of virulence genes. In *V. cholera*, the *gacA* ortholog was shown to be required for the production of cholera toxin and colonization of the murine intestine [26]. In several plant pathogens such as *E. carotovora* and *Pseudomonas syringae*, the *gacA* ortholog was found to be required for extracellular enzyme production and for virulence [27,28]. In *Pseudomonas aeruginosa*, the *gacA* ortholog, was shown to be required for quorum-sensing [29], extracellular virulence factor production [30], biofilm formation [31], and virulence in plant, animal and nematode models [32,33]. In uropathogenic *E. coli*, *gacA* ortholog was demonstrated to be required for growth in urine [34] and in *S. typhimurium*, *gacA* and *gacS* homologous genes were found to be required for type III secretion and invasion functions [35,36].

Recently, the *L. pneumophila gacA* homologous gene was isolated using a genetic screen and designated *letA* [37]. The mutant strain isolated was shown to be impaired for some virulence traits including motility, macrophage infectivity, sodium sensitivity, evasion of phagosome-lysosome fusion, and cytotoxicity, but it was shown to be dispensable for intracellular replication in murine bone marrow macrophages [37]. We were interested to determine the role of the *L. pneumophila* LetA regulator in two main aspects of *L. pneumophila* virulence. Namely, intracellular growth in its natural host *A. castellanii* as well as in HL-60 derived human macrophages, and its involvement in the expression of genes required for intracellular growth (*icm*/*dot* genes). Our results indicate that LetA plays a significant role in *L. pneumophila* pathogenicity. The LetA regulator was found to be required for intracellular multiplication in the protozoan host *A. castellanii*, and it was found to affect the level of expression of three *icm* genes required for intracellular growth.

2. Results

2.1. Construction of a L. pneumophila letA deletion substitution mutant

The *L. pneumophila letA* gene was identified by homology to the *Pseudomonas gacA* gene and its coding sequence was amplified by PCR from *L. pneumophila* JR32 chromosome and cloned. To gain insight into the involvement of the LetA regulator in the regulation of *icm*/*dot* genes and its potential role in *L. pneumophila* pathogenicity, a deletion substitution was constructed in it. The resulting strain (OG2001, Fig. 1), grows well on bacteriological media with similar growth rate as the wild-type strain (data not shown).

2.2. LetA is required for intracellular growth in A. castellanii but is dispensable for intracellular multiplication in HL-60 derived human macrophages

In the environment, *L. pneumophila* replicates intracellularly within protozoan hosts, such as *A. castellanii* [38], while in humans, during infection, the bacteria grow inside alveolar macrophages [2]. Therefore, the role of the *L. pneumophila* LetA regulator during intracellular multiplication in amoebae, and in HL-60 derived human macrophages was examined. The wild-type strain (JR32), the *letA* mutant strain (OG2001), and a strain that does not multiply intracellularly (25D) were used for infection (Fig. 2). The *letA* mutant strain (OG2001) was found to multiply similarly to the wild-type strain in HL-60 derived human macrophages (Fig. 2(B)). However, its intracellular growth in *A. castellanii* was severely attenuated (Fig. 2(A)). To confirm that the growth defect in amoebae, occurred due to the insertion in the *letA* gene, a complementation experiment was performed. As can be seen in Fig. 2(A), a plasmid (pOG-Mgac, Fig. 1) containing the *L. pneumophila letA* gene, fully complemented the intracellular growth defect of the *letA* mutant strain (OG2001). These results indicate that

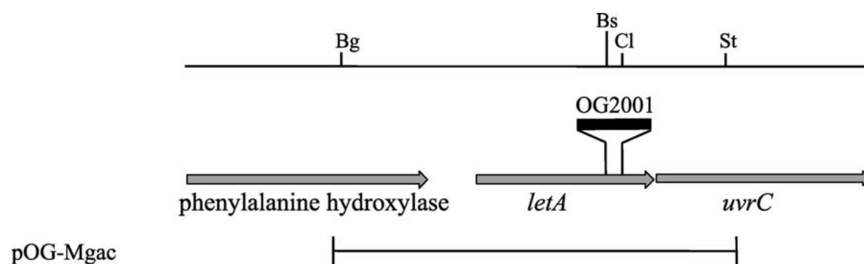

Fig. 1. Linkage map of the *gacA* locus. The arrows indicate open reading frames (phenylalanine-hydroxylase; *letA* and *uvrC*). The site of the kanamycin cassette in the *letA* deletion substitution mutant (OG2001) is indicated. The thin line indicates the region covered by the complementation plasmid pOG-Mgac (1410 bp). The restriction enzymes are Bg, *Bgl* I; Bs, *Bsr*GI; Cl, *Cla* I; St, *Stu* I (only relevant sites are shown).

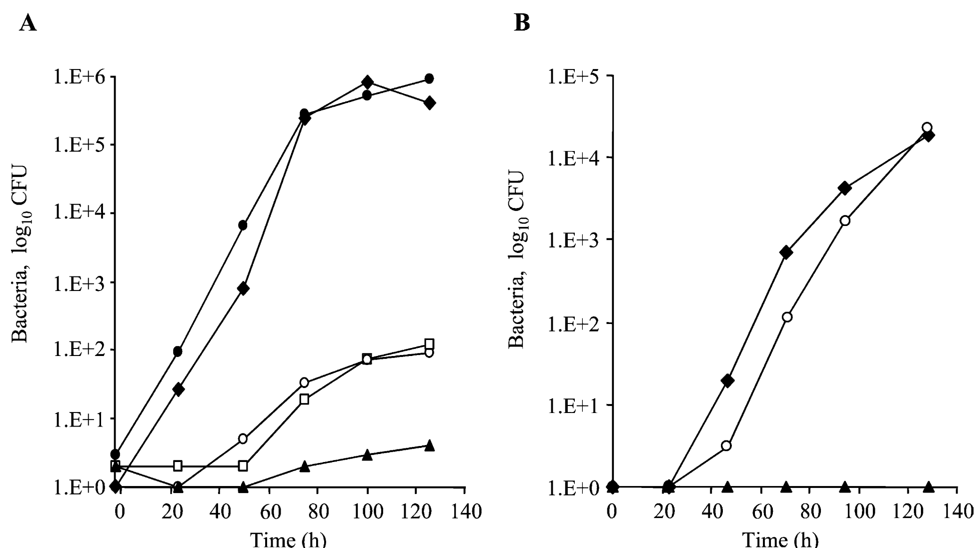

Fig. 2. LetA is required for *L. pneumophila* intracellular growth in *A. castellanii* but not in HL-60 derived human macrophages. (A) Intracellular growth assay in *A. castellanii*. Closed diamond, *L. pneumophila* wild type (JR32); closed triangle, mutant strain 25D; open circle, *letA* mutant strain (OG2001); closed circle, OG2001 containing the *L. pneumophila letA* gene (pOG-Mgac); and open box, OG2001 containing the vector pMMB207. The experiment was performed as described in Section 4. The experiment was performed three times, (as well as with three independent isolates) and the same results were obtained. (B) Intracellular growth in HL-60 derived human macrophage. The experiment was performed as described in the Section 4. Closed diamond, *L. pneumophila* wild type (JR32); closed triangle, mutant strain 25D; open circle, *letA* mutant strain (OG2001).

LetA is required for intracellular multiplication in *A. castellanii*, but it is dispensable for intracellular growth in HL-60 derived human macrophages.

2.3. LetA affects the flaA gene expression

The effect of GacA on motility and on the expression of the flagellar regulon has been previously demonstrated in several bacteria [39–41]. In addition, recently a *L. pneumophila letA* mutant strain was shown to express a *flaA::gfp* fusion poorly and to be attenuated in motility [37]. To examine this phenotype in the new *letA* mutant strain constructed (OG2001), the level of expression of a *flaA::lacZ* fusion was compared between a *L. pneumophila* wild-type strain and the *letA* mutant strain (JR32 and OG2001, respectively). The level of expression of the *flaA::lacZ* fusion was examined at stationary phase, as the highest expression of the flagella genes occurs at this growth phase [42]. The level of expression of the *flaA::lacZ* fusion was reduced 7 fold from 7361 ± 1557 Miller unit (MU) in the wild-type strain, to 1070 ± 289 MU in the *letA* mutant strain. These data confirm the previous results indicating that in *L. pneumophila* LetA is required for optimal expression of the flagellum subunit gene, *flaA*.

2.4. LetA affect the icm genes expression

Based on the results presented above, indicating that LetA is required for intracellular growth in the protozoan host *A. castellanii*, the involvement of LetA in the regulation of the *icm/dot* virulence genes was examined. A series of nine *icm::lacZ* translational fusions (*icmT::lacZ*, *icmR::lacZ*, *icmQ::lacZ*, *icmP::lacZ*, *icmM::lacZ*, *icmJ::lacZ*, *icmF::lacZ*, *icmW::lacZ* and *icmV::lacZ*) that has been constructed before [43], were used for this analysis. Plasmids containing these *icm::lacZ* fusions were introduced into the *letA* mutant strain (OG2001), and their levels of expression were compared to the ones obtained with the wild-type strain. As can be seen in Fig. 3(A) and (B), at stationary phase ($OD_{600} = 3–4$), the level of expression of three *icm::lacZ* fusions was affected. The level of expression of the *icmT::lacZ* fusion, increased by 40% in OG2001 (from 690 ± 107 to 970 ± 98 MU), while the level of the *icmR::lacZ* fusion increased by 54% in OG2001 (from 2435 ± 339 to 3753 ± 367 MU). An opposite effect was found with *icmP::lacZ* fusion, as its level of expression decreased by 23% in OG2001 (from 756 ± 102 to 581 ± 30 MU). The other six *icm::lacZ* fusions retain a similar level of expression as in the wild-type strain (Fig. 3(A) and (B)). The higher levels of expression found with the *icmT::lacZ* and *icmR::lacZ* fusions in OG2001, were also observed at exponential phase ($OD_{600} = 0.5–1$), as shown in Fig. 3(C) and (D). The level of expression of the *icmT::lacZ* fusion increased by 55% in OG2001 (from 259 ± 57 to 403 ± 37 MU), and the level of expression of the *icmR::lacZ* fusion increased by 77% in OG2001 (from 1157 ± 159 to 2052 ± 247 MU). However, the level of expression of the *icmP::lacZ* fusion was unaffected, at this growth phase (Fig. 3(D)). In addition, similar differences in the levels of expression of the *icmT::lacZ* and *icmR::lacZ* fusions were also obtained at late exponential phase ($OD_{600} = 1.5–2$), and early stationary phase ($OD_{600} = 2–2.5$). In contrast, the small decrease in the level of expression of the *icmP::lacZ* fusion was observed only

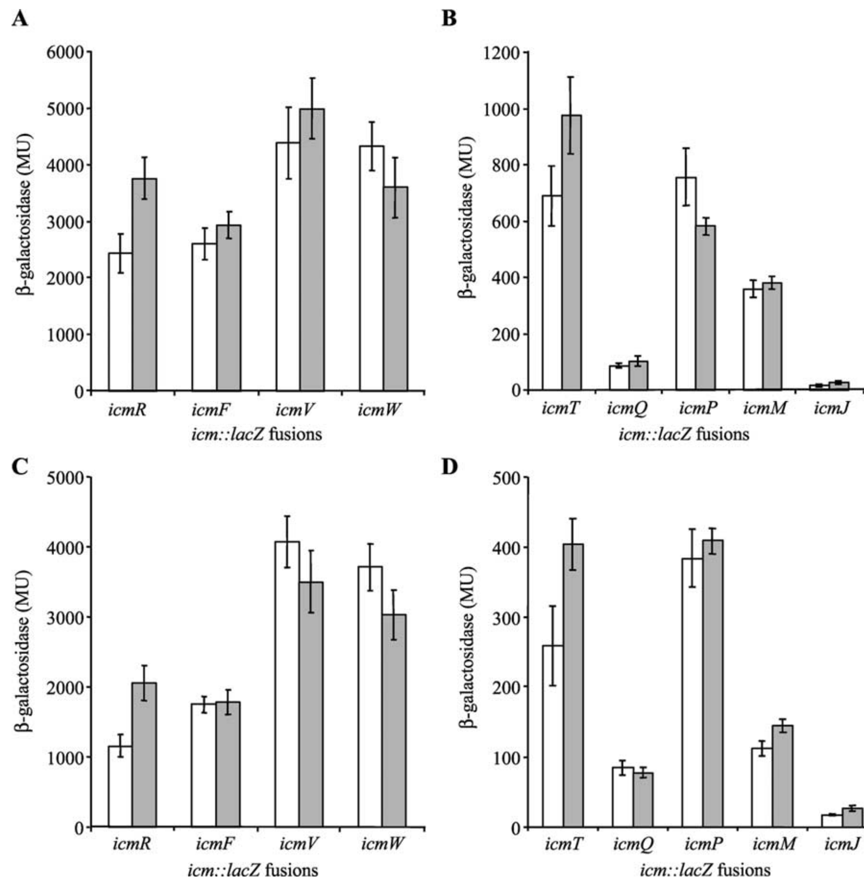

Fig. 3. LetA affects the expression of the *icmR::lacZ*, *icmT::lacZ* and *icmP::lacZ* fusions. The expression of nine *icm::lacZ* fusions (*icmT*, *icmR*, *icmQ*, *icmP*, *icmM*, *icmJ*, *icmF*, *icmV*, and *icmW*) was examined in *L. pneumophila* wild-type strain JR32 (white) and *letA* deletion substitution mutant OG2001 (gray). β-galactosidase activity was measured as described in Section 4. The data presented in panels A and B was obtained at stationary phase and in panels C and D at exponential phase. Four *icm::lacZ* fusions that had high β-galactosidase activities are shown in panels A and C, and five that had low activities are shown in panels B and D. The data is presented in Miller units (MU) and is an average of at least three different experiments. The error bars represent standard deviation. The level of expression of the vector pGS-lac-01 was 7.3 ± 2.4 MU at stationary phase and 13.2 ± 2.7 MU at exponential phase. The *Y*-axis in the four panels is different.

during stationary phase. These results indicate that the LetA regulator has negative as well as positive effects on the *icm*/*dot* gene expression. The negative effect was more pronounced at exponential phase and the positive effect was observed only at stationary phase.

3. Discussion

The GacA regulator was shown to be involved in the expression of virulence traits and required in order to cause disease by several gram-negative bacteria that belong to the gamma subdivision of the *Proteobacteria* including *Pseudomonas* species, *E. carotovora*, *E. coli*, *V. cholera* and *S. typhimurium*. Yet, in each bacteria, the virulence genes that *gacA* regulates are different (reviewed in [22]). Recently, it was shown that a mutant in the *L. pneumophila gacA* homolog (*letA*) is attenuated for some virulence traits including motility, macrophage infectivity, sodium sensitivity, evasion of phagosome-lysosome fusion, and cytotoxicity, but it was shown to be dispensable for intracellular growth in murine bone marrow macrophages [37].

We have shown here that the LetA regulator has a host specific phenotype in *L. pneumophila*. A strain containing a deletion substitution in the *letA* gene was severely attenuated for intracellular growth in the protozoan host *A. castellanii*. This phenotype was fully complemented by introducing the *L. pneumophila letA* gene on a plasmid. As opposed to that, LetA was found to be dispensable for intracellular growth in HL-60 derived human macrophages. This result is in agreement with an observation reported before, indicating that the intracellular growth of a *letA* mutant in murine bone marrow macrophages was unaffected [37]. A similar phenomenon was previously described for the stationary phase sigma factor, RpoS, and the type II general secretion system, in *L. pneumophila*. A strain harboring a knockout in the *rpoS* gene, lost its ability to grow in the protozoan host *A. castellanii*, but it was found to have no intracellular growth defect in HL-60 derived human macrophages and THP-1 cells [44]. Likewise,

L. pneumophila strain containing a deletion substitution mutation in the *lspGH* genes (that are part of the type II secretion system), was defective for intracellular multiplication in *A. castellanii*, but its growth within HL-60 derived human macrophages was unaffected [45]. Recently, the stage during infection when *L. pneumophila* exit from the host cell was shown to be different between protozoan hosts and human macrophages [46], and the *icmT* gene was shown to participate in this process in amoebae. Bacteria containing a mutation in the *icmT* gene (*rib* mutants) were found to be trapped inside the phagosome in the amoebae [47,48], but the cytotoxin required for the bacterial exit from the phagosome was not identified. Because that our intracellular growth experiments determined the number of bacteria that multiply intracellularly and exit from the amoebae, it could have been that the phenotype observed with the *letA* mutant is related to the regulation of the yet unrecognized cytotoxin. However, we did not observed bacteria that were trapped inside the amoebae, during the intracellular growth experiments (data not shown). We think that the *letA* intracellular growth phenotype in amoebae is related to another difference between the two hosts, which probably require genes that are under the regulation of LetA.

It seems that there is a tight correlation between the stationary phase sigma factor RpoS and LetA pathways in *L. pneumophila* as well as in other bacteria. It has been shown that in *E. coli*, *P. fluorescens* and *P. aeruginosa*, the GacA orthologs, are involved in the expression of *rpoS* [49–51]. In *L. pneumophila*, *letA* mutation affects virulence traits, which are correlated with the entry of the bacteria into stationary phase [37,52]. In addition, as was shown here, the level of expression of *icmP::lacZ* fusion decreased in the absence of LetA at stationary phase. Similar effect on the level of expression of this *icmP::lacZ* fusion was also observed in *L. pneumophila rpoS* and *relA* mutant strains, in which the level of expression of this fusion was lower in comparison to the wild-type strain at stationary phase [43]. Taking all these data together, it is possible that, the *L. pneumophila* LetA is involved in the signal transduction cascade leading to the induction of RpoS.

Recently, we began to explore the regulation of the *icm*/*dot* genes, that were shown to be required for phagosome establishment and intracellular growth, and identified 12 regulatory sites in the upstream region of eight of them [19]. Seven of these sites were found to constitute the -10 promoter elements of the *icm* genes, where the other five, are expected to serve as binding sites for regulatory factors. The results presented here indicate that LetA is involved in the expression of three *icm* genes (*icmT*, *icmP* and *icmR*), but not in the same manner. Considering that in the absence of *letA*, the expression of the *icmT::lacZ* and *icmR::lacZ* fusions increased, we speculate that LetA has a negative effect on the expression of these genes in the wild-type strain. However, analysis of the regulatory regions of these two genes did not reveal any similar regulatory elements [19]. In the case of the *icmP::lacZ* fusion, its level of expression decreased at stationary phase in the *letA* deletion substitution mutant, suggesting that LetA has a positive effect on the level of expression of *icmP* in the wild-type strain. Usually, GacA is known to activate the transcription of its target genes (as was demonstrated here for the *L. pneumophila flaA* gene), however, GacA was shown recently to function as a negative regulator as well. The GacA orthologs from *E. coli* and *S. enterica* serovar Typhimurium were shown to have repressing effects on the expression of flagellar genes in these species [40]. In addition, the moderate effect of the *letA* deletion on the *icmT*, *icmP* and *icmR lacZ* fusions might indicate that its effect was indirect. This possibility is in agreement with previous assumptions about the GacA regulation of virulence related genes [22]. It is important to note that all the *icm::lacZ* fusions used were translational fusions that contain the promoter, the ribosomal binding site as well as the region that codes for the first seven amino acids of the *icm* genes fused to the *lacZ* reporter. Due to that, possible post-transcriptional regulation (that was described for the *Pseudomonas* GacA regulator [24,25]) of LetA on the *icm::lacZ* mRNA, should have been identified with our system as well as regulation at the transcriptional level.

In summary, the results presented here, indicate that LetA regulates several *L. pneumophila* genes including the flagella regulon, three *icm* genes and most important some unknown *L. pneumophila* host specific virulence factors, required for intracellular growth in the protozoan host *A. castellanii*. It is possible that LetA and RpoS play a major role in the expression of *L. pneumophila* virulence genes that are host specific.

4. Materials and methods

4.1. Bacterial strains, plasmids, primers, and media

L. pneumophila strains used in this work were JR32 - a streptomycin resistant, restriction negative mutant of *L. pneumophila* Philadelphia-1 which is a wild-type strain in terms of intracellular growth [53]. A mutant that cannot grow intracellularly 25D [54], and a *letA* deletion substitution mutant OG2001 which is a JR32 derivative. Plasmids used in this work are described in Table 1. Bacterial media, plates and antibiotic concentrations were used as described before [21].

4.2. Plasmid construction

The primers gacA-F (5′-TTATTAGATAT-GAAAATGCCTGGG-3′) and gacA-R (5′-AAGGCTT-CAAAAGTCTGCTGCC-3′) were designed according to the *L. pneumophila* genome sequence information (http://genome3.cpmc.columbia.edu/~legion/index.html). These primes were used to amplify a 1452 bp DNA fragment containing most of *letA* gene, and part of the *uvrC* gene,

Table 1
Plasmids used in this study

Plasmid	Features	Reference or source
pGS-flaA-03	*L. pneumophila flaA* regulatory region in pGS-lac-01	[43]
pGS-lac-01	pAB-1 with a promoter less *lacZ* gene	[43]
pGS-reg-F3	The *icmF::lacZ* fusion in pGS-lac-01	[43]
pGS-reg-M3	The *icmM::lacZ* fusion in pGS-lac-01	[43]
pLAW344	*sacB* MCS *oriT*(RK2) Cm^r *oriR*(colEI) Ap^r	[55]
pMMB207	RSF1010 derivative, *IncQ* Cm^r *oriT* MCS	[56]
pOG-gacA-1	The *gacA* region in pUC18	This study
pOG-gacA-2	pOG-gacA-1 with the Km cassette in the *gacA* gene	This study
pOG-gacA-3	Insert of pOG-gacA-sin pLAW344	This study
pOG-J-122	The *icmJ::lacZ* fusion in pGS-lac-01	[43]
pOG-Mgac	Insert of pOG-Ugac in pMMB207	This study
pOG-P-121	The *icmP::lacZ* fusion in pGS-lac-01	[43]
pOG-Q-126	The *icmQ::lacZ* fusion in pGS-lac-01	[43]
pOG-R-125	The *icmR::lacZ* fusion in pGS-lac-01	[43]
pOG-T-120	The *icmT::lacZ* fusion in pGS-lac-01	[43]
pOG-Ugac	The complete *gacA* gene in pUC18	This study
pOG-V-123	The *icmV::lacZ* fusion in pGS-lac-01	[43]
pOG-W-124	The *icmW::lacZ* fusion in pGS-lac-01	[43]
pUC18	*oriR* (colEI) MCS Ap^r	[57]

located immediately downstream to it. This fragment was cloned into pUC18 digested with *Hin*cII to generate pOG-gacA-1. To knockout the *letA* gene, the kanamycin resistance cassette (Pharmacia) was cloned into the *letA* gene, instead of an internal 53 bp *Bsr*GI-*Cla* I fragment, after fill-in, to generate pOG-gacA-2. The plasmid pOG-gacA-2 was digested with *Pvu* II and the insert was cloned into the *Eco* RV site of the allelic exchange vector pLAW344, to generate pOG-gacA-3. This plasmid was used for allelic exchange, as was previously described [21]. Several isolates were analyzed by Southern-blot to confirm that the right change occurred on the chromosome (data not shown).

To construct a *letA* complementing clone the primers gacA-Up (5′-TAAAGGGCTTAGAGCATACGG-3′) and gacA-Down (5′-GGATAGGATTTGTCATCACGC -3′) were used. These primers were used to amplify a 1410 bp DNA fragment that was cloned into pUC18 digested with *Hin*cII to generate pOG-Ugac. The plasmid pOG-Ugac was digested with *Hin*dIII and *Eco* RI and the insert was cloned into pMMB207 digested with the same enzymes to form pOG-Mgac. This plasmid was used for the complementation experiment.

4.3. Intracellular growth in A. castellanii

Intracellular growth assays were performed in a similar way to what was previously described [12]. 1.5×10^5 amoebae in PYG were added to wells of a 24-well microtiter dish and the amoebae were incubated for 1 h at 37 °C, to let the amoebae adhere. Then the PYG was aspirated, and the wells were washed once with 0.5 ml of warm (37 °C) Ac-buffer, and 0.5 ml of warm Ac-buffer was added to the wells. Then, *L. pneumophila*, in Ac-buffer, were added to the wells at a multiplicity of infection (MOI) of approximately 0.1. The plate was incubated for 30 min at 37 °C, then the Ac-buffer was aspirated, and the wells were washed three times with 0.5 ml of warm Ac-buffer, and 0.6 ml of warm Ac-buffer was added to the wells. The supernatant of each well was sampled (50 μl) at intervals of about 24 h and colony forming unit (CFU) were determined by plating on ACES buffered charcoal yeast extract (ABCYE) plates.

4.4. Intracellular growth in HL-60 derived human macrophages

Intracellular growth assays were performed in a similar way to what was previously described [12]. Wells of a 24-well microtiter dish containing 6×10^6 differentiated HL-60 derived macrophages were used for infection. *L. pneumophila* were added to the wells at a MOI of approximately 0.1 and the infected HL-60 derived macrophages were incubated for 1 h at 37 °C under CO_2 (5%). Then the wells were washed three times, and 0.6 ml of RPMI containing 2 mM Glutamin, and 10% normal human serum, was added to the wells. The supernatant of each well was sampled (50 μl) at intervals of about 24 h and CFU were determined by plating on ABCYE plates.

4.5. β-galactosidase assays

β-galactosidase assays were performed as described elsewhere [58]. *L. pneumophila* strains were grown on ABCYE plates containing chloramphenicol for 48 h. The bacteria were scraped of the plate, suspended in AYE broth and bacteria optical density at 600 nm (OD_{600}) was calibrated to 0.1 in AYE. The resulting cultures were grown on a roller drum for 17–18 h until reaching an OD_{600}

of about 3.2 (stationary phase) and 50 μl from these cultures were taken for the assay. To test the levels of expression at exponential phase, the cultures were diluted to an OD_{600} of 0.1 and grown for additional 6–7 h until reaching an OD_{600} of about 0.7 (exponential phase). From these cultures 50 or 100 μl were taken for the assay. The substrate for β-galactosidase hydrolysis was *o*-nitrophenyl-β-D-galactopyranoside.

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