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The *Legionella pneumophila* global regulatory protein LetA affects DotA and Mip

Chunwei Shi, Vera Forsbach-Birk, Reinhard Marre, Tamara L. McNealy*

Department of Medical Microbiology and Hygiene, University of Ulm, Albert-Einstein Allee 11, D-89081Ulm, Germany

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Abstract

Several genes have been identified in *Legionella pneumophila* which are necessary for its virulence properties. These genes include the *dot/icm* type IV secretion system (T4SS), *mip* and *letA*. Genes of the *dot/icm* system, in particular *dotA*, have been found to be essential for intracellular growth. The macrophage infectivity protein (Mip) is also necessary for full virulence of the bacteria. Although these genes are well characterized, the regulation of such virulence factors is not. The LetA transcriptional activator interacts with the global regulator CsrA in controlling the switch from the replicative, non-infectious to the transmissive, highly infectious form of *L. pneumophila*. Regulation by LetA of the *dot/icm* genes has also been previously postulated. Here we show that the *letA* mutation exerts effects not only on DotA but on a substrate of the secretion system, RalF as well. LetA was found to be necessary for full transcriptional expression of the *dotA* and *ralF* genes. Although at the transcriptional level *dotA* was reduced, this did not result in a decrease of DotA protein in whole cell lysates. The *letA* mutation, however, does result in decreased amounts of the DotA protein found in the membrane and increased amounts in the culture supernatant. Additionally, the *letA* mutation dramatically decreased the secretion of Mip. This work demonstrates the participation of the global regulatory protein LetA in the regulation of an essential part of the *dot/icm* T4SS. Also shown is the presence of secreted Mip and a decrease in this secretion in the *letA*(−) strain. Exactly how LetA is regulating these virulence factors remains to be elucidated but it obviously occurs at both transcriptional and post-transcriptional levels.
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Keywords: *Legionella pneumophila*; *LetA*; *DotA*; *Mip*; Regulation

Introduction

Many pathogenic bacteria adapt to the various local environmental conditions they encounter by coordinate regulation of gene expression and modulation of phenotype. *Legionellae* have a distinct biphasic life cycle alternating between the 'replicative phase' and the 'transmissive phase.' Replicative-phase bacteria are sodium resistant, sensitive to environmental stress, such as acidic pH, and display reduced cytotoxicity (Hammer et al., 2002). Transmissive-phase bacteria then are more resistant to biocides and antibiotics, more invasive and virulent to host cells as well as competent to avoid phagosome–lysosome fusion upon the next infection cycle (Barker et al., 1992; Hammer and Swanson, 1999). The ability to perform this phenotypic switch contributes to the adaptation of the bacteria to the harsh

*Corresponding author. Albany Medical College, Center for Immunology and Microbial Disease, 47 New Scotland Ave, MC-151, Albany, NY 12208, USA.
E-mail address: mcnealt@mail.amc.edu (T.L. McNealy).

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environmental situations and to the transmission of the pathogen into a new host cell. In *Legionella pneumophila*, the causative agent of Legionnaires' disease, the global regulatory genes *csrA*, *letA* and *rpoS* are responsible for maintenance and induction of either the replicative-focused genes or the genes necessary for invasion and virulence.

The global regulator CsrA has been shown to repress the expression of virulence traits such as flagella (Fettes et al., 2001; Forsbach–Birk et al., 2004) during the replicative phase. CsrA is also essential for bacterial growth within macrophages (Molofsky and Swanson, 2003). In late replication phase, facing amino acid depletion, *Legionella* begin a stringent response-like mechanism involving ppGpp, the *letA/letS/letE* genes as well as RpoS and FliA (Hammer and Swanson, 1999). The sigma factors RpoS and FliA induce the transcription of genes of the transmission regulon, while the two-component regulator LetA/LetS and the *letE* locus cooperate to overcome post-transcriptional repression by CsrA. LetA-RpoS regulation of both exponential- and stationary-phase gene expression has been shown in several studies (Bachman and Swanson, 2004; McNealy et al., 2005) (Broich et al., submitted). All together these regulators form a network with the goal of allowing *L. pneumophila* an exquisite adaptation ability ensuring its survival, replication and virulence for new host cells (Bachman and Swanson, 2001, 2004; Fettes et al., 2001; Forsbach-Birk et al., 2004; Hammer and Swanson, 1999; Heuner et al., 1997; Lynch et al., 2003; Molofsky and Swanson, 2003; Zusman et al., 2002).

Virulence of *Legionella* is due in part to secreted proteins. To date, four major secretion systems have been described in *Legionella* pathogenesis: the LSS type I, Lsp type II and Dot/Icm and Lvh type IV secretion systems (T4SS) (Jacobi and Heuner, 2003; Rossier and Cianciotto, 2001; Segal et al., 1999.) The Dot/Icm T4SS exports virulence factors which are essential in the inhibition of phagosome–lysosome fusion. Dot/Icm genes have also been shown to be necessary for induction of apoptosis and host cell killing (Berger et al., 1994; Molmeret et al., 2004; Nagai et al., 2002; Roy et al., 1998; Segal et al., 1998; Vogel and Isberg, 1999). DotA, one of the essential genes of the Dot/Icm secretion system, is a structural component of the Dot/Icm secretion system as well as a substrate of the system (Nagai and Roy, 2001). DotA along with an additional substrate, RalF have been shown to be involved in regulation of phagosome biogenesis and transport (Nagai and Roy, 2001; Roy et al., 1998).

Legionellae also possess various other secreted as well as cell-associated virulence factors. Secreted factors such as the *proA* protease, phospholipase A, and lysophospholipase A have been shown to be associated with the pathogenesis of the bacteria (Flieger et al., 2000, 2001, 2002). Most of these secreted activities are through the Lsp type II system as the *lsp* mutant demonstrates loss of these activities as well as decreased virulence (Aragon et al., 2002; Banerji et al., 2005). It has also been recently shown that *letA* and *rpoS* play a role in the regulation of some but not all of these activities (Broich et al., submitted). Cell-associated factors such as the macrophage infectivity protein (Mip) have also been shown to be necessary for full virulence of *L. pneumophila*. Mip has been shown to contribute to infection of both protozoan and macrophage cells (Cianciotto and Fields, 1992). Recently, regulation of *mip* was examined and found to be repressed in early stages after infection (Wieland et al., 2002). This study and others support the concept that expression of Mip is important for adherence and invasion but not for intracellular replication.

Regulation of various proteins of the Dot/Icm system has been examined through reporter gene fusion analysis demonstrating some regulation due to the transcriptional regulator LetA (Gal-Mor and Segal, 2003). Previously we have reported that a *L. pneumophila letA* insertion mutant exhibited reduced transcript expression of *dotA* as compared to wild-type cells (Lynch et al., 2003). In contrast to the dotA mutants which no longer are capable of macrophage intracellular replication, the *letA* mutants of the JR32 strain are affected in replication only in amoebae species (Lynch et al., 2003). We were interested in examining in more detail the influence of *letA* on the function of the Dot/Icm secretion system, in particular on DotA, as data on regulation of DotA and other secreted and cell-associated factors are still scarce. During this line of experimentation we also examined the expression of Mip in the *letA(−)* background. LetA was found to be necessary for full transcriptional expression of the *dotA* and *ralF* genes and affected the normal distribution of DotA and secretion of Mip.

Materials and methods

Bacterial strains and growth conditions

L. pneumophila JR32 is a serogroup 1 isolate, *letA*:kana and *letA* pMMBletA were derived from the JR32 wild-type strain and have been described previously (Lynch et al., 2003). All *Legionella* were cultured on buffered charcoal yeast extract (BCYE) agar plates or were grown in buffered yeast extract (BYE) broth supplemented with α-ketoglutarate, L-cysteine and ferric pyrophosphate (Edelstein, 1981) at 37 °C. All experiments described in this report were performed with the same batch of *Legionella* frozen at −70 °C. *Escherichia coli* DH5α cultures were grown in Luria-Bertani (LB)

medium with or without agar. When required, antibiotics were added as follows: kanamycin (Km), 25 μg/ml (*L. pneumophila*) and 50 μg/ml (*E. coli*); chloramphenicol (Cm), 7.5 μg/ml (*L. pneumophila*) and 20 μg/ml (*E. coli*).

RNA isolation and analysis

For slot blot and Northern analysis total RNA was isolated from wild-type, mutant and complemented *L. pneumophila* JR32 grown in BYE broth at 37 °C using Trizol reagent (Life Technologies). Samples were collected either at early exponential ($OD_{578\,nm} = 1.2$; ~12 h), mid-exponential phase ($OD_{578} = 1.5$; ~16 h), post exponential phase ($OD_{578} = 1.8$; ~20 h) or late stationary ($OD_{578} = 2.0$; 24 h). The isolated RNA was used immediately or stored at −70 °C before use. For Northern analysis, 20 μg RNA for *dotA* and 40 μg RNA for *ralF* analysis was size fractionated by electrophoresis through a 1.2% agarose gel containing formaldehyde (Sambrook et al., 1989). RNA was transferred overnight to a nylon membrane and hybridization was conducted overnight with PCR-generated probes specific for *dotA* (DotA_F: 5′-GTGGCCAAATCTGCATGTTG; DotA_R: 5′-AGACGTGGTTGTTGAAAACG) or *ralF* (RalF_F: 5′-CAATGCGGATGCTGCCTATC; RalF_R: 5′-TGATTTTGGTTGATCTACAG) labeled with digoxigenin (DIG)-dUTP (Roche). Slot blots were performed with samples of 10 μg RNA for *dotA* transcript detection according to the manufacturer's instructions. RNA was transferred to a nylon membrane using vacuum transfer and hybridization was carried out overnight with PCR-generated probes labeled with digoxigenin (DIG)-dUTP. Dig-labeled probes were visualized using the DIG Luminescent Detection Kit (Roche). Northern and slot blot experiments were repeated at least three times with independent RNA isolates.

Reporter gene fusion and luciferase assay

The *ralF* promotor region was amplified from *L. pneumophila* JR32 DNA using primers RalF Luc F (5′-CGCGGATCCCAGTTAAATGCCTCACCAC) and RalF Luc R (5′-CCCAAGCTTCTGAGGAACCGCTGGATTCC) and cloned into pBCluc2 generating the plasmid pBCluc2-RalF which was then electroporated into *L. pneumophila* JR32 and *letA*(−). Transformants were selected on BCYE plates containing chloramphenicol. In order to quantify luciferase activity, *L. pneumophila* strains harboring the *ralF* promoter-*luc* fusion were inoculated at an $OD_{578\,nm}$ of 0.1 in BYE medium. The culture was then grown in an orbital shaker at 37 °C. Samples were taken at 2-h intervals and the $OD_{578\,nm}$ was measured. A 150-μl aliquot was held on ice until measured for luciferase activity. The reservoirs of the "Flash n Glow" luminometer (Berthold) were filled with 2.5 × assay buffer (62.5 mM glycyl-glycine, pH 7.8, 25 mM $MgCl_2$, 12.5 mM ATP, 2.4 mM acetyl-CoA, 0.025% H_2O_2) and 330 mM D-luciferin, freshly dissolved in water. After placing the sample into the luminometer, 200 μl of 2.5 × assay buffer followed by 200 μl of the luciferin solution were automatically added. Luminescence was immediately measured for 15 s at 22 °C. Adjustments for pumping of liquids and washing of tubing were according to manufacturer's instruction. The luciferase activity of an *L. pneumophila* strain containing the pBCluc2 plasmid, which was just above background levels, was subtracted from each measurement resulting in luciferase activity expressed as relative light units (RLUs).

Protein analysis

Whole cell extracts were prepared as described (Forsbach-Birk et al., 2004). Briefly, bacterial cell pellets were resuspended in 250 μl lysis buffer (50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 1% NP-40 and 0.5% Na-deoxycholate) and sonicated for 10 s. Lysates were then shaken for 2 h at 4 °C and centrifuged for 10 min at 7600*g*. The protein-containing supernatant was removed and protein concentration was estimated using a commercial kit (Biorad). Different bacterial samples were disrupted and lysates were fractionated into soluble proteins and membrane-associated proteins as described previously (Roy and Isberg, 1997). For analysis of supernatant proteins, culture supernatants were collected at the above stated time points and precipitated with 10% trichloroacetic acid. SDS-PAGE was performed on all fractions as described previously (Laemmli, 1970). Western blots for identification of DotA (antibody provided by C. Roy, Yale University, New Haven, CT), Mip (antibody provided by Klaus Heuner, University of Wuerzburg, Wuerzburg, Germany) and CsrA (antibody provided by Tony Romeo, Emory University Atlanta, GA) were carried out as described elsewhere (Towbin et al., 1979).

Results

LetA regulation of the expression of *dotA* and *ralF* genes

The *L. pneumophila letA*(−) mutant was found to be defective for efficient infection in macrophages (Hammer et al., 2002) and intracellular replication within *Acanthamoebae castellanii*. Additionally, LetA has been shown to exhibit some regulation of various components of the Dot/Icm T4SS (Gal-Mor and Segal, 2003; Lynch et al., 2003). Lynch et al. (2003) demonstrated a reduced

transcriptional expression of *dotA* in the *letA*(−) mutant. We therefore wanted to examine more closely, through protein analysis as well as Northern blotting at various time points in the growth curve, the effect of LetA on the expression of DotA as well as on an additional substrate of the T4SS, RalF.

In slot blot analysis of *dotA*, clear differences were apparent in *dotA* transcription levels between the *letA*(−) mutant and the wild-type strain JR32, at both exponential and stationary phase (Fig. 1a). The amount of transcription in JR32 at $OD_{578\,nm}$ 1.8 (post exponential phase) was notably higher than that in the *letA*(−) mutant. Further confirmation was obtained by Northern blot analysis. In this analysis, RNA samples of *letA/C*, in which the *letA* mutation was complemented with plasmid pMMB207 *letA*, were also analysed. The reduction of *dotA* transcript seen in *letA*(−) was returned to wild-type levels with the introduction of the *letA* gene in trans (Fig. 1b). The transcript size of the signal seen on the Northern blot was ~1 kb which is smaller than predicted from the sequence and predicted polycistronic transcription of *icmV-dotA*. Therefore, probes were designed for the beginning of the *icmV* gene, an overlapping set from the end of *icmV* to the beginning of *dotA* and the end of the *dotA* gene. All probes gave a signal at the same location on the blot (data not shown), confirming first that the entire transcript is present at this location and second that *icmV* and *dotA* are indeed on one transcript.

In order to determine the transcription dynamics of the *ralF* gene, we first examined *ralF* expression with luciferase reporter gene fusions. In the wild-type background, *ralF* is transcribed beginning at the transition between exponential and stationary phase with expression dropping after entry into stationary phase. This result matches the hypothesis that proteins injected into host cells by the Dot/Icm apparatus are upregulated as exponentially growing bacteria enter stationary phase (Byrne and Swanson, 1998). In *letA*(−), *ralF* transcription was also found to be induced at this transition to stationary phase. However, the maximum level of *ralF* transcription in *letA*(−) was found to be reduced by approximately 80% (Fig. 2a). Confirmation of the differential expression of *ralF* in *letA*(−) was obtained by Northern blot analysis. Clear differences in expression level were seen between wild type and *letA*(−). A reversion to normal expression levels could be obtained for *letA*(−) by expressing LetA in trans (*letA*/C) (Fig. 2b). Using multiple methods, it was shown that the *letA* defect leads to reduced transcription of *dotA* and *ralF*, the protein products of which are substrates of the Dot/Icm secretion system.

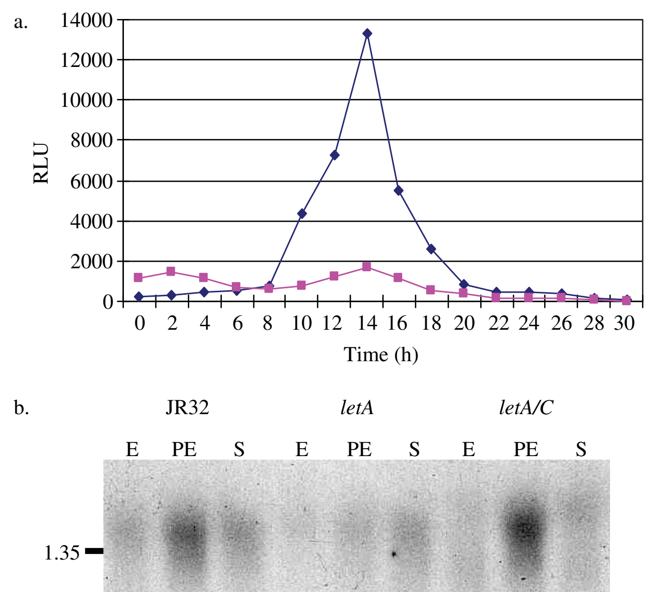

Fig. 2. Expression of the Dot/Icm substrate RalF is reduced in the *letA*(−) background: (a) Luciferase reporter gene expression of the *ralF* promoter in JR32 (◆) and *letA*(−) (■) shows post-exponential-phase expression of *ralF*. (b) Northern blot of RNA from JR32, *letA*(−) and *letA/C* showing post-exponential-phase expression of *ralF*, which is reduced in the *letA*(−) background. Both experiments were repeated three times using independent RNA samples. $E = OD_{578}1.5$, $PE = OD_{578}1.8$ and $S = OD_{578}2.0$.

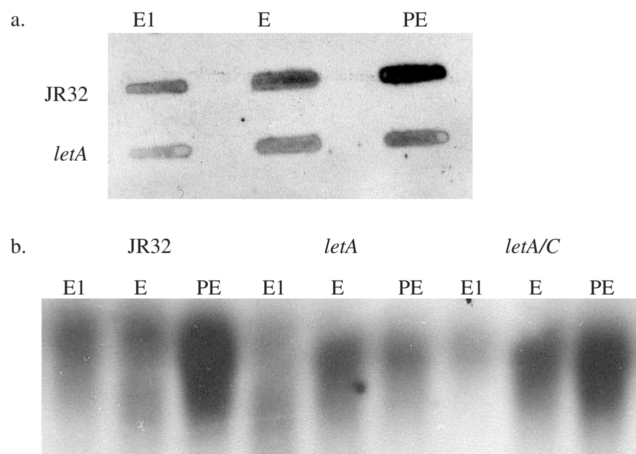

Fig. 1. Expression of *dotA* in the *letA*(−) background is reduced in comparison to wild-type levels during all time points of the growth curve. RNA from JR32 wild type, *letA*(−) and *letA/C* (*letA*(−) complemented in trans) at OD_{578} of 1.2 (E1, early exponential), 1.5 (E, exponential) and 1.8 (PE, post exponential) was hybridized with a dotA-specific probe: (a) slot blot; (b) northern blot. Both experiments were repeated three times using independent RNA samples.

Altered protein expression of DotA and Mip in a *letA(−)* background

DotA is an integral component of the Dot/Icm T4SS. Not only is the protein a structural component but it

is also secreted through the system. Because of the multiple roles this protein appears to play in *Legionella* virulence we examined total soluble protein levels, membrane protein levels and secreted proteins levels in order to determine whether all were proportionately affected by the transcriptional reduction associated with the *letA* mutation.

Cellular levels of DotA protein were determined for wild-type *L. pneumophila* JR32, *letA*(−) and *letA/C* by immunoblot analysis. *L. pneumophila* cell lysates were probed for DotA protein using mouse monoclonal antibodies specific for DotA. In comparison to the wild type and *letA/C*, an increase in the cellular concentration of DotA protein at $OD_{578\,nm}$ 1.8 and 2.0 was observed in *letA*(−) (Fig. 3a). In the whole cell extracts two bands were observed, the first being a 120-kDa band which is the size that has been previously reported. The second band appears as a slightly larger, growth phase-dependent band. An apparent shift between the two bands can be also correlating advancement from exponential to stationary phase. It is interesting to note that this shift appears to occur earlier in the *letA*(−) strain than in the wild-type or complemented strains. The appearance of two reactive bands has not been reported in previous work with this antibody. However,

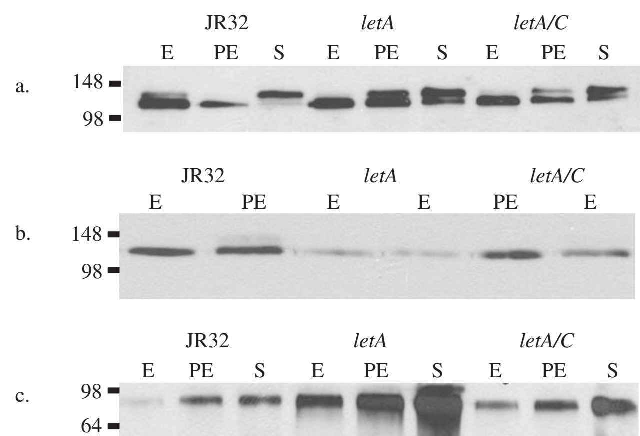

Fig. 3. Western blot analysis of DotA protein in cell lysates, membrane fraction and supernatant of JR32, *letA*(−) and *letA/C*: (a) Cell lysates show an increase in DotA accumulation in the *letA*(−) mutant. Additionally, a shift in size of the protein is seen in PE cultures which appears to occur earlier in the *letA*(−) mutant. (b) Decreased presence of DotA in the membrane fraction of the *letA*(−) mutant. Membrane-associated Dot A levels are restored in the mutant complemented with the *letA* gene in trans. Stationary-phase cultures demonstrated the same pattern as seen in *E* and PE cultures (not shown). (c) Supernatant protein precipitates show an increase in DotA in *letA*(−) cultures. The increase is most dramatic in stationary-phase culture supernatants. $E = OD_{578}1.5$, $PE = OD_{578}1.8$ and $S = OD_{578}2.0$. The experiment was repeated three times using independent protein samples.

the earlier work was completed using the Lp02 *Legionella* wild type which may account for differences. The meaning of this double band and apparent shift remains to be explained.

It has been shown that an overabundance of DotA protein is present in most of the *dot/icm* mutants; however, there is no significant increase in the *dotA* transcription level compared with wild-type *L. pneumophila* (Nagai and Roy, 2001) and it was concluded that DotA secretion is dependent upon a functional Dot/Icm apparatus. We originally hypothesized that the reduction at the transcriptional level and the intracellular accumulation of DotA in *letA*(−) mutant could indicate that LetA affects functions of one or more components of the Dot/Icm system, which then affect the transport of DotA protein.

The data above led us to question whether the *letA* defect affects DotA integration into the bacterial inner membrane and/or its secretion, which could explain why the *letA*(−) bacteria contain more cellular DotA protein. We first examined the amount of DotA protein in membrane fractions by immunoblot. It was found that the amount of membrane-associated DotA protein was reduced in the *letA*(−) mutant, compared to wild type and could be fully restored with complementation in *letA/C* (Fig. 3b). Therefore, LetA affects the ability of the DotA protein to efficiently integrate into or to remain in the inner membrane. With this information, it was interesting to next ask if this membrane defect reduced the secretion of DotA.

It has been reported that the DotA protein is abundantly secreted into the culture supernatant during growth of *L. pneumophila* (Nagai and Roy, 2001). Before secretion, the intact DotA protein is predicted to be 120 kDa as seen in the soluble protein samples in Fig. 3a. However, after export, the C-terminal DotA epitope is processed by the MspA protein (zinc metalloprotease) and the remaining DotA molecule is about 80 kDa. In analysis of culture supernatants using a protein secretion assay to determine the amount of the secreted DotA protein, it was found that the culture supernatant of *letA*(−) actually contained higher levels of DotA, at 80 kDa, than wild type and *letA/C* (Fig. 3c), contrary to our expectations.

In order to ensure the reliability of our secretion assay protocol we chose to analyze two additional proteins as controls. The Mip, is a 24-kDa protein and is required for maximum virulence of the bacteria. It has previously been reported that Mip is exposed on the cell surface of *L. pneumophila*. To date there have been no reports of secretion of Mip. However, during our investigation we found that Mip was also secreted or shedded into the supernatant. Interestingly, the amount of supernatant Mip is dramatically reduced in the culture supernatant of *letA*(−) compared to the wild type and fully restored

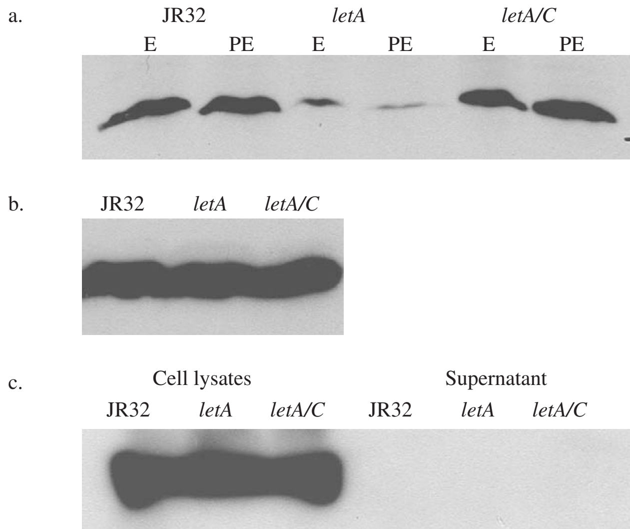

Fig. 4. Western blot analysis of protein expression in JR32, *letA*(−) and *letA/C*: (a) Mip is found in culture supernatants of JR32 wild type and *letA/C* but drastically reduced in *letA*(−). (b) Amounts of membrane-associated Mip are unchanged in *letA*(−) PE-phase cultures. (c) In order to verify that there is no contamination of supernatant precipitates with cellular proteins, cell lysates and supernatants from PE cultures were probed with an antibody against CsrA. CsrA is found in the cytosol in significant amounts at all growth phases. No signal was seen in culture supernatant precipitates. $E = OD_{578}\,1.5$, $PE = OD_{578}\,1.8$. The experiment was repeated three times using independent protein samples.

in *letA/C* (Fig. 4a). However, in membrane fractions no apparent differences between wild type, *letA* and *letA/C* were observed (Fig. 4b). Therefore, it appears that the *letA* defect results in reduction of, most likely, the secretion of Mip but not its localization to the cell membrane. These data however, necessitated an additional protein as a control for the protein secretion assay.

As a control to ensure that we were not recovering soluble cellular proteins in the secreted protein precipitates, we blotted for the presence of the global regulatory protein CsrA. CsrA is a small cytosolic protein (7 kDa) present in the cell at all growth phases and acts as a global repressor of *Legionella* transmissive phenotypes. We performed Western blot analysis with the CsrA antibody with supernatant protein samples and whole cell extract samples at $OD_{578\,nm}$ of 1.8 in parallel. Corresponding signals at 7 kDa were obtained in all whole cell extract samples, while no signals were found in the supernatant protein samples (Fig. 4c) Therefore, the results from the secretion assay for DotA and Mip can be said to represent truly secreted proteins without contamination of intracellular cytosolic soluble proteins.

Discussion

The *gacA* locus is highly conserved among Gram-negative bacteria and functions as a global regulator of stationary-phase gene expression (reviewed by Heeb and Haas (2001)). This conservation allowed for the identification of the *L. pneumophila gacA* homologue *letA* (Hammer et al., 2002; Lynch et al., 2003). In *L. pneumophila*, LetA functions as an activator of the transmission phase genes. The induction of these virulence traits correlates with the ability of *L. pneumophila* to infect and replicate within eukaryotic host cells. Homologues of LetA in various Gram-negative bacteria such as *Salmonella enterica* (Johnston et al., 1996), *Vibrio cholerae* (Wong et al., 1998) and *Pseudomonas aeruginosa* (Rahme et al., 1995), are involved in regulation of virulence. These homologues control production of various secreted factors such as in the pathogenic *P. aeruginosa* strain PAO1 the secretion of pyocyanin, cyanide and lipase (Reimmann et al., 1997). Secreted lipase and protease activities have also been shown to be regulated by LetA homologues.

Formation of the replicative vacuole is essential for intracellular growth of *Legionella* and is attributed to proteins secreted into the host (Chen et al., 2004; Conover et al., 2003; Luo and Isberg, 2004; Nagai et al., 2002) by a T4SS encoded by the *dot/icm* loci (Andrews et al., 1998; Berger et al., 1994; Marra and Shuman, 1992; Purcell and Shuman, 1998; Segal and Shuman, 1997; Segal et al., 1998; Vogel et al., 1998). Null mutants in *dot/icm* genes are defective in entry and intracellular multiplication in macrophage and amoeba hosts, lead to a delay of the phagosome–lysosome fusion and affect the induction of host cell apoptosis and killing (Berger and Isberg, 1993; Hilbi et al., 2001; Segal et al., 1998; Zink et al., 2002). However, regulation of the Dot/Icm T4SS is still unclear. As the structures and functions of more Dot/Icm proteins are clarified and an increasing number of substrates of this T4SS identified, there is also a need to understand the regulatory mechanism of the system. Recently, 12 regulator sites were identified in the upstream region of *dot/icm* genes and five of them are expected to serve as binding sites for regulatory factors (Gal-Mor et al., 2002). Based on LetA properties, acting as a global regulator in *Legionella* infection and intracellular growth, we therefore investigated if LetA may be involved in the regulation of the Dot/Icm T4SS.

Analysis of *dotA* expression showed that, under the growth conditions tested, the transcription of this gene was reduced in the *letA* mutant compared with wild type. Because loss of LetA directly or via other regulatory genes such as *csrA* reduces *dotA* transcription but does not completely abolish it, the *dotA* gene is probably activated or repressed through multiple genes in addition to *letA*. In order to determine whether the

entire *icmV-dotA* mRNA transcript was affected or only *dotA* or *icmV*, Northern blots were performed to determine the transcript size. Transcription was examined using probes specific for *icmV*, *dotA* or the region spanning the end of *icmV* and the start of *dotA*. The hybridization signals obtained with these three probes (*dotA*, *icmV*, *icmV-dotA*) were found at identical positions (data not shown). Therefore, the LetA defect leads to the reduced expression of the *icmV-dotA* transcriptional unit. However, the detailed mechanism of regulation, whether it is direct or indirect, needs further research.

Expression of the *ralF* gene was also found to be reduced in the *letA*(−) mutant. This gene encodes a protein with a 200-amino-acid region homologous to the catalytic domain found in all eukaryotic proteins that function as guanine nucleotide exchange factors (GEFs) and activates ADP-ribosylation factor (ARF) by stimulating the exchange of GDP for GTP. This *Legionella* ARF-GEF, RalF, was found to be required for the recruitment of ARF to the *Legionella* phagosome for aiding the biogenesis of *Legionella* 'replicative organelles' (reviewed by Roy (2002)). Analysis of *ralF* expression using a luciferase reporter gene fusion and Northern blot showed that, under the growth conditions tested, the transcription of this gene in wild type is induced at the transition from the exponential stage of growth to the stationary phase. The time point of expression matches the genetic studies which show that expression of the Dot/Icm complex was required very early after contact with macrophages and dispensable following the successful evasion of fusion with endocytic compartments (Roy et al., 1998).

In the *letA* mutant, expression of RalF at the transition time point was reduced in comparison to the wild type. Although it has been shown that the expression level of RalF protein in the wild type and in the *dotA* mutant is unchanged (Nagai et al., 2002), we cannot exclude the possibility that in the *letA* mutant a disfunctional Dot/Icm complex due to the reduced expression of *dotA* represses the export of RalF protein and then through a negative feedback mechanism reduces the transcription of *ralF*. It may also be that defects in other components of the Dot/Icm system due to the loss of *letA* lead to the reduced *ralF* expression. The expression kinetics of the *ralF* gene in *dot*/*icm* mutants has not been determined. Naturally, there is the possibility that LetA acts directly on *ralF*. Furthermore, because of limited experimental conditions, we have carried out the luciferase assay to examine *ralF* expression dynamics in broth culture, but not in eukaryotic host cells with an ARF background. Since RalF functions as a regulator of host ARF, it remains to be determined if our result reflects *ralF* transcription in vivo.

The observation that DotA is both integrated into the inner membrane and secreted implies that the role for this protein is intricate. In Western blot analysis we found higher levels of cellular DotA in the *letA(−)* mutant than in the wild type, which does not fit with the results of LetA transcriptional regulation seen in the RNA blotting analysis. However, this phenomenon corresponds with the observation that DotA secretion from *L. pneumophila* is a specific Dot/Icm-dependent export process, and the disruption of Dot/Icm transporter function results in cellular accumulation of the DotA protein (Nagai and Roy, 2001). Furthermore, analyzing immunoblots of whole cell extracts, two bands close to each other, which both react with the anti-DotA antibody, were observed, and it seems that an apparently growth phase-associated and possibly strain-specific band shift occurs. However, the meaning of the double band and band shift remains to be explained. One possibility is that the DotA protein is complexing tightly with some of the smaller components of the Dot/Icm system.

An immunoblot analysis of membrane-associated proteins indicated that DotA protein integration into the inner membrane was severely reduced in the *letA*(−) mutant and this may be a partial reason for the intracellular accumulation of DotA protein. There are at least two possible explanations for the decrease in DotA found in the membrane fractions: (1) the protein is not integrated into the membrane, perhaps due to abnormal complex formation with other components, or (2) it is integrated into the membrane complex but the membrane is prone to increased vesicle release and DotA protein is lost to the supernatant of the cultures. It appears that the loss of DotA in the membrane fractions of *letA*(−) correlates with the increase seen in the supernatants (Figs. 3b and c). Various Gram-negative bacteria including *Legionella* have been shown to release membrane vesicles (Beveridge, 1999; Flesher et al., 1979). Additionally, *Legionella* membrane vesicle production and composition appears to correlate with growth phase and virulence (M. Swanson, personal communication). Results from supernatant analysis for presence of DotA revealed increased amounts of DotA in the *letA*(−) strain. However, the size of this protein corresponds to the smaller version of DotA said to be partially degraded by the active protease of *Legionella* (Nagai and Roy, 2001). Dependent on where and how the protease is acting upon DotA, either one of the two above mentioned hypotheses could still be supported by the results. Although Nagai and Roy (2001) have shown that vesicle production is not involved in DotA secretion, vesicles produced by instability of the *letA*(−) membrane may be containing an increased amount of the secretion apparatus, contributing to an increased presence of DotA in the supernatant. Whether this DotA is then vulnerable to the protease, therefore

accounting for the size of the protein found in the *letA*(−) supernatants remains to be determined.

It may also be that release or exportation of DotA via a homologous T4SS occurs. In addition to the Dot/Icm T4SS, another T4SS, *lvh*, has been identified and found to be able to substitute for some components and functions of the Dot/Icm system (Segal et al., 1999). It has not yet been determined if in the *lvh* system a homolog to DotA exists or if the system is capable of secreting the known substrates of the Dot/Icm system. It has been suggested that experiments using the LpO2 *Legionella* strain, which has lost the lvh system, could help in testing this hypothesis. However, the *letA* isogenic mutant of this strain differs in its phenotype for regulation of secreted and associated virulence factors such as phospholipase A, lysophospholipase A and acyltransferase (Broich et al., submitted). In light of these differences for the role of LetA, it is questionable if these experiments would confirm our hypothesis. Recently, an additional *dot* gene, *dotU*, which is directly upstream of *icmF*, was characterized. *DotU* and *icmF* were found to be functionally important for the stability of the Dot/Icm complex. These two genes encode proteins, which prevent degradation of some Dot/Icm proteins and therefore stabilize the Dot/Icm complex. It has been shown that instability of the Dot/Icm T4SS correlated with virulence as *Legionella* enter into stationary phase (Sexton et al., 2004). Therefore, *dotU* and *icmF* are possible targets for examination of LetA regulation.

Interestingly, in analysis of control proteins, Mip was found to be dramatically reduced in *letA*(−) supernatant but present in normal amounts in the membrane. To date, there has been no report of Mip being a secreted virulence factor but it is known to form homodimers in solution (Schmidt et al., 1994). It is not known through which system Mip is secreted but apparently this secretion is positively regulated by LetA. This evidence of LetA regulation of secreted virulence factors also fits with the recent report of LetA and RpoS regulation of secreted phospholipase A, lysophospholipase A, protease, and phosphatase (Broich et al., submitted). Although these activities are dependent on the lsp type II pathway, other type II-dependent activities were not affected, indicating that the LetA regulation does not work directly on the type II machinery. Recently, a Tat secretion pathway has been reported and characterized in *L. pneumophila* (De Buck et al., 2005; Rossier and Cianciotto, 2005). This pathway was shown to play a role in the formation of a respiratory complex, growth under low-iron conditions, the secretion of a phospholipase C activity, and intracellular infection. Additionally, substrates were identified which were either type II dependent or independent. Whether LetA has any effect on regulation of substrates secreted via this pathway remains to be determined.

Although LetA is an important global regulator of *Legionella* transmissive phenotypes, up to now not much is known about its target genes. However, it is becoming obvious that *Legionella letA*, like its homologs in other bacteria, plays an important role in regulating secreted virulence factors. We have investigated the regulatory role of LetA on DotA as well as on Mip. LetA exerts positive regulation on the transcription of *dotA* and *ralF*. Additionally, it affects the membrane integration and/or stability and possibly the secretion of DotA. Mip, which we have shown to be a secreted as well as a cell-associated factor, is also regulated by LetA. Exactly how LetA is regulating these virulence factors remains to be elucidated but it obviously occurs at both transcriptional and post-transcriptional levels. Both the *dot/icm* system and Mip are recognized virulence factors of *L. pneumophila*, and clarification of the regulation of these factors will add to our understanding of the unique adaptation ability of this bacterial species.

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