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# The Global Regulatory Proteins LetA and RpoS Control Phospholipase A, Lysophospholipase A, Acyltransferase, and Other Hydrolytic Activities of *Legionella pneumophila* JR32

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***Legionella pneumophila*** **possesses a variety of secreted and cell-associated hydrolytic activities that could be involved in pathogenesis. The activities include phospholipase A, lysophospholipase A, glycerophospholipid: cholesterol acyltransferase, lipase, protease, phosphatase, RNase, and *p*-nitrophenylphosphorylcholine (p-NPPC) hydrolase. Up to now, there have been no data available on the regulation of the enzymes in *L. pneumophila* and no data at all concerning the regulation of bacterial phospholipases A. Therefore, we used *L. pneumophila* mutants in the genes coding for the global regulatory proteins RpoS and LetA to investigate the dependency of hydrolytic activities on a global regulatory network proposed to control important virulence traits in *L. pneumophila*. Our results show that both *L. pneumophila rpoS* and *letA* mutants exhibit on the one hand a dramatic reduction of secreted phospholipase A and glycerophospholipid: cholesterol acyltransferase activities, while on the other hand secreted lysophospholipase A and lipase activities were significantly increased during late logarithmic growth phase. The cell-associated phospholipase A, lysophospholipase A, and p-NPPC hydrolase activities, as well as the secreted protease, phosphatase, and p-NPPC hydrolase activities were significantly decreased in both of the mutant strains. Only cell-associated phosphatase activity was slightly increased. In contrast, RNase activity was not affected. The expression of *plaC*, coding for a secreted acyltransferase, phospholipase A, and lysophospholipase A, was found to be regulated by LetA and RpoS. In conclusion, our results show that RpoS and LetA affect phospholipase A, lysophospholipase A, acyltransferase, and other hydrolytic activities of *L. pneumophila* in a similar way, thereby corroborating the existence of the LetA/RpoS regulation cascade.**

*Legionella pneumophila* is an intracellular bacterial pathogen which infects protozoa, such as amoebae, present in fresh water sources. When inhaled by susceptible humans, the bacteria infect and multiply in human lung macrophages and cause the potentially fatal pneumonia Legionnaires' disease (20). When nutrients become rare after replication in a modified phagosome (1), *L. pneumophila* exits the spent host cell by disruption of the eukaryotic phagosomal and cell membranes and infects a new host (49, 67). Accordingly, the life cycle of *L. pneumophila* can be differentiated into two phases where the bacterium needs to adapt to specific conditions: intracellular replication within the host cell and host cell exit and transmission to a new host (50). Furthermore, it was shown that *L. pneumophila* develops a mature infectious form that is different from in vitro grown stationary phase cells with regard to infectivity and resistance to antibiotics (30).

Adaptation between replicative and transmissive phases in *L. pneumophila* is strictly regulated on the genetic level. Virulence properties such as motility (8, 14, 36, 37), cytotoxicity (2, 14, 33), and resistance to stress such as nutrient limitation (6, 8, 32, 44) that are needed for host cell exit, invasion of a new host, and establishment of the replicative vacuole, are only expressed in the transmissive form. Conversely, those factors are repressed in the replicative form (see reference 50 and references therein). However, some *L. pneumophila* strains have been shown to be infectious even in the mid-logarithmic growth phase (61).

Various regulators have been identified to control the phenotypic changes within the life cycle of *L. pneumophila*, including the two-component system LetA/S (29, 33, 44) and the alternative sigma factor RpoS (6, 32). For example in enteric bacteria, such as *Escherichia coli*, RpoS is central to the regulation of stationary-phase growth and stress response (for a recent review see reference 35). In pathogenic *Vibrio* species, RpoS is involved in the regulation of stress response and additionally in the expression of secreted virulence factors in both the stationary and exponential growth phases (17, 39, 53, 65). *Borrelia burgdorferi* RpoS also plays a role in the regulation of virulence traits, for example the outer surface proteins OspA and OspC (15, 38), and is expressed during mid-logarithmic growth phase (15). The findings of *rpoS* expression before stationary phase in *Vibrio* spp. and *B. burgdorferi* are in contrast to the function of RpoS as a master regulator of adaptation to stationary phase and of global environmental stress response in *E. coli* and related bacteria (35).

Likewise, *L. pneumophila* RpoS seems not to be the central regulator of stationary phase growth, as a knockout mutation in *rpoS* does not affect resistance to oxidative, osmotic, and acidic stresses (6, 32). On the other hand, RpoS was shown to be necessary for intracellular infection and replication of the bacterium in amoebae and expression of virulence traits

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present in the transmissive growth phase (6, 8, 32, 33). The infectivity and replication behavior of an *L. pneumophila* JR32 *rpoS* mutant in HL60-derived macrophages remained unchanged; however strain Lp02 (12) needs *rpoS* for efficient replication in bone marrow-derived macrophage cells (6). Furthermore, it was described that *rpoS* mRNA is predominantly present in the exponential growth phase (8, 44), while the RpoS protein is abundant during the postexponential growth phase (32).

The phosphorelay response regulator GacA (*g*lobal *ac*tivator), a protein homologous to LetA of *L. pneumophila*, is involved in the regulation of virulence genes in various gram-negative bacteria, including *Pseudomonas* and *Vibrio* species (reviewed in reference 34). In the opportunistic lung pathogen *Pseudomonas aeruginosa*, GacA regulates quorum sensing and various secreted virulence factors (57). Furthermore, GacA influences the accumulation of RpoS in *Pseudomonas fluorescens* (68) and is involved in the regulation of exoenzymes in the phytopathogen *Erwinia carotovora* (16).

In *L. pneumophila*, LetA is another protagonist of the regulation cascade. It forms a two-component signaling system with the sensor kinase LetS and the transcription enhancer LetE (7, 8, 29, 33, 42, 44). LetA furthermore affects the expression of different genes belonging to the *icm*/*dot* virulence region (29, 44, 63). This genetic region is important for *L. pneumophila* intracellular replication (12, 46) and cytotoxicity (64). In *L. pneumophila* JR32, LetA was shown to regulate the transcription of *rpoS* and to be essential for efficient infection of amoebae (44). However, *letA* mutants still multiply efficiently in macrophage host cells (29, 33). Furthermore, the virulence traits of the transmissive phase, such as motility, cytotoxicity, and resistance to stresses, are dependent on the LetA/S system (33, 44, 51). Combined, these results show that a mutation in *letA* produces a phenotype that is arrested in the exponential growth phase, preventing the expression of cytotoxicity and motility.

Since secreted lipase and protease activities in other bacteria are regulated by GacA (LetA) and RpoS, and since secreted hydrolytic activities of *L. pneumophila* are expressed at peak levels during late logarithmic growth phase and entry into stationary phase (24), it is possible that the *L. pneumophila* enzymes are regulated in a similar manner. *L. pneumophila* possesses a range of secreted and cell-associated hydrolytic activities: phospholipase A (PLA), lysophospholipase A (LPLA), glycerophospholipid: cholesterol acyltransferase (GCAT), lipase/esterase, protease, phosphatase, *p*-nitrophenylphosphorylcholine (p-NPPC) hydrolase, and RNase. Several proteins have been characterized for *L. pneumophila* that are associated with these activities and are listed in Table 1.

With respect to transmission phase cytotoxicity, host cell membrane degrading and affecting activities such as PLA, acyltransferase, and protease may be upregulated when the bacterium prepares itself for cell exit. Furthermore, phospholipases have been shown to be important virulence factors of other bacteria, such as *P. aeruginosa* (11, 21, 52, 54, 62) and *Listeria monocytogenes* (43, 47). It is also interesting that *L. pneumophila* type II secretion system, *rpoS*, and *letA* mutants show similar phenotypes with respect to the defect in bacterial infection of amoebae, which implies furthermore a regulation effect on secreted activities. Due to the fact that there are no data available on the regulation of bacterial phospholipase A or acyltransferase activities and *L. pneumophila* hydrolytic activities, we elucidated the impact of LetA and RpoS on the regulation of *L. pneumophila* hydrolytic enzymes. Here we provide data that LetA and RpoS are indeed important regulators of phospholipases A and other hydrolytic activities of *L. pneumophila*.

TABLE 1. Proteins corresponding to hydrolytic activities of *Legionella pneumophila*

| Activity | Localization | Type II secreted | Corresponding enzyme (reference) |
|---|---|---|---|
| Phospholipase A | Secreted | Yes | Major activity unidentified; PlaC (10) |
| | Cell associated | | PlaB (27) |
| Lysophospholipase A | Secreted | Yes | PlaA (26), PlaC (10) |
| | Cell associated | | PlaB (27) |
| Acyltransferase | Secreted | Yes | PlaC (10) |
| Lipase | Secreted | Yes | LipA, LipB (5), PlaA (26), PlaC (10) |
| Protease | Secreted | Yes | ProA (31) |
| Phosphatase | Secreted | Yes | MapA (3) |
| | Cell associated | | Pho (41) |
| p-NPPC hydrolase | Secreted | Yes | PlcA (5), another unidentified activity |
| RNase | Secreted | Yes | Unidentified |

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *L. pneumophila* sg1 JR32 (60) (kindly provided by H. Shuman, Columbia University, New York) served as a wild-type control. Isogenic *L. pneumophila* JR32 *rpoS* mutants (32) (kindly provided by H. Shuman) and JR32 *letA* mutants (44) contained a gentamicin or kanamycin resistance cassette insertion, respectively, in their chromosomal genes. For complementation studies of the JR32 *rpoS* mutant, vector pLM845 (32) (kindly provided by H. Shuman), containing the complete *rpoS* gene, was electroporated into the mutant strain. For complementation studies of the *L. pneumophila* JR32 *letA* mutant, strain K44, containing the complete *letA* in *trans*, was used (44).

For RNase activity studies, *L. pneumophila* strain 130b (American Type Culture Collection strain BAA-74, also known as Wadsworth or AA100) and its direct derivative NU258, containing an insertion mutation in the *Legionella lspDE* genes (58) (kindly provided by N. Cianciotto, Northwestern University Medical School, Chicago), were used as a control.

*L. pneumophila* was routinely grown on buffered charcoal yeast extract (BCYE) agar for 2 to 3 days at 37°C (19). For detection of RNase activity, a modified growth agar was used which was designated buffered starch yeast extract (BSYE) and in which charcoal was replaced by the same amount of starch (VWR, Germany) and 1% (wt/vol) RNA type VI from *Torula* yeast (Sigma-Aldrich, Taufkirchen, Germany) was added. For extracellular growth in laboratory growth medium, *L. pneumophila* was cultured in buffered yeast extract (BYE) broth at 37°C with shaking at 300 to 320 rpm. Bacterial growth was checked by determining the optical density of the culture at 660 nm ($OD_{660}$, spectrophotometer DU520, Beckman Coulter, Unterschleissheim, Germany). Cultures were inoculated with bacteria grown on BCYE agar plates for 2 days at an $OD_{660}$ of 0.2 to 0.3. When needed, media were supplemented with antibiotics at final concentrations as follows: kanamycin, 25 μg/ml; chloramphenicol, 6 μg/ml; and gentamicin, 5 μg/ml.

**Preparation of culture supernatants and cell lysates.** *L. pneumophila* culture supernatants for assessment of hydrolytic activities were obtained at mid-logarithmic growth phase ($OD_{660}$ of 1.0) and late logarithmic growth phase ($OD_{660}$ of 2.0) by centrifugation for 5 min at 5,000 × *g*. Cell lysates were produced by adding lysozyme and Triton X-100 as described previously (10, 26) and were resuspended in the original culture volume in 40 mM Tris-HCl, pH 7.5 (25°C).

Cell lysates were diluted for the enzymatic assay of lipolytic activities at 1:5 and 1:20 with 40 mM Tris-HCl, pH 7.5 (25°C), for the mid-logarithmic growth phase and late logarithmic growth phase, respectively, and at 1:2 for both the phosphatase activity assay and the p-NPPC hydrolase activity assay. Cell culture supernatants and cell lysates were stored at 4°C overnight before being used in the activity assays.

**Enzymatic assay for lipolytic activities.** Enzymatic activities were detected as described previously (26, 27). In short, different lipids were incubated with the same volume of bacterial culture supernatant or cell lysate in a mixture containing 6.7 mM substrate [1,2-dipalmitoylphosphatidylcholine (DPPC), 1,2-dipalmitoylphosphatidylglycerol (DPPG), 1-monopalmitoyllysophoshatidylcholine (MPLPC), 1-monopalmitoyllysophosphatidylglycerol (MPLPG), and 1-monopalmitoylglycerol (1-MPG)], 3 mM $NaN_3$, 0.5% (vol/vol) Triton X-100, and 40 mM Tris-HCl pH 7.5 (25°C). Incubation with bacterial cell culture supernatants or cell lysates was performed at 37°C with continuous shaking at 170 rpm for 5 h. Free fatty acids as a marker of lipolytic activity were determined by means of a Nefa-C-kit (WAKO Chemicals, Neuss, Germany) according to the instructions of the manufacturer. BYE broth or 40 mM Tris-HCl, pH 7.5 (25°C), was incubated, treated like the cultures, and subsequently used as a negative control.

For measuring the glycerophospholipid:cholesterol acyltransferase (GCAT) activity of cell culture supernatants, 50 μl cholesterol in ethanol (10 mg/ml) was added to 1 ml of DPPG mixture prior to sonication (26). All lipids, including standards for thin-layer chromatography (TLC), were obtained from Sigma-Aldrich (Taufkirchen, Germany) or Avanti Polar Lipids Inc. (Alabaster, Ala.). Prior to incubation, the lipid substrates were vortexed for 15 min at 37°C and then exposed to ultrasonication using a probe (Sonoplus, Bandelin, Berlin, Germany) three times for 15 s each at cycle 4 and 10% and power set to 65% (27). After the incubation period, the samples were processed by lipid extraction and analyzed by thin-layer chromatography.

**Lipid extraction and thin-layer chromatography.** For the detection of distinct apolar lipids, including cholesterol esters, reaction mixtures of lipids and cholesterol with culture supernatant and BYE broth as a negative control were subjected to a lipid extraction (13). The chloroform phase was used for separation of lipids by thin-layer chromatography. For detection of cholesterol esters, silica gel plates (Merck, Darmstadt, Germany) were developed in tanks containing solvent 1, petroleum ether–diethylether–glacial acetic acid in a ratio of 90:10:1 (vol/vol/vol), For the separation of the uncharacterized cholesterol-independent substance, we used solvent 2, *n*-hexane–diethylether–glacial acetic acid in a ratio of 70:30:4 (vol/vol/vol) (45). Finally, silica gel plates were stained with 0.2% naphthol blue black (Sigma-Aldrich, Taufkirchen, Germany) (26, 55).

**Assay for detection of p-nitrophenylphosphorylcholine hydrolase activity.** For the determination of p-NPPC hydrolase, 100 μl *L. pneumophila* culture supernatant or lysate (diluted at 1:2 with 40 mM Tris-HCl, pH 7.5, 25°C) were incubated with the same volume of substrate mixture containing 10 mM p-NPPC (Sigma-Aldrich, Taufkirchen, Germany), 6 mM $NaN_3$, 1% (vol/vol) Triton X-100, and 10 mM $MnCl_2$ in 40 mM Tris-HCl (pH 7.5, 25°C) at 37°C and with continuous agitation at 100 to 170 rpm for 20 to 50 h. Subsequently the release of *p*-nitrophenol (p-NP) was measured optically at a wavelength of 405 nm and compared to p-NP (Sigma-Aldrich, Taufkirchen, Germany) standard solutions (9, 22). BYE broth and 40 mM Tris-HCl, pH 7.5 (25°C), were used as negative controls. When precipitation occurred, samples were centrifuged at 350 × *g* for 10 min and sample supernatants were used for p-NP readings.

**Assay for the detection of phosphatase activity.** For the determination of phosphatase activity, 50 μl *L. pneumophila* culture supernatant or cell lysate (diluted at 1:2 with 40 mM Tris-HCl (pH 7.5, 25°C) was incubated with the same volume of substrate mixture, containing 10 mM *p*-nitrophenylphosphate (p-NPP) (Sigma-Aldrich, Taufkirchen, Germany), 6 mM $NaN_3$, 1% (vol/vol) Triton X-100 in 40 mM Tris-HCl at 37°C and with continuous agitation at 170 rpm for 1 h as previously described (24). Subsequently the release of p-NP was measured optically at a wavelength of 405 nm and compared to p-NP (Sigma-Aldrich, Taufkirchen, Germany) standard solutions. BYE broth and 40 mM Tris-HCl, pH 7.5 (25°C), were used as negative controls.

**Assay for detection of protease activity.** For the detection of protease activity, 50 μl *L. pneumophila* culture supernatant was incubated with the double volume of substrate mixture, containing 1.33% (wt/vol) azocasein (Sigma-Aldrich, Taufkirchen, Germany) in 40 mM Tris-HCl, pH 7.5 (25°C) at 37°C and with continuous agitation at 170 rpm for 1 h as previously described (24). After treatment as described by Prestidge (56), samples were measured at a wavelength of 420 nm and compared to a reaction product of a protease standard solution (*Bacillus polymyxa* protease, Sigma-Aldrich, Taufkirchen, Germany; concentrations used in experiment: 0.1, 0.25, and 0.5 U/mg). BYE broth was used as a negative control.

TABLE 2. Primer pairs used for RT-PCR

| Primer | Sequence 5′–3′ | Product length (bp) |
|---|---|---|
| plaC_s2_f | TGGATTGAGTATTTGGCAGAA | 462 |
| plaC_s2_r | CACGTCACGATCAGTAGTTT | |
| RTgyr_a1f | CACATATGGCCGGCTTTAGAG | 470 |
| RTgyr_b1r | TCGCGCTTGTTTTGCTGAG | |

**Assay for detection of RNase activity.** For the detection of RNase activity, *L. pneumophila* strains were grown on BCYE agar plates for 2 to 3 days. Bacterial cells were resuspended in phosphate-buffered saline (137 mM NaCl; 2.7 mM KCl; 4.3 mM $Na_2HPO_4$; 1.5 mM $KH_2PO_4$) and adjusted to an $OD_{660}$ of 0.3; 15 μl of this suspension was spotted on an RNA-containing BSYE agar plate and bacteria were grown for 1 to 2 days at 37°C. After the bacterial film was visible, approximately 15 ml of 10% (wt/vol) trichloroacetic acid (4°C) was applied to the agar plate. After 3 to 5 min, clear zones around the bacteria indicated RNA hydrolysis (28).

**Investigation of *plaC* expression.** For preparation of mRNA, obtained from *L. pneumophila* JR32 wild-type, *letA* mutant, *rpoS* mutant, and complemented strains, bacteria were grown in BYE broth to an $OD_{660}$ of 2.0 and diluted to an $OD_{660}$ of 0.3 with BYE broth in order to adjust comparable bacterial numbers. An aliquot of 700 μl was mixed with 1,300 μl RNAprotect bacteria reagent (QIAGEN, Germany) and incubated for 5 min at room temperature. Then bacteria were pelleted by centrifugation for 10 min at 5,000 × g and pellets were stored at −80°C until RNA isolation. RNA was isolated using the RNeasy minikit (QIAGEN, Germany) according to the manufacturer's instructions.

Possible DNA contamination was removed with RQ1 RNase-Free DNase (Promega, Germany). Briefly, RNA (0,5 to 2 μg) was mixed with 1 μl 10× DNase buffer, 1 μl RQ1 DNase, and 1 μl RNaseOUT RNase inhibitor (Invitrogen, Germany) and incubated overnight at 37°C. Subsequently, 1 μl DNase STOP buffer was added to the mixture and incubated for 15 min at 65°C to inactivate DNase. reverse transcription (RT) PCR was performed by means of the QIAGEN OneStep RT-PCR kit. Control reactions to check for DNA contamination and for the presence of RNA were performed with specific primers which amplified a part of *gyr*, the gene coding for the constitutively expressed gyrase subunit B of *L. pneumophila*. Gene-specific primer pairs are based on the sequence found in the *L. pneumophila* Philadelphia-1 genome project (http://genome3.cpmc.columbia.edu/~legion) and are listed in Table 2.

## RESULTS

**Lipolytic activities of *L. pneumophila* JR32 *letA* and *rpoS* mutants.** *L. pneumophila* possesses several secreted and cell-associated lipolytic activities: PLA, LPLA, GCAT, and lipase activity (4, 5, 10, 22, 24, 26, 27) (Table 1). In order to examine the role of the global regulator proteins RpoS and LetA with regard to the lipolytic activities, we tested late logarithmic ($OD_{660}$ of 2.0) and mid-logarithmic ($OD_{660}$ of 1.0) culture supernatants and cell lysates of the *L. pneumophila* JR32 wild-type strain and its isogenic *rpoS* and *letA* mutants with different substrates for lipolytic activities. Specifically, for detection of PLA activities, bacterial samples were incubated with diacylphospholipids (DPPG and DPPC), for LPLA activities with lysophospholipids (MPLPG and MPLPC), and for lipase activities with a nonphospholipid (1-MPG).

Late-logarithmic-phase culture supernatants of *letA* and *rpoS* mutants showed significantly reduced PLA activities in comparison to the wild-type strain (16 and 7% for DPPG, respectively, and 37 and 30% for DPPC, respectively, compared to 100% for JR32) (Fig. 1A). In contrast to the findings for PLA, the LPLA activity of *letA* and *rpoS* mutants was significantly higher in late-logarithmic-phase culture supernatants compared to the wild-type strain (167 and 156% for MPLPG, respectively; 175 and 178% for MPLPC, respectively,

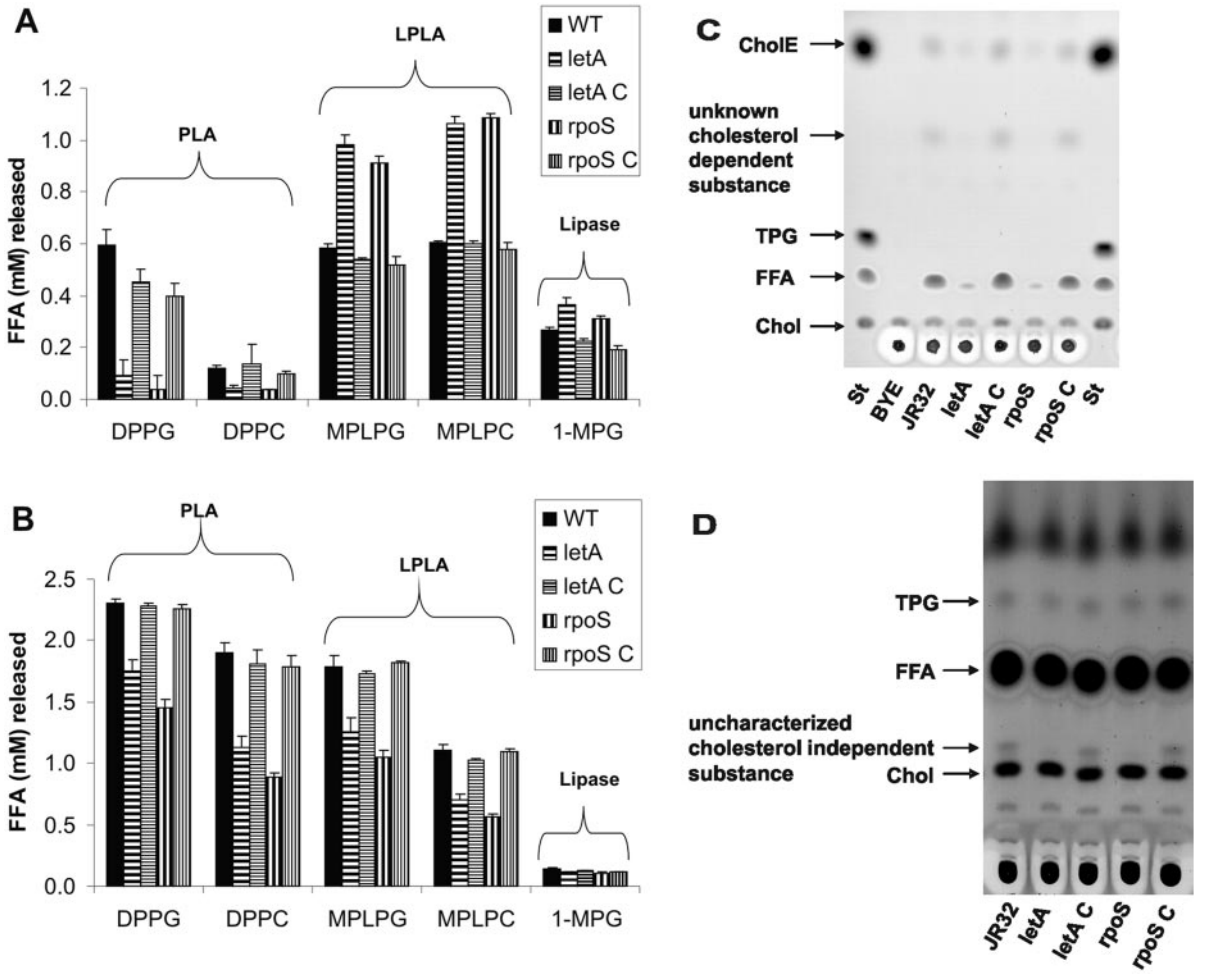


FIG. 1. Lipolytic activities of *L. pneumophila* JR32 wild-type and Δ*letA* and Δ*rpoS* mutant, complemented Δ*letA*, and complemented Δ*rpoS* strains. Late-logarithmic (A) culture supernatants as well as late-logarithmic (B) cell lysates were incubated with DPPC, DPPG, MPLPG, MPLPC, and 1-MPG for 5 h at 37°C. The release of free fatty acids was quantified. Data are expressed as differences between the amount of free fatty acids released by culture samples and the amount released by BYE broth for supernatant samples and 40 mM Tris-HCl (pH 7.5, 25°C) for cell lysates. The results represent the means and standard deviations of duplicate cultures and four reactions and are representative of three independent experiments. For all substrates, Δ*letA* and Δ*rpoS* mutants were significantly different from the wild type ($P < 0.01$; Student's *t* test, $n = 4$), except for 1-MPG hydrolysis by cell lysates. (C) TLC analysis of GCAT activity of *L. pneumophila* JR32 wild-type, Δ*letA* and Δ*rpoS* mutant, and complemented Δ*letA* and Δ*rpoS* culture supernatants. Culture supernatants were incubated with a mixture of DPPG and cholesterol for 23 h at 37°C, and then lipids were extracted and applied to TLC. A mixture of BYE and the lipids was also incubated and served as a negative control (BYE). An apolar solvent mixture (solvent 1) was employed for the separation of the apolar lipids, in particular for cholesterol ester. For the qualitative identification of the lipid spots, lanes containing lipid standards are marked St. The results are representative of three independent experiments. (D) TLC analysis of an uncharacterized cholesterol-independent reaction product appearing in the acyltransferase assay with *L. pneumophila* JR32 wild-type, Δ*letA* and Δ*rpoS* mutant, and complemented Δ*letA* and Δ*rpoS* cell lysates. Cell lysates of the strains were incubated with a mixture of DPPG and cholesterol for 23 h at 37°C, and then lipids were extracted and applied to TLC. An apolar solvent mixture (solvent 2) was employed for the separation of the apolar lipids. The results are representative of three independent experiments. Abbreviations: CholE, cholesterolester; TPG, tripalmitoylglycerol; FFA, free fatty acids; Chol, cholesterol; JR32, *L. pneumophila* JR32; letA, *L. pneumophila* JR32 Δ*letA*; rpoS, *L. pneumophila* JR32 Δ*rpoS*; letA C, complemented *L. pneumophila* JR32 Δ*letA*; rpoS C, complemented *L. pneumophila* JR32 Δ*rpoS*.

compared to 100% for JR32) (Fig. 1A). Lipase activity was also increased to 137 and 117% in the *letA* and the *rpoS* mutant, respectively (Fig. 1A). The *rpoS* and *letA* mutant lipolytic phenotypes could be complemented by introduction of the wild-type genes into the mutant strains (Fig. 1A).

The dramatic reduction of secreted PLA activity, as observed in the *rpoS* and *letA* mutants, is caused by either the direct or indirect influence of the regulator proteins LetA and RpoS on transcription of genes coding for PLAs or on post-transcriptional events controlling PLA activities. The enzyme that is responsible for the major secreted PLA activity in *L. pneumophila* could not be identified until now. Nevertheless, there is one candidate gene known to contribute to the secreted PLA activity, as recently Banerji et al. characterized the secreted *L. pneumophila* enzyme PlaC, which possesses PLA, LPLA, GCAT, and lipase activities (10).

The secreted LPLA activity of *L. pneumophila* in late-logarithmic-phase cultures was shown to depend >90% on the major secreted LPLA PlaA (26). It is likely that the expression or the activity of this enzyme is regulated negatively by LetA

and RpoS in a direct or indirect manner. Furthermore, the slight increase of lipase activity could be due to the influence of the regulator proteins on *plaA* and *plaC* transcription, because the PlaA and PlaC enzymes additionally show lipase activity towards 1-MPG (10, 26).

Next, we tested whether *L. pneumophila* cell-associated lipolytic activities depend on RpoS and LetA. Late-logarithmic-phase cell lysates of *L. pneumophila letA* and *rpoS* mutant strains both showed a significant reduction of PLA and of LPLA activities in comparison to the wild-type strain (76 and 63% for DPPG, respectively; 60 and 47% for DPPC, respectively; 70 and 59% for MPLPG, respectively; 64 and 51% for MPLPC, respectively, compared to 100% for JR32) (Fig. 1B). The reductions were restored by complementation of the mutant strains with the wild-type genes.

Since the cell-associated PLA and LPLA activities of *L. pneumophila* are mainly dependent on the PLA/LPLA PlaB (27), we conclude that LetA and RpoS are involved in the regulation of this enzyme in a positive way. Cell-associated lipase activity is not affected by RpoS and LetA (Fig. 1B). This suggests that the existence of a cell-associated lipase distinct from PlaB is possible for *L. pneumophila*.

Samples of *L. pneumophila letA* and *rpoS* mutants harvested in the mid-logarithmic growth phase ($OD_{660}$ of 1.0) showed a similar reduction trend in secreted PLA and both cell-associated PLA and LPLA activities, but to a smaller extent (data not shown).

*L. pneumophila* possesses a major secreted GCAT activity that is coded by *plaC* (10, 26). This activity is predominantly expressed in the late logarithmic growth phase, but only at a low level in the mid-logarithmic growth phase (Banerji et al., unpublished data). In order to elucidate whether mutations in *letA* or *rpoS* affect GCAT activity, culture supernatants of *L. pneumophila* wild-type and mutant strains were incubated with DPPG and cholesterol and analysis of the GCAT reaction product cholesterol ester was performed by TLC. Indeed, for both of the mutant strains a quantitatively reduced spot of cholesterol ester appeared on the TLC plate in comparison to the wild-type strain JR32, showing that GCAT activity is positively regulated by LetA and RpoS (Fig. 1C). An as yet uncharacterized cholesterol-dependent product of this reaction (10) also disappeared in *letA* and *rpoS* reaction samples (Fig. 1C).

*L. pneumophila* cell lysates did not exhibit a detectable GCAT activity. However, another uncharacterized spot appeared in the wild-type strain, but not in the *rpoS* and *letA* mutant strains (Fig. 1D). In contrast to the first uncharacterized cholesterol-dependent product, this spot was not dependent on cholesterol in the acyltransferase reaction assay (data not shown). The generation of the cholesterol-independent product was found to be positively regulated by LetA and RpoS.

The reduction in GCAT activity, the disappearance of the uncharacterized cholesterol-dependent, and the cholesterol-independent substances could be restored by complementation of the mutant strains with the wild-type genes (Fig. 1C and D). As a result, LetA and RpoS are involved in the positive regulation of the secreted GCAT activity of *L. pneumophila* which is dependent on PlaC (10) and of two yet unknown activities generating two uncharacterized reaction products.

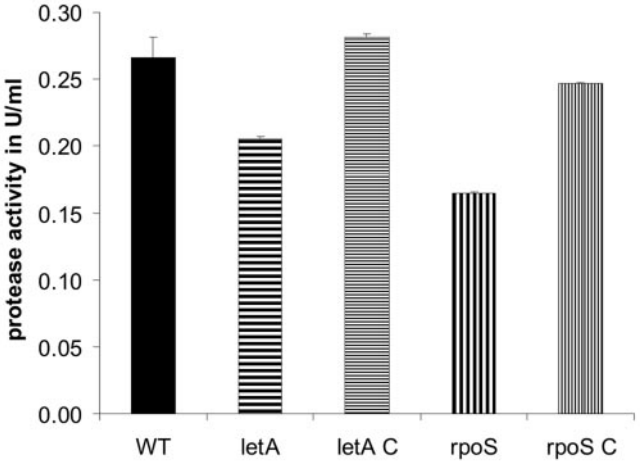


FIG. 2. Protease activities of late-logarithmic-phase culture supernatants of *L. pneumophila* JR32 wild-type, Δ*letA* and Δ*rpoS* mutant strains, and complemented Δ*letA* and Δ*rpoS* strains. Late-logarithmic-phase culture supernatants were incubated with azocasein for 1 h at 37°C and azocasein hydrolysis was evaluated. BYE was treated like culture supernatant samples and served as a negative control. Data are expressed as differences between $OD_{420}$ values of culture samples and the $OD_{420}$ value of BYE broth. The results represent the means and standard deviations of duplicate cultures and are representative of three independent experiments. Δ*letA* and Δ*rpoS* mutants were significantly different from the wild type ($P < 0.01$; Student's *t* test, $n = 4$). Abbreviations: JR32, *L. pneumophila* JR32; letA, *L. pneumophila* JR32 Δ*letA*; rpoS, *L. pneumophila* JR32 Δ*rpoS*; letA C, complemented *L. pneumophila* JR32 Δ*letA*; rpoS C, complemented *L. pneumophila* JR32 Δ*rpoS*.

Taken together, LetA and RpoS play a role in the regulation of lipolytic activities especially in the late logarithmic growth phase of *L. pneumophila*. Secreted PLA and GCAT activities, as well as cell-associated PLA and LPLA activities were regulated in a positive manner, whereas secreted LPLA and lipase activities were negatively regulated (Fig. 1A, B, and C).

**Protease activity of *L. pneumophila* JR32 *letA* and *rpoS* mutant strains.** It was shown that *L. pneumophila* possesses a secreted protease, ProA, which is cytotoxic for macrophages and contributes to virulence of *L. pneumophila* in a guinea pig model of infection, but is not essential for bacterial multiplication in host cells (18, 31, 40, 48). Banerji et al. showed that ProA is necessary for activation of *L. pneumophila* PlaC, the secreted enzyme responsible for GCAT, PLA, LPLA, and lipase activities (10). We examined whether mutations in *letA* or *rpoS* affect *L. pneumophila* protease activity by incubating late-logarithmic-phase culture supernatants of *L. pneumophila* wild-type and mutant strains with azocasein as a protease substrate. Protease activity in *letA* and *rpoS* mutants was reduced to 62 and 57%, respectively, in comparison to 100% activity in *L. pneumophila* JR32. This reduction could be restored by complementation of the mutant strains (Fig. 2). There was no detectable protease activity present in *L. pneumophila* cell lysate samples (data not shown). With regard to our data, LetA and RpoS play a role in the positive regulation of the secreted protease activity of *L. pneumophila*, ProA.

**Phosphatase activity of *L. pneumophila* JR32 *letA* and *rpoS* mutants.** *L. pneumophila* possesses two secreted phosphatase activities, the major one of which is coded by the *map* gene (3),

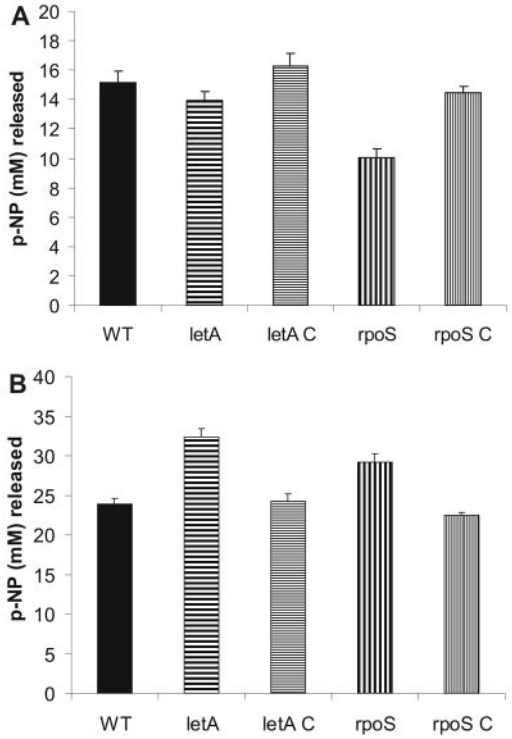


FIG. 3. Phosphatase activities of late-logarithmic-phase culture samples of *L. pneumophila* JR32 wild-type, Δ*letA* and Δ*rpoS* mutant strains, and complemented Δ*letA* and Δ*rpoS* strains. Late-logarithmic (A) culture supernatants, as well as late-logarithmic (B) cell lysates were incubated with p-NPP for 1 h at 37°C and hydrolysis of p-NPP was evaluated. BYE and 40 mM Tris-HCl (pH 7.5, 25°C) were treated in the same way as the bacterial samples and served as negative controls for culture supernatants and cell lysates, respectively. Data are expressed as differences between the amount of p-NP released by culture samples and the amount released by BYE broth for supernatant samples and 40 mM Tris-HCl (pH 7.5, 25°C) for cell pellet lysates. These results represent the means and standard deviations of duplicate cultures and are representative of three independent experiments. Δ*letA* and Δ*rpoS* mutants were significantly different from the wild type in all experiments ($P < 0.01$; Student's *t* test, $n = 4$) except the Δ*letA* culture supernatant. Abbreviations: JR32, *L. pneumophila* JR32; letA, *L. pneumophila* JR32 Δ*letA*; rpoS, *L. pneumophila* JR32 Δ*rpoS*; letA C, complemented *L. pneumophila* JR32 Δ*letA*; rpoS C, complemented *L. pneumophila* JR32 Δ*rpoS*.

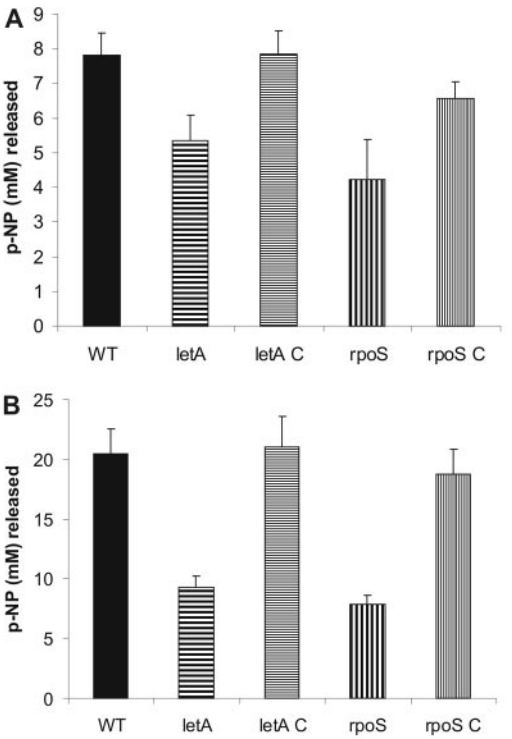


FIG. 4. p-NPPC hydrolase activities of late-logarithmic-phase culture samples of *L. pneumophila* JR32 wild-type, Δ*letA* and Δ*rpoS* mutant strains, and complemented Δ*letA* and Δ*rpoS* strains. Late-logarithmic (A) culture supernatants, as well as late-logarithmic (B) cell lysates were incubated with p-NPPC for 44 h at 37°C and hydrolysis of p-NPPC was evaluated. BYE and 40 mM Tris-HCl (pH 7.5 25°C) were treated in the same way as the bacterial samples and served as negative controls for culture supernatants and cell pellet lysates, respectively. Data are expressed as differences between the amount of p-NP released by culture samples and the amount released by BYE broth for supernatant samples and 40 mM Tris-HCl (pH 7.5 25°C) for cell lysates. The results shown here represent the means and standard deviations of duplicate cultures and are representative of three independent experiments. Δ*letA* and Δ*rpoS* mutants were significantly different from the wild type in all experiments ($P < 0.01$; Student's *t* test, $n = 4$). Abbreviations: JR32, *L. pneumophila* JR32; letA, *L. pneumophila* JR32 Δ*letA*; rpoS, *L. pneumophila* JR32 Δ*rpoS*; letA C, complemented *L. pneumophila* JR32 Δ*letA*; rpoS C, complemented *L. pneumophila* JR32 Δ*rpoS*.

and the cell-associated alkaline phosphatase activity Pho (41). In order to investigate whether LetA and RpoS are involved in the regulation of this activity, late-logarithmic-phase culture supernatants and cell lysates were incubated with p-NPP and phosphatase activity was monitored by release of p-NP. *letA* and *rpoS* mutant culture supernatants showed a minor reduction in phosphatase activity in comparison to the wild-type strain (92 and 66%, respectively, compared to 100% in JR32). The reduction was restored by complementation of the mutant strains (Fig. 3A). On the other hand, *L. pneumophila letA* and *rpoS* mutant late-logarithmic cell lysates had a higher phosphatase activity than the wild-type strain (135 and 122%, respectively, compared to 100% in JR32). This effect was as well restored by complementation (Fig. 3B). LetA and RpoS therefore play a positive role in regulation of secreted phosphatase activity and a negative role in the regulation of cell-associated phosphatase activity.

**p-NPPC hydrolase activity of *L. pneumophila* JR32 *letA* and *rpoS* mutants.** *L. pneumophila* secretes a p-NPPC-hydrolyzing activity into its culture supernatant and the PlcA protein contributes to this activity (5, 9, 22). p-NPPC is an artificial substrate for phosphodiesterases, for example, phospholipases C, and has additionally been shown to be cleaved by phosphatases (22, 23, 66). In *L. pneumophila* it remains to be investigated which biochemical activities (PLC or other phosphodiesterases) hydrolyze p-NPPC. To date, it has not been determined whether *L. pneumophila* possesses a cell-associated p-NPPC hydrolase activity.

For investigation of the regulatory effect of LetA and RpoS on this hydrolytic activity, late-logarithmic-phase culture supernatants and cell lysates were incubated with p-NPPC and hydrolysis

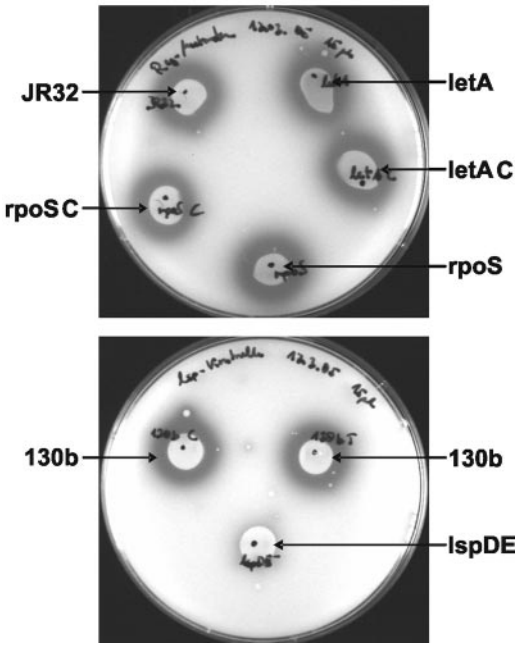


FIG. 5. RNase activities of *L. pneumophila* JR32, Δ*letA* and Δ*rpoS* mutant strains, and complemented Δ*letA* and Δ*rpoS* strains. Bacterial cell suspensions of the strains were applied to RNA-containing BSYE agar plates and grown for 2 days at 37°C. Then, plates were covered with 10% trichloroacetic acid in order to visualize clear zones in which RNA was hydrolyzed. *L. pneumophila* strain 130b and its derivate Δ*lspDE* which is impaired in RNase secretion were used as a negative control by which the effect of the lacking RNase should be demonstrated. The results shown here are representative of three independent experiments. Abbreviations: JR32, *L. pneumophila* JR32; letA, *L. pneumophila* JR32 Δ*letA*; rpoS, *L. pneumophila* JR32 Δ*rpoS*; letA C, complemented *L. pneumophila* JR32 Δ*letA*; rpoS C, complemented *L. pneumophila* JR32 Δ*rpoS*; 130b, *L. pneumophila* 130b; lspDE, *L. pneumophila* 130b Δ*lspDE*.

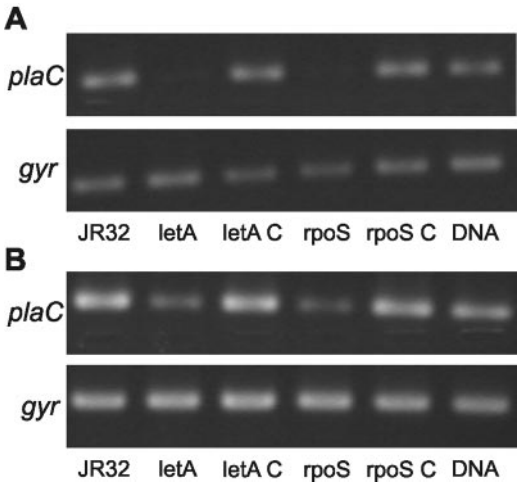


FIG. 6. Expression of *plaC* in *L. pneumophila* JR32, Δ*letA* and Δ*rpoS* mutant strains, and complemented Δ*letA* and Δ*rpoS* strains. RNA was isolated from *L. pneumophila* late logarithmic growth phase cultures and used for quantitative RT-PCR with specific primers for *L. pneumophila plaC*. Specific primers for *L. pneumophila gyr*, the constitutively expressed gene coding for subunit B of gyrase, were used as an internal control. The quantity of RNA applied in the experiment was 0.25 μg. A: 24 PCR cycles; B: 28 PCR cycles. The results shown here are representative of two independent experiments. JR32, *L. pneumophila* JR32; letA, *L. pneumophila* JR32 Δ*letA*; rpoS, *L. pneumophila* JR32 Δ*rpoS*; letA C, complemented *L. pneumophila* JR32 Δ*letA*; rpoS C, complemented *L. pneumophila* JR32 Δ*rpoS*; DNA, JR32 genomic DNA.

was monitored by release of p-NP. In *letA* and *rpoS* mutant strain culture supernatants and cell lysates, the p-NPPC-hydrolyzing activity was reduced significantly compared to the wild-type strain (69 and 54% for supernatants, respectively; 45 and 38% for cell lysates, respectively, compared to 100% in JR32) (Fig. 4A and B). The phenotypes could be restored by complementation (Fig. 4A and B). Therefore, LetA and RpoS positively affect p-NPPC hydrolase activity found in both cell lysate samples and culture supernatant samples.

**RNase activity of *L. pneumophila* JR32 *letA* and *rpoS* mutants.** *L. pneumophila* secretes an RNase activity into its culture supernatant which is dependent on translocation by the Lsp type II secretion system (4, 59). In order to elucidate whether LetA and RpoS possess regulatory functions for this secreted activity, *L. pneumophila* JR32 and *letA* and *rpoS* mutants were grown on BSYE agar plates containing RNA. *L. pneumophila letA* and *rpoS* mutant strains showed a clear zone development around the bacteria, demonstrating no differences in RNase activity compared to JR32 (diameter of bacterial growth and clearing zones for JR32, 19.28 ± 0.85 mm; Δ*letA*, 18.85 ± 2.27 mm; Δ *letA* C, 19.64 ± 0.84 mm; Δ*rpoS*, 19.24 ± 0.28 mm; and Δ*rpoS* C, 19.19 ± 0.94 mm). There was no clear zone visible around a *L. pneumophila* 130b *lspDE* mutant (58) which was used as a negative control for the RNase test in comparison to wild-type *L. pneumophila* 130b (130b, 19.26 ± 0.61 mm; 130b Δ*lspDE*, 11.38 ± 0.17 mm) (Fig. 5). Therefore, *L. pneumophila* RNase activity is an example for a type II secreted activity that is not regulated by LetA and RpoS.

**Gene expression of *plaC* in *L. pneumophila letA* and *rpoS* mutant strains.** In order to investigate whether the changed activity levels of hydrolytic enzymes in *L. pneumophila letA* and *rpoS* mutant strains correspond to modified expression of a candidate gene coding for a lipolytic enzyme, we evaluated mRNA quantities for the *L. pneumophila* PLA, LPLA, and acyltransferase PlaC by RT-PCR. Our data show that *plaC* expression is reduced in *letA* and *rpoS* mutant strains during the late logarithmic growth phase compared to wild-type JR32 and the corresponding complemented strains (Fig. 6). As a standard for constitutive gene expression, the gene coding for subunit B of *L. pneumophila* gyrase was used. Here, no differences in expression levels were observed between the *L. pneumophila* strains (Fig. 6). The RNA used for RT-PCR experiments was proved to be free of DNA by leaving out the reverse transcription step of the PCR (data not shown).

## DISCUSSION

The life cycle of the pathogen *L. pneumophila* can be divided into two phases, the replicative phase and the transmissive phase (50). Similar to the situation in other gram-negative bacteria, components of a global regulation system have been identified in *L. pneumophila* (29, 32, 33, 37, 42, 44). Here, we examined whether two of these proteins, LetA (29, 33, 44) and RpoS (32), influence secreted and cell-associated hydrolytic activities of *L. pneumophila*.

Here we found that the secreted PLA activity was dramatically decreased in *L. pneumophila letA* and *rpoS* mutant strains, similar to the reduction in a type II secretion mutant that is not able to replicate within an amoebae infection model but remains infectious for macrophage cell lines (31, 58). Since this phenotype is also known for the *rpoS* and *letA* mutant strains of *L. pneumophila* JR32 (32, 44), our data point to an important role of PLA activity in infectivity and perhaps also in cytotoxicity of *L. pneumophila*. The protein that represents the major secreted PLA activity however still needs to be identified.

On the other hand, LPLA activity was significantly increased in *L. pneumophila letA* and *rpoS* mutant strains. This increase could be due to a more prominent activity of the major secreted LPLA activity PlaA, but it is also possible that another yet unidentified enzyme that contributes to the secreted LPLA activity is upregulated in the *L. pneumophila letA* and *rpoS* mutants. A further possibility is that a cofactor or an activator that is needed for LPLA activity is upregulated in the mutant strains and leads to increased LPLA activity.

We found that many type II secreted activities were decreased in the *letA* and *rpoS* knockout mutants (PLA, GCAT, protease, phosphatase, and p-NPPC hydrolase) and this leads to the question of whether the Lsp secretion apparatus may be directly affected in the mutants. However, the increased secreted LPLA activity, which also depends on the type II secretion system Lsp (25, 26), conflicts with this theory. Additionally, secreted RNase activity, which is not present in a type II secretion mutant (58), was not affected by the mutations in *letA* and *rpoS*. It is however possible that the Lsp apparatus is indeed influenced by the *letA* and *rpoS* mutations and that redundant enzymes translocated by other secretion systems are upregulated and balance the loss of type II secreted activities. Furthermore, LetA or RpoS could affect some sort of chaperone or assistant molecule required by the reduced activities, in order that the enzymatic proteins arrive, translocate, or be processed properly. In addition, it is also possible that mutations in *letA* and *rpoS* affect a building block of the Lsp type II secretion system required by some translocated molecules but not by others. Furthermore, the predominant reduction in hydrolytic activities observed in the *letA* mutant might result from locking the strain in the exponential growth phase, where hydrolytic enzymes of wild-type *L. pneumophila* would normally increase with growth. We found that the decrease in acyltransferase activity in *L. pneumophila letA* and *rpoS* mutants was indeed caused by a decrease in *plaC* gene expression. However, it still needs to be examined whether LetA or RpoS plays a direct role in the regulation of *plaC* or whether other members of the regulation cascade exist.

Since *L. pneumophila letA* and *rpoS* mutant strains showed very similar phenotypes with respect to the activities investigated, it is likely that these two regulators form a regulation cascade. This has already been hypothesized in several studies (29, 44). Additionally, mid-logarithmic-phase activities of *letA* and *rpoS* mutants showed the trend of alterations present in the late-logarithmic activities, but to a smaller extent (data not shown). As it was recently shown that *rpoS* is already expressed during the exponential growth phase (8), the regulation of hydrolytic enzymes seems to be effective or starts to build up a hydrolytic potential already in the replicative phase.

Here, we showed that the regulation proteins RpoS and LetA, which play a role in the control of bacterial virulence factors of *L. pneumophila*, control PLA, LPLA, acyltransferase, and other hydrolytic activities of *L. pneumophila*. Whether LetA and RpoS influence the hydrolytic enzyme activities directly, via regulation of structural gene transcription, or indirectly, via the expression control of activators, cofactors, or components of secretion apparatuses, still needs to be elucidated in future studies. Additionally, the identification of the major enzyme contributing to the secreted PLA activity of *L. pneumophila* is of importance, because the severe decrease in secreted PLA activity in the *L. pneumophila rpoS* and *letA* mutants, as well as in a type II secretion mutant, which are all defective for bacterial replication in amoebae, points to an important role of this enzyme for *L. pneumophila*'s virulence properties.

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