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FliA Expression Analysis and Influence of the Regulatory Proteins RpoN, FleQ and FliA on Virulence and *in vivo* Fitness in *Legionella pneumophila*

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Abstract

In *Legionella pneumophila* the regulation of the flagellum and the expression of virulence traits are linked. FleQ, RpoN and FliA are the major regulators of the flagellar regulon. We demonstrated here that all three regulatory proteins mentioned (FleQ, RpoN and FliA) are necessary for full *in vivo* fitness of *L. pneumophila* strains Corby and Paris. In this study we clarified the role of FleQ for *fliA* expression from the level of mRNA towards protein translation. FleQ enhanced *fliA* expression, but FleQ and RpoN were not necessary for basal expression. In addition, we identified the initiation site of *fliA* in *L. pneumophila* and found a putative σ^{70} promoter element localized upstream. The initiation site was not influenced in the Δ*fleQ* or Δ*rpoN* mutant strain. We demonstrated that there is no significant difference in the regulation of *fliA* between strains Corby and Paris, but the FleQ-dependent induction of *fliA* transcription in the exponential phase is stronger in strain Paris than in strain Corby. In addition, we showed for the first time the presence of a straight hook at the pole of the non-flagellated Δ*fliA* and Δ*fliD* mutant strains by electron microscopy, indicating the presence of an intact basal body in these strains.

Introduction

The bacterial pathogen *Legionella pneumophila* lives in natural and man-made water systems and replicates intracellularly within aquatic protozoa (Rowbotham 1986). After inhalation of *L. pneumophila* by immunocompromised humans, bacteria survive and replicate within alveolar macrophages (Horwitz and Silverstein 1980). Within the host cell, the bacteria establish a specialized *Legionella*-containing vacuole (LCV) in which *L. pneumophila* starts to replicate (Horwitz 1983; Horwitz and Maxfield 1984; Sturgill-Koszycki and Swanson 2000). Finally, in the late replicative phase, when the LCV merges with lysosomes and nutrients become limiting, the bacteria enter the transmissive phase of the biphasic life cycle. In this phase *L. pneumophila* expresses many virulence-associated traits, including the flagellum (Brüggemann et al. 2006; Byrne and Swanson 1998; Heuner and Albert-Weissenberger 2008; Molofsky et al. 2005; Sauer et al. 2005a; Sauer et al. 2005b). Bacterial flagella are highly complex molecular machines. They are assembled from over 40 different protein components and mediate bacterial motility. Bacteria use hierarchical regulatory networks involving transcriptional and post-transcriptional mechanisms to ensure maximal efficiency and accuracy during flagellar biogenesis. These mechanisms regulate the ordered expression of the various components of the flagellum. In general, bacterial flagellar genes can be classified based upon their temporal gene expression and on their dependence on different transcriptional regulators (McCarter 2006).

As mentioned above, there is a genetic link between the mechanisms of life cycle regulation and flagellar gene expression in *L. pneumophila*, and several studies have been undertaken to understand these mechanisms (Heuner and Albert-Weissenberger 2008; Molofsky and Swanson 2004). The two-component system LetA/LetS plays an important role in the regulation of the life cycle switch as well as in flagellar gene expression (Gal-Mor and Segal 2003; Hammer et al. 2002; Molofsky and Swanson 2004). It is believed that LetA/LetS responds to the alarmone (p)ppGpp and the intracellular concentration of (p)ppGpp is

regulated by RelA and SpoT (Dalebroux et al. 2010a; Edwards et al. 2009; Dalebroux et al. 2009; Hammer and Swanson 1999; Hammer et al. 2002; Zusman et al. 2002). LetA seems to be involved in the expression of two small regulatory RNAs (RsmY and RsmZ) that are substrates for the RNA-binding protein CsrA. As a consequence of RsmY and RsmZ binding, CsrA is released from its target mRNAs inducing the expression of transmissive traits (Rasis and Segal 2009; Sahr et al. 2009; Zusman et al. 2002; Molofsky and Swanson 2003). Flagellar gene expression is thought to be regulated by this CsrA-dependent pathway. However, RpoS and the response regulator LqsR were also shown to be involved in the expression of the flagellin gene (*flaA*) (Tiaden et al. 2007; Bachman and Swanson 2001, 2004).

Beneath these global regulators influencing flagellin expression, the flagellar regulon of *L. pneumophila* is organized as a regulatory cascade exhibiting four different classes of genes (Albert-Weissenberger et al. 2010; Heuner and Albert-Weissenberger 2008). The master regulator FleQ (sigma factor σ^{54} activator protein, class I gene) and the alternative σ^{54} factor (RpoN) together enhance the expression of the flagellar class II genes (predominantly basal body genes). The alternative σ^{28} factor FliA (class III gene) directly controls the expression of the flagellar class IV genes (e.g. *flaA*) (Albert-Weissenberger et al. 2010; Brüggemann et al. 2006; Jacobi et al. 2004; Heuner and Albert-Weissenberger 2008). It is known that *L. pneumophila rpoN*, *fleQ*, *fliA* and *flaA* mutant strains are non-flagellated and non-motile (Heuner et al. 1995; Heuner et al. 2002; Heuner et al. 1997; Jacobi et al. 2004; Molofsky et al. 2005). Conflicting data suggested different regulation mechanisms in strains Corby and Paris for *fliA* expression. It seems as if *fliA* belongs to the class III genes in strain Paris, but is not directly linked to the flagellar regulon in strain Corby (Albert-Weissenberger et al. 2010; Jacobi et al. 2004). Both strains are virulent *L. pneumophila* Sg1 isolates with only small differences within their genomes (Cazalet et al. 2004; Jepras et al. 1985; Glöckner et al. 2008). Our intention was to clarify the regulation of *fliA* expression in *L. pneumophila* and to examine the differences between both strains. In this work the transcriptional start site

of *L. pneumophila fliA* was determined for the first time. Furthermore, we studied the influence of FleQ, RpoN and FliA on the transcription of various flagellar genes in a semi-quantitative manner and the growth phase-dependent expression of FleQ, FliA and FlaA on the protein level in *L. pneumophila* strain Corby.

In addition, it has been demonstrated that FleQ, RpoN and FliA are involved in the invasion process of *L. pneumophila* Paris and of strain Corby. FliA is required for the *in vivo* fitness (Albert-Weissenberger et al. 2010; Dietrich et al. 2001; Hammer et al. 2002; Heuner et al. 2002; Jacobi et al. 2004; Molofsky et al. 2005). Furthermore it is thought that motility but not flagellin promotes contact with host cells and increases infectivity (Molofsky et al. 2005). Moreover, FleQ and RpoN seemed not to be reduced in their infection cycle within *Acanthamoeba castellanii* (Albert-Weissenberger et al. 2010; Jacobi et al. 2004). This is surprising since not only do FleQ and RpoN influence the expression of flagellar genes, but they are also involved in the expression of various known virulence genes of *L. pneumophila* Paris (Albert-Weissenberger et al. 2010). Therefore we hypothesized that there must be a yet unidentified effect of FleQ and RpoN on the pathogen-host interaction. For further analysis, we used a more sensitive replication/survival assay recently published to investigate the role of FleQ, RpoN and FliA regarding the *in vivo* fitness of *L. pneumophila* Corby and Paris in more detail (Eylert et al. 2010).

Materials and Methods

Bacterial strains and amoeba

Experiments were done with *L. pneumophila* Sg1 strain Corby (Jepras et al. 1985) and *L. pneumophila* Sg1 strain Paris (Cazalet et al. 2004) and their isogenic Δ*rpoN*, Δ*fleQ* and Δ*fliA* mutant strains (Heuner et al. 2002; Jacobi et al. 2004; Albert-Weissenberger et al. 2010; Brüggemann et al. 2006). Further Corby strains used in this study were Δ*flaA*, Δ*motA* and

Δ*fliD* (Dietrich 2000; Heuner and Albert-Weissenberger 2008). All strains are listed in Table 1. *E. coli* strain DH5α was used as host for recombinant plasmids (Hanahan 1983). *Acanthamoeba castellanii* ATCC 30010 (Hall and Voelz 1985) was used for infection assays.

Media and growth conditions

L. pneumophila was cultured in AYE medium [1% yeast extract, supplemented with 1% N-(2-acetamido)-2-aminoethanesulfonic acid (ACES), 0.025% ferric PP_i, and 0.04% L-cysteine] at 37°C with agitation at 250 rpm or on ACES-buffered charcoal yeast extract (BCYE) agar for 3 days at 37°C (Edelstein 1981). Bacterial growth in broth was monitored by determining the optical density at 600 nm (OD_{600}) with a Thermo Scientific GENESYS 10 Bio spectrophotometer (VWR, Darmstadt, Germany). Growth phases were defined as follows: OD_{600} of 1.0 corresponded to the exponential (E), additional growth for 8 hours and OD_{600}~1.7 to the late exponential (LE), additional growth for 4 hours and OD_{600}~1.8 to the post exponential (PE) and additional growth for 8 hours and OD_{600}~2.0 to the stationary (S) growth phase. *E. coli* was cultivated in Luria-Bertani (LB) medium or LB agar at 37°C for 1 day (Hanahan 1983).

When appropriate, media were supplemented with antibiotics at final concentrations suitable for *L. pneumophila* or *E. coli* as follows: kanamycin at 8 or 40 µg/ml and gentamycin at 8 or 30 µg/ml, respectively; and ampicillin at 100 µg/ml for *E. coli*. For cultivation of *L. pneumophila* on agar plates, the final concentrations of kanamycin and gentamycin used were at 12.5 µg/ml and 10 µg/ml, respectively.

Acanthamoeba castellanii ATCC 30010 was cultured in PYG 712 medium [2% proteosepeptone, 0.1% yeast extract, 0.1 M glucose, 4 mM $MgSO_4$, 0.4 M $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2$ x 6 H_2O, 2.5 mM NaH_2PO_4 and 2.5 mM K_2HPO_4] at 20 °C.

DNA techniques and sequence analysis

Genomic and plasmid DNAs were prepared according to standard protocols (Sambrook and Russell 2000). PCR was carried out using a TRIO-Thermoblock (Biometra, Göttingen, Germany) and Taq DNA polymerase (Qiagen, Hilden, Germany). Plasmid DNA was introduced into bacterial strains by electroporation with a gene pulser (Bio-Rad, Munich, Germany). *E. coli* was electroporated at 1.7 kV, 100 Ω and 25 mF. Both strands of plasmid DNA or a PCR product were sequenced with infrared, dye-labeled primers by using an automated DNA sequencer (LI-COR-DNA4000; MWG-Biotech, Ebersberg, Germany). Oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt a.M., Germany).

Legionella Δ*fliD* and Δ*flaA* knockout mutant construction

To generate a Δ*fliD* mutant in *L. pneumophila* Corby we amplified the insert of plasmid pGEM-Δ*fliD*-gent [kindly provided by Michele S. Swanson, Ann Abor, Michigan, USA (Molofsky et al. 2005)] using the primers M13U and M13R. The plasmid contains the *fliD* gene disrupted by a gentamycin resistance cassette (Gm^r). Natural transformation of *L. pneumophila* Corby was done as described before with modification (Stone and Kwaik 1999). In brief, 3 ml of a culture exponentially grown overnight were transferred to a plastic tube and incubated with the PCR product for 3 days at 37°C without agitation. Subsequently, bacteria were grown on antibiotic selective medium for 4 more days at 37°C and 5% CO_2. Screening for Gm^r mutants obtained by homologous recombination was performed by PCR with primers FliD_Mut_Test 1U, 2R, 3U and 4R. The construct pKH3 for the *flaA* mutant has been described before (Dietrich et al. 2001). A new flagellin-deficient mutant strain was created via natural transformation instead of electrotransformation. pKH3 contained the *flaA* gene which was disrupted by a Km^r cassette. The insert was amplified by using the primers M13U and

M13R. The generated *Legionella* mutants were confirmed via PCR with primers FlaA-F and FlaA-R and Western blot analysis (data not shown).

Intracellular multiplication in *A. castellanii*

The *A. castellanii* infection assay for *in vivo* growth of *Lp* strains Corby and Paris and their derivatives was done as described before (Brüggemann et al. 2006). In brief, 3-day-old cultures of *A. castellanii* were washed in AC buffer [PYG 712 medium without proteose-peptone, glucose and yeast extract], adjusted to 1 x 10^5 cells per ml and incubated in 24-well plates for 2 hours at 37°C and 5% CO_2. Stationary-phase *Legionella* bacteria grown on BCYE agar were diluted in sterile phosphate-buffered saline (PBS) and mixed with *A. castellanii* at a multiplicity of infection of 0.01. After invasion for 2 h at 37°C, the *A. castellanii* layer was washed twice with PBS, defining the start point of the time course experiment. The colony-forming units (CFU) of *L. pneumophila* were determined by plating on BCYE agar. Each infection was carried out in duplicate wells and was done at least three times.

Intracellular multiplication and survival in *A. castellanii*

The intracellular multiplication was carried out as described recently (Eylert et al. 2010). In brief, after 3 days of infection *A. castellanii* cells were resuspended, 100 µl aliquots were lysed and serial dilutions were spread on BCYE agar to determine the number of CFU. The infection was repeated weekly with fresh amoebae to study the replication rates. Therefore, remaining solution was incubated at 37°C for further 4 days and diluted in AC buffer (1:1,000). The number of CFU was determined by plating the remaining dilution on BCYE agar. 1 ml of the remaining dilution was used to reinfect fresh amoeba cultures as described above. Four rounds of infection were performed. Each infection was carried out in duplicate wells and done at least three times.

Intracellular multiplication in competition

The infection procedure was done as described recently (Eylert et al. 2010). It is similar to the assay described above, but infection was done with a 1:1 mixture of *L. pneumophila* wild type and one of its isogenic mutant strains. The number of CFU was determined by plating the remaining dilution on BCYE with and without kanamycin. To determine the number of wild-type bacteria, the CFU on BCYE-kanamycin agar were subtracted from the CFU on BCYE plates without kanamycin. Each infection was carried out in duplicate wells and was done at least three times. For the *L. pneumophila* Paris Δ*fleQ* mutant and the *Lp* Corby Δ*rpoN*, Δ*fleQ* and Δ*fliA* mutants, two mutant strains generated independently were investigated, showing similar results.

RT-PCR analysis

RNA preparation and reverse transcription (RT-)PCR analysis was done as described previously with modification (Jacobi et al. 2004). In brief, bacteria were grown to specific optical density at 600 nm (see above) and harvested. Total RNA was extracted by using a FastRNA Pro Blue Kit (MP biomedicals, Illkirch, France) as described by the manufacturer. Additionally, purified RNA was incubated with 100 U of DNase I per ml (Qiagen, Hilden, Germany) at room temperature for 20 min and then repurified by using an RNeasy Mini Kit (Qiagen). PCR with primers specific for the *rpoN* gene was done to check the isolated mRNA for the absence of genomic DNA. RT-PCRs were performed with gene-specific primers (see Table 1), using a OneStep RT-PCR Kit (Qiagen) according to the instructions of the manufacturer. The RT reaction was performed at 50°C for 30 min with 10 ng of total RNA, calibrated with a Nano Drop 2000 spectrophotometer (Thermo Scientific, Schwerte, Germany). PCR amplification was performed in the same tube, with each primer at a concentration of 0.5 µM, each deoxynucleoside triphosphate at a concentration of 400 µM, 5 x OneStep RT-PCR buffer containing 12.5 mM $MgCl_2$ and 1 µl of OneStep RT-PCR enzyme

mixture in a 25 µl (total volume) reaction mixture. After reverse transcription, initial denaturation was performed at 95°C for 15 min (activation step) and final extension was performed at 72°C for 10 min. The cycling conditions were 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. For all gene products, several numbers of cycles were performed [24, 27 and 30 cycles (see Online Resource 1)]. The *rpoN* gene served as loading control. On a 2% agarose gel the RT reaction was visualized and analyzed semi-quantitatively. Experiments were carried out at least in two biological replicates.

SDS-PAGE and Western blot analysis

Total cell extracts of *L. pneumophila* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed as described previously (Laemmli 1970). *Legionella* was grown in AYE medium at 37°C for 1 day. The optical density at 600 nm (OD_{600}) and the time of growth defined the specific growth phases at which the bacteria were harvested and suspended in PBS (see above and Fig. 1). The OD_{600} was adjusted to 1. Bacteria (8 ml) were pelleted and the protein concentration was determined with Roti nanoquant solution [Roth, Karlsruhe, Germany (Bradford 1976)]. 30 µg of whole-cell lysates were suspended in Laemmli buffer and loaded onto a 12% SDS polyacrylamide gel. Western blotting was carried out by using polyclonal antibodies diluted in 1% milk/TBS specific for *L. pneumophila* Corby FliA protein (1:100), *P. aeruginosa* FleQ protein [1:500; (Jacobi et al. 2004)] and the FlaA protein [1:1,000; (Jacobi et al. 2004)]. The major outer membrane protein (Momp) was detected by a commercially available MONOFLUO anti-*Legionella* Staining Reagent (1:100; Biorad, Munich, Germany) and served as loading control (Helbig et al. 2007). The proteins FliA, FleQ, FlaA and Momp are specifically detected in Western blot analysis with a molecular mass of 27, 53, 48 and 25 kD, respectively. The anti-FliA antibody was generated by R. Schade (Institut für Pharmakologie und Toxikologie der Humboldt-Universität zu Berlin, Germany), using a

standard protocol for immunization of two rabbits with a C-terminal peptide (NH_2-CISQILSQATHRIRSR-COOH) of FliA of *L. pneumophila* Corby, as described before (Heuner et al. 2001). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were used as the secondary antibodies (1:1,000 for FleQ, FliA and FlaA and 1:5,000 for Momp; Dianova, Hamburg, Germany). Momp, FleQ and FlaA were visualized by incubation of the blot with 50 ml of color reaction solution (47 ml TBS, 3 ml 4-chloro-1-naphthol and 80 µl H_2O_2). The blot for FliA detection was incubated with commercially available ECL Detection Solution (GE Healthcare, Munich, Germany) and Millipore Advanced Detection Solution (Millipore, Schwalbach, Germany) mixed in a ratio of 10:1 for 1 min and exposed to X-ray film (Fujifilm, Tokyo, Japan). Experiments were carried out in two biological replicates.

Identification of the transcriptional start site (5'RACE)

Experimental procedure was done as described previously (Schunder et al. 2010). The transcriptional start site of *fliA* was determined with total RNA from late exponentially grown *L. pneumophila* Corby wild type and the mutant strains Δ*fleQ* and Δ*rpoN* using the 2nd Generation 5'/3' RACE Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. In brief, first strand cDNA synthesis from 2-3 µg of RNA was carried out in a reverse transcription reaction for 60 min at 55°C with the gene-specific primer FliA_RACE_4R. The cDNA was purified using the High Pure Purification Kit (Roche Applied Science). A homopolymeric A-tail was added to the 3' end of the cDNA, using terminal deoxynucleotidyltransferase and dATP. PCR was carried out with a (dT-)anchor primer and the gene-specific primer FliA_RACE_5R. In a second PCR with the PCR anchor primer and the specific primer FliA_RACE_6R, 1 µl of a 1:10 dilution from the first PCR was further amplified. The PCR product was analyzed on a 2% agarose/TAE gel (5 µl) and

purified using the High Pure Purification Kit (45 µl). The purified PCR product was sequenced directly using primer FliA_RACE_6R.

Electron microscopy

Experiments were performed at room temperature as described before with modifications (Jacobi et al. 2004). Bacteria were grown for 1 day in AYE medium at 30°C and low agitation (125 rpm) to keep the flagellar structure intact. Then bacteria were harvested by gentle centrifugation at 1,000 rpm for 20 min and suspended in distilled water. For negative staining electron microscopy concentrated bacteria were adsorbed on Pioloform-F-coated, carbon-stabilised and glow-discharged copper grids. The adherent bacteria were washed three times with distilled water. After negative staining with 0.5% uranyl acetate (pH 4-4.5) the samples were analyzed using a TEM 902 (Zeiss, Oberkochen, Germany) at 80 kV and the images digitized using a slow scan CCD-camera (Proscan, Scheuring, Germany). Analysis was done with three biological replicates.

Results and Discussion

FleQ, RpoN and FliA are necessary for full *in vivo* fitness of *L. pneumophila*

We recently presented a comparative microarray analysis of the Δ*rpoN* and Δ*fleQ* mutants of *L. pneumophila* strain Paris and their isogenic wild-type strain, showing that various virulence genes are affected in both mutants (Albert-Weissenberger et al. 2010). Surprisingly, no intracellular growth defect has yet been reported for these mutants, using a murine alveolar macrophage cell line (MH-S) or *A. castellanii* (Albert-Weissenberger et al. 2010; Jacobi et al. 2004). We hypothesized that there might be a yet unidentified effect of FleQ and RpoN on the pathogen-host interaction. Therefore, we now used a more sensitive replication/survival assay recently published (Eylert et al. 2010), to investigate the role of

RpoN, FleQ and FliA for the *in vivo* fitness of *L. pneumophila*. This assay is able to detect even small limitations in the fitness of a mutant strain, because the repeated pathogen-host interaction cycles are carried out in competition with the wild-type strain. First, we performed a control experiment showing that all mutants investigated grew similarly in AYE medium (Fig. 1a). Then we performed common infection assays with *A. castellanii* and could corroborate that the Δ*fleQ* and Δ*rpoN* mutant strains exhibited no significant defect regarding intracellular replication in this assay (Fig. 1b). Only the Δ*fliA* mutant strain showed less survival and establishment of infection at the early stage of infection, but was still able to replicate intracellularly (Fig. 1b), as described previously (Heuner et al. 2002; Molofsky et al. 2005). To investigate the above-mentioned accumulative effects during re-infection of amoebae, we then performed the replication/survival assays. As expected, all strains examined were still able to replicate within *A. castellanii* even after 4 rounds of infection (Fig. 2a). To investigate the role of the flagellin and motility for *in vivo* fitness, we also performed these experiments with a Δ*flaA* and a Δ*motA* mutant strain. Both mutants behaved like the wild-type strain (Fig. 2a). Then we performed experiments with successive rounds of infection using a mutant strain and the wild-type strain in competition. In this assay, the Δ*rpoN* and Δ*fleQ* mutant strains were outcompeted by the wild type, respectively (Fig. 2b, 2c). This indicates that the Δ*rpoN* and Δ*fleQ* mutant strains are less able to survive in direct competition to the wild-type strain, demonstrating that they are reduced in their virulence or fitness. However, the Δ*fliA* mutant exhibited only a slightly reduced fitness (Fig. 2d). A reduced fitness was also seen in the assays using Δ*flaA* and Δ*motA* mutant strains, respectively (Fig. 2e, 2f). The results indicate that in long-time co-culture studies of *L. pneumophila* Corby with *A. castellanii* motility is an important factor for the fitness of *L. pneumophila*. To reassess these results, we also analyzed *L. pneumophila* strain Paris and the isogenic Δ*rpoN*, Δ*fleQ* and Δ*fliA* mutant strains in our competitive replication/survival assay. The results for the Δ*rpoN,* Δ*fleQ* and Δ*fliA* mutant strains were similar to those for strain

Corby, but the effects observed were more pronounced (Fig. 2g-i). All three mutants of *L. pneumophila* Paris were outcompeted by the isogenic wild-type strain. As hypothesized from microarray data, this means the mutant strains were reduced in their *in vivo* fitness in co-culture with *A. castellanii*. This reduced fitness of the Δ*fleQ* and Δ*rpoN* mutants was not detected before using the common infection assay (Albert-Weissenberger et al. 2010; Jacobi et al. 2004). The reduction in the *in vivo* fitness of the Δ*rpoN* and Δ*fleQ* mutant strains (Paris and Corby) verified in the present study may be explained by the low expression of the regulator target genes as demonstrated recently via microarray analysis (Albert-Weissenberger et al. 2010). The genes most repressed in the Δ*rpoN* mutant belonged to the arginine biosynthesis pathway, but also effectors of the *dot*/*icm* type IV secretion system (*sidE*, *sdeD*, *sdcA*, *sidF*) were affected, as well as known virulence factors like *proA*, *htpB*, *enhA*, *enhC* and *argR*. In the Δ*fleQ* mutant strain, genes of the histidine operon were the genes most repressed, but also known virulence factors (*gspA*, *mip*, *lvgA*) were affected (Albert-Weissenberger et al. 2010). In both mutants, virulence genes as well as genes involved in the metabolism of *L. pneumophila* may be responsible for the reduced fitness observed. The observed reduction in the *in vivo* fitness of the Δ*fliA* mutant strains was more pronounced in strain Paris compared to strain Corby. This may be explained by the differences in *fliA* expression observed between both strains (see below).

Expression analysis of flagellar regulators of *L. pneumophila* Corby

As mentioned in the introduction, it is known for *L. pneumophila* strain Paris that *fliA* transcription is mainly FleQ-dependent, whereas in *L. pneumophila* strain Corby *fliA* transcription seems to be independent of FleQ (Albert-Weissenberger et al. 2010; Jacobi et al. 2004). FliA is a virulence-associated factor of *L. pneumophila* and is the direct and main regulator of flagellin expression. However, less is known about the expression of FliA itself. To clarify the above-mentioned differences and to further investigate *fliA* expression in strain

Corby, we performed semi-quantitative reverse transcriptase (RT-)PCR experiments and Western blot analysis of the Δ*fliA* as well as of the Δ*fleQ*, Δ*rpoN* and Δ*flaA* strains at the exponential (E), late exponential (LE), post exponential (PE) and stationary (S) phases (Fig. 1a). In addition, we investigated the transcriptional initiation site of *fliA* by 5´RACE experiments. As a control experiment, we verified flagellation for all *L. pneumophila* Corby mutant strains investigated in this study by electron microscopy.

Expression of the alternative σ^{28} factor FliA and transcriptional start site determination of *fliA* and adjacent genes

The amount of of *fliA* transcript increased from the E to LE phase and decreased from the LE to PE phase (Fig. 3a, *fliA*/wt). A similar expression pattern was detected on the protein level (Fig. 3b, αFliA/wt). In the Δ*rpoN* strain transcription of the *fliA* gene and the amount of FliA protein were downregulated in the E phase but upregulated from the PE to S phase, when compared with the expression pattern of the wild-type strain (Fig. 3a *fliA*/Δ*rpoN* and Fig. 3b αFliA/Δ*rpoN*). However, in the Δ*fleQ* strain *fliA* expression in the LE phase was lower than that in the wild-type strain, but transcription of *fliA* then was not decreasing further as observed for the wild-type strain (Fig. 3a, *fliA*/Δ*fleQ*). This expression pattern was corroborated by Western blot analysis (Fig. 3b, αFliA/Δ*fleQ*). These results demonstrated that in the Δ*rpoN* and Δ*fleQ* mutants *fliA* is only expressed at a basal level during early growth phase and then induced from the PE to S phase.

To further analyze *fliA* expression of strain Corby, we did 5'RACE experiments to identify the transcriptional initiation site of *fliA*. FliA is located within a region containing the flagellar genes *flhA-flhF-fleN-fliA-motA-motB* (Fig. 4a). Yet it is unclear which of these genes are transcribed as a mono- or polycistronic mRNA. Microarray analysis of strain Paris revealed that *flhF* and *fleN* are expressed, most likely, RpoN-dependently, whereas *fliA*

transcription in the Δ*rpoN* mutant strain is FleQ- but not RpoN-dependent (Albert-Weissenberger et al. 2010).

In our work, we analyzed the transcription initiation site of *fliA* in *L. pneumophila* Corby. The results showed a guanine residue 73 bp upstream of the GTG start codon of *fliA* as the transcriptional start site (Fig. 4b and Online resource 2). The Δ*fleQ* and Δ*rpoN* mutant strains exhibited identical initiation sites of *fliA* (Fig. 4b), confirming that in *L. pneumophila* Corby the expression of Δ*fliA* is not directly regulated by FleQ or RpoN. Taken together, the gene *fliA* was found to be regulated differently and therefore separately from the adjacent genes *flhF* and *fleN*. Directly upstream of the initiation site of *fliA* the sequence revealed a σ^{70}-like promoter element (GGGACA-N_{16}-TATACT). Between the +1 site and the -10 site an A/T-rich region could be identified. Therefore, *fliA* might be controlled by the housekeeping σ^{70} factor RpoD. This could be explained by a model of DksA-dependent basal *fliA* expression recently published. It was hypothesized that basal *fliA* promoter activity during the E phase in *L. pneumophila* may be mediated by DksA independently of ppGpp, whereas DksA cooperates with ppGpp for the activation of *fliA* from the E to PE phase. In this model an A/T-rich discriminator site (A/T-rich sequence between the -10 and +1 site) is described and typically found in DksA/ppGpp-modulated, RpoD-dependent promoters (Dalebroux et al. 2010a; Dalebroux et al. 2010b). But neither the promoter nor the initiation site of *fliA* were determined in these studies (Dalebroux et al. 2010a; Dalebroux et al. 2010b). In the present work we could now provide the experimental basics of this model by demonstrating the presence of a discriminator site within the identified promoter region of the *fliA* gene (Fig. 4b). Altogether, the results suggested that in *L. pneumophila* FleQ acts as an activator protein of *fliA* expression, independently of RpoN (Albert-Weissenberger et al. 2010). In the Δ*fleQ* mutant strain the activator protein is missing, and the basal expression of *fliA* in the E phase, probably mediated by DksA, is then upregulated by increasing levels of ppGpp from the PE to S phase (Fig. 3). It was also published that *fliA* transcription depends on RpoS and that the

transcription of RpoS is induced in the E phase, whereas a higher amount of the protein seems to be present in the S phase, resulting in an inductive effect in later growth phases (Bachman and Swanson 2001; Hales and Shuman 1999). However, we could not yet rule out that RpoS may be a direct regulator of *fliA* expression, since the promoter element identified may also be recognized by RpoS in the PE phase because of similarities between both consensus sequences (Lacour and Landini 2004; Potvin et al. 2008). Therefore, it is likely that the expression of *fliA* depends on σ^{70}, whereas FleQ acts as an enhancer protein of *fliA* transcription in a manner yet unknown. However, FleQ is not essential for the basal activity of the *fliA* promoter in *L. pneumophila*. Altogether, the results demonstrate that there is no major difference in the regulation of *fliA* between the strains Corby and Paris. However, it seems as if the positive effect of FleQ on *fliA* expression is stronger in strain Paris than in strain Corby.

Transcription of the alternative σ^{54} factor *rpoN*

The *rpoN* gene of *L. pneumophila* Corby was nearly constitutively expressed and thus in good agreement with the results published for strain Paris (Brüggemann et al. 2006; Albert-Weissenberger et al. 2010). Therefore, *rpoN* served as loading control for the RT-PCR analysis. The transcription of *rpoN* was not significantly influenced in the Δ*fleQ* and Δ*flaA* mutant strains and seemed to be slightly induced in the Δ*fliA* mutant strain (Fig. 3a, *rpoN*). This effect can be explained by a modified RNA polymerase availability. Via deletion of the sigma factor (σ^{28}) FliA, other sigma factors like σ^{70} have access to a higher amount of free core RNA polymerases (Nyström 2004).

Expression of the master regulator *fleQ* and presence of FleQ

We found that the expression of the *fleQ* gene increased from the E to LE phase and decreased from the LE to PE phase in *L. pneumophila* Corby wild type (Fig. 3a, *fleQ*/wt). However, the highest amount of FleQ protein was detected in the E phase, whereas a lower,

but constant amount of FleQ protein was detectable from the LE to S phase (Fig. 3b, αFleQ/wt). This is in agreement with the role of FleQ as the regulator of the flagellar class II and III genes expressed early (Albert-Weissenberger et al. 2010). The expression of *fleQ* was upregulated in the Δ*rpoN* strain, especially in the PE phase (Fig. 3a, *fleQ*/Δ*rpoN*). Additionally, we could demonstrate this also on protein level by the detection of a higher amount of FleQ protein in the Δ*rpoN* mutant compared to the wild-type strain (Fig. 3b, αFleQ/Δ*rpoN*). The transcription of *fleQ* was not significantly influenced in the Δ*fliA* and Δ*flaA* mutant strains (Fig. 3a, *fleQ*), but the amount of FleQ protein seemed to be higher in the Δ*fliA* strain in the LE to S phase when compared with the wild-type strain (Fig. 3b, αFleQ/Δ*fliA*). The results underline the importance of FleQ during the early growth phases and the ability to regulate the assembly of the basal body and the hook structure (Jacobi et al. 2004).

Expression of *flaA* and presence of flagellin (FlaA)

As a control, we also investigated the expression of *flaA* and the presence of FlaA in *L. pneumophila* strain Corby. The amount of *flaA* transcript increased at the LE phase and was still high until reaching the S phase (Fig. 3a, *flaA*/wt). As published earlier, the *flaA* transcript as well as FlaA protein were hardly detectable in a Δ*fliA* mutant strain (Fig. 3a and 3b, Δ*fliA*) (Heuner et al. 2002). Only at 30 cycles of RT-PCR a *flaA* transcript was detected (Online Resource 1). In the Δ*rpoN* and Δ*fleQ* mutant strain the *flaA* transcription was similar to that in the wild-type strain, whereas the amount of flagellin protein in both mutant strains did not decrease from the LE to S phase, as observed for the wild-type strain (Fig. 3a wt, Δ*rpoN*, Δ*fleQ* / *flaA* and Fig. 3b wt, Δ*rpoN* Δ*fleQ* / αFlaA). The results are in agreement with the observed transcription pattern of *fliA* in Δ*rpoN* and Δ*fleQ* mutant strains, since FliA is known to be the major positive regulator of *flaA* expression (Albert-Weissenberger et al. 2010; Heuner et al. 2002; Heuner et al. 1997). Jacobi et al. reported that *fliA* and *flaA* gene

transcription are not reduced in the Δ*fleQ* and Δ*rpoN* mutant strains of *L. pneumophila* Corby, but a reduction of FlaA protein was observed in both mutant strains (Jacobi et al. 2004). In contrast, our Western blot analysis showed an induction of the amount of flagellin from the LE phase in the Δ*fleQ* and Δ*rpoN* mutant strains. The differences may be explained by the usage of agar-grown bacteria (30°C) for Western blot analysis (Jacobi et al. 2004). Our data for the growth phase-dependent protein expression of FleQ, FliA and FlaA corroborated our RT-PCR results and demonstrate for the first time the regulatory relationships in *L. pneumophila* from transcription to translation.

Electron microscopy of the flagellin filament and identification of a straight hook in *L. pneumophila*

As a control experiment, we tested the *L. pneumophila* mutant strains used in this study for flagellation by electron microscopy. The flagellar apparatus consists of a membrane-anchored basal body with motor proteins, a hook structure, a filament consisting of FlaA monomers and a cap structure (FliD) (Heuner and Albert-Weissenberger 2008). As shown earlier, the wild type is a monopolar flagellated strain (Fig. 5a). As expected and shown before, the Δ*fliA* strain was non-flagellated, but we observed a short needle-like structure yet unidentified at one pole of the cells. At a higher magnification, the observed structure of the Δ*fliA* strain was identified as a straight hook, missing the filament of the flagellum (Fig. 5b). The hook was also observed at the pole of the Δ*flaA* strain (data not shown). It has been described that Δ*fliD* mutants generally secrete the flagellin into the medium through the intact basal body and hook structure, whereas the level of flagellin in the cell is comparable to that of the wild-type strain (Shimizu et al. 2003; Molofsky et al. 2005). To compare the structures, we generated a Δ*fliD* mutant strain of *L. pneumophila* Corby and also detected a similar straight hook structure on the surface of this mutant strain (Fig. 5c). In the Δ*fleQ* and Δ*rpoN* mutant strains no hook structure was present (data not shown). As described before, straight

hook structures can be prepared artificially under cold or acidic buffer conditions (Hirano et al. 1994; Kagawa et al. 1979; Potvin et al. 2008). Therefore, we tested preparation methods at room temperature and at different pH from neutral to acidic. In all cases we observed these straight hook structures (data not shown). In the Δ*fliA* and Δ*flaA* mutant strains flagellin is not expressed and therefore could not be assembled into a flagellum. In the case of the Δ*fliD* mutant strain, flagellin is expressed but is not assembled into a filament because of the lack of the filament cap protein (FliD). In conclusion, we could demonstrate that the flagellar basal body structures are built up independently from *fliA*, *flaA* and *fliD*. The lack of the hook structure in the Δ*fleQ* and Δ*rpoN* mutant strains demonstrated that RpoN and FleQ are important already in the first steps of the flagellar assembly cascade.

Conclusions

Although it was known that RpoN, FleQ and FliA are regulatory proteins influencing the flagellum assembly and other virulence factors, no intracellular replication defect of the *Legionella ΔrpoN* and *ΔfleQ* mutant strains has been reported (Albert-Weissenberger et al. 2010; Jacobi et al. 2004). In our competitive replication/survival assay we could detect a reduced fitness of these mutant strains in *A. castellanii* for the first time. The effect was more pronounced in strain Paris than in strain Corby. In addition, we were able to corroborate the important role of motility for the fitness of *L. pneumophila*. Furthermore, the initiation site of *fliA* has been identified, exhibiting a putative σ^{70}-dependent promoter element, and basal expression of *fliA* is independent of FleQ and RpoN. However, growth phase-dependent transcription and protein expression analysis demonstrate that there is no major difference in the regulation of *fliA* between the strains Corby and Paris, but the positive effect of FleQ on *fliA* expression seems to be stronger in strain Paris than in strain Corby. Electron microscopy revealed the presence of a straight hook structure yet unknown at the pole of the *ΔfliA* mutant

strain, indicating that the temporal expression and assembly of the basal body and the hook is functional and therefore independent of FliA.

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Figure legends

Fig. 1 Replication of *L. pneumophila* Corby (*Lp*C) wild-type and mutant strains in (a) liquid medium and (b) infection assay with *A. castellanii*. (a) Bacteria were grown in AYE medium, and samples for reverse transcription (RT-)PCR and Western blot analysis were taken at various time points and growth phases (arrows). (b) Monolayers of *A. castellanii* were infected with bacteria at a multiplicity of infection of 0.01, washed after 2 hours and incubated for 4 days at 37°C. The inoculum and the resuspended bacteria at 2 h, 24 h, 48 h, 72 h and 96 h were plated onto BCYE agar plates to determine CFU per ml. Results are mean +/- standard deviations of duplicate samples and are representative of at least three independent experiments. Symbols: filled circle (●), *Lp*C wild type (wt); down-pointing filled triangle (▼), *Lp*C Δ*rpoN*; up-pointing filled triangle (▲), *Lp*C Δ*fleQ*; filled square (■), *Lp*C Δ*fliA*; open diamond (◊), *Lp*C Δ*motA*; filled diamond (♦), *Lp*C Δ*flaA*; arrows, various time points when samples were taken.

Fig. 2 Replication/survival assay of *L. pneumophila* strains Corby and Paris and orthologue mutant strains in *A. castellanii*. Monolayers of *A. castellanii* were infected with (a-f) *L. pneumophila* Corby wild type (*Lp*C wt) and mutant strains (*Lp*C Δ*rpoN, Lp*C Δ*fleQ, Lp*C Δ*fliA*, *Lp*C Δ*flaA, Lp*C Δ*motA*) or with (g-i) *L. pneumophila* Paris wild type (*Lp*P wt) and mutant strains (*Lp*P Δ*rpoN, Lp*P Δ*fleQ, Lp*P Δ*fliA*) at a multiplicity of infection of 0.01 for 3 days, resuspended, incubated for a further 4 days, diluted 1:1,000 and used to infect fresh amoebae. Four rounds of infection were performed. Except for one experiment (a) all infection assays (b-h) were performed via co-infection of amoeba with a wild-type strain and a mutant strain together (in competition). In the former experiment amoebae were infected with each strain separately. At various time points postinoculation, bacteria were quantitated by plating aliquots on BCYE agar with and without kanamycin to determine the CFU per ml.

Results are mean +/- standard deviations of duplicate samples and are representative of at least three independent experiments. Experiments d-f and h were performed with two independent mutant strains of each gene investigated, showing similar results. Symbols: filled circle (●), *L. pneumophila* Corby (*Lp*C) wild-type; down-pointing filled triangle (▼), *Lp*C Δ*rpoN*; up-pointing filled triangle (▲), *Lp*C Δ*fleQ*; filled square (■), *Lp*C Δ*fliA*; filled diamond (♦), *Lp*C Δ*flaA*; open diamond (◊), *Lp*C Δ*motA*; open circle (○) *L. pneumophila* Paris (*Lp*P) wild-type; down-pointing open triangle (▽), *Lp*P Δ*rpoN*; up-pointing open triangle (△), *Lp*P Δ*fleQ*; open square (□), *Lp*P Δ*fliA*.

Fig. 3 Reverse transcription (RT-)PCR (a) and Western blot (b) analysis of *L. pneumophila* Corby wild-type and different mutant strains (Δ*rpoN*, Δ*fleQ*, Δ*fliA*, Δ*flaA*). Bacteria were grown in AYE medium, and samples were taken during different growth phases and prepared for RT-PCR as well as Western blot analysis. (a) For RT-PCR experiments whole-cell RNA was isolated and equal amounts (10 ng) were used for amplification. To analyze the rate of amplification semi-quantitatively, different numbers of cycles were performed (Online Resource 1). For the genes *rpoN*, *fleQ* and *fliA* 27 cycles are shown and for the gene *flaA* 24 cycles. (b) For Western blot analysis equal amounts (30 µg) of whole-cell lysates were analyzed by using antibodies against FleQ (arrow 2, 53 kD), FliA (arrow 3, 27 kD) and FlaA (arrow 4, 48 kD). The major outer membrane protein Momp (arrow 1, 25 kD) served as loading control. Abbreviations: E, exponential growth phase; LE, late exponential growth phase; PE, post exponential growth phase; S, stationary growth phase.

Fig. 4 Determination of the transcriptional start site of *fliA*. (a) Genomic organization of the sequence containing *fliA* (black arrow). FliA is located within a region containing the flagellar genes *flhA-flhF-fleN-fliA-motA-motB*. (b) Transcriptional start site of *fliA* was determined by 5'RACE. RNA from late exponentially grown *L. pneumophila* Corby wild type (wt) and *L.*

pneumophila Corby Δ*rpoN* and Δ*fleQ* mutant strains was transcribed in cDNA, polyA-tailed and amplified with a PCR-anchor primer and the gene-specific primer. The PCR product was sequenced using gene-specific primers and revealed the transcriptional start sites (+1). The raw data for the 5'RACE experiments are given in Online resource 2. The identified transcriptional start site of *fliA* revealed a putative RpoD (σ^{70}) promoter region with -10 and -35 boxes, linked by a spacer element of 16 nucleotides, followed by an A/T-rich discriminator site (ds). Neither a deletion of *fleQ* nor of *rpoN* resulted in a different start site. Abbreviation: RBS, ribosome-binding site. Arrow, start codon methionine.

Fig. 5 Electron microscopy of *L. pneumophila* Corby strains. Bacteria were grown in AYE medium at 30°C and low agitation for 1 day. After negative staining with 0.5% uranyl acetate (pH 4-4.5) the samples were analyzed using a TEM 902. (a, a') *L. pneumophila* Corby wild type (wt); (b, b') *Lp*C Δ*fliA* mutant strain; (c, c') *Lp*C Δ*fliD* mutant strain. Arrows indicate polar flagellar or hook structures. Bars (a-c): 200 nm ; bars (a'-c'): 1 µm.

Table 1 Strains, oligonucleotides, plasmids and antibodies used in this study

Name	Sequence or relevant characteristics[a]	Source or Reference
Strains		
Escherichia coli DH5α	K-12 DH5α F⁻ Φ80dΔ*lacZ*M15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 glnV44 thi-1 gyrA96 relA1*	(Hanahan 1983)
L. pneumophila (*Lp*)		
Corby	Virulent *L. pneumophila* serogroup 1, strain Corby	(Jepras et al. 1985)
Δ*rpoN* mutant	*Lp* Corby *rpoN*::Km^r	(Jacobi et al. 2004)
Δ*fleQ* mutant	*Lp* Corby *fleQ*::Km^r	(Jacobi et al. 2004)
Δ*fliA* mutant	*Lp* Corby *fliA*::Km^r	(Heuner et al. 2002)
Δ*flaA* mutant	*Lp* Corby *flaA*::Km^r	This study
Δ*fliD* mutant	*Lp* Corby *fliD*::Gm^r	This study
Δ*motA* mutant	*Lp* Corby *motA*::Km^r	(Dietrich 2000; Heuner and Albert-Weissenberger 2008)
Paris	Virulent *L. pneumophila* serogroup 1, strain Paris	(Cazalet et al. 2004)
Δ*rpoN* mutant	*Lp* Paris *rpoN*::Km^r	(Albert-Weissenberger et al. 2010)
Δ*fleQ* mutant	*Lp* Paris *fleQ*::Km^r	(Albert-Weissenberger et al. 2010)
Δ*fliA* mutant	*Lp* Paris *fliA*::Km^r	(Brüggemann et al. 2006)
Oligonucleotides		
PCR		
M13U	5' – GTAAAACGACGGCCAGT – 3'	Promega, Mannheim, Germany
M13R	5' – GGAAACAGCTATGACCATG – 3'	Promega, Mannheim, Germany
FliD_Mut_Test 1U	5' – AGCCAATTCTACACTATGAAATCAACC – 3'	This study
FliD_Mut_Test 2R	5' – GCTAATGGCTTTGCCAATGTGT – 3'	This study
FliD_Mut_Test 3U	5' – CGGACAAGATGCTACGTCTGC – 3'	This study
FliD_Mut_Test 4R	5' – CACCAAAGCAATTCACCGTCTT – 3'	This study
FlaA-F	5' – CATGATGCAAACATCGATCCA – 3'	This study
FlaA-R	5' – CTGCTACTTCTGTTCCTGTTG – 3'	This study
RT-PCR		
RpoN_RNA_1F	5' – GCGATATTGACCGAGTCATTGCT – 3'	This study
RpoN_RNA_2R	5' – CAGACTATTGTTTGCGCGTTGGAT – 3'	This study
FleQ_RNA_F	5' – GCGCGGATCGAAATATCGTTGATT – 3'	This study
FleQ_RNA_R	5' – GTTACCTGGCCAGCTGTATTCG – 3'	This study
FliA_RNA_F	5' – CAACAAACCCAGGAAGCTCTGG – 3'	This study
FliA_RNA_R	5' – GCGATGCGTTGCTTGACTTAGAAT – 3'	This study
FlaA_RNA_1F	5' – GCTAACGATGGTATCTCCCTATCA – 3'	This study
FlaA_RNA_2R	5' – CTGCTTTGGCATAGGCAGACG – 3'	This study
5'RACE		
neo2/rev	5' – GCTGCCTCGTCCTGCAGTTC – 3'	Roche, Mannheim, Germany
neo3/for	5' – GATTGCACGCAGGTTCTCCG – 3'	Roche, Mannheim, Germany
Oligo d(T)-anchor[b]	5' – GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV– 3'	Roche, Mannheim, Germany
FliA_RACE_4R	5' – GGCTGCTAATGGAATCTTGCAAC – 3'	This study
FliA_RACE_5R	5' – GACACACCCAACTCTTCAGCAA – 3'	This study
FliA_RACE_6R	5' – GCTCCAATATGCGCACAGCAT – 3'	This study
Plasmids		
pGEM-Δ*fliD*-gent	pGEM-fliD with 1.3-kb Km^r cassette disrupting internal *Eco*RI site at base 641 of 1,626-base FliD ORF; Ap^r, Gm^r	(Molofsky et al. 2005)
pKH3	pKS vector containing the cloned *flaA* gene disrupted by replacement of an internal 109-bp *Eco*RI-*Hind*III fragment with a Km^r cassette	(Dietrich et al. 2001)
Antibodies		
αMomp	MONOFLUO anti-*Legionella* Staining Reagent; mouse monoclonal antibodies for the epitope on the outer membrane protein Momp (1:100)	Biorad, Munich, Germany; (Helbig et al. 2007)
αFleQ	Rabbit polyclonal antibodies specific for *P. aeruginosa* FleQ (1:500)	Reuben Ramphal, Gainesville, Florida, USA; (Jacobi et al. 2004)
αFliA	Rabbit polyclonal antibodies specific for *L. pneumophila* FliA (1 :100)	This study
αFlaA	Rabbit polyclonal antibodies specific for *L. pneumophila* FlaA (1 :1,000)	(Jacobi et al. 2004)
αMouse-HRP	Goat antibodies specific against mouse; IgG (H+L); conjugated to horseradish-peroxidase (1:5,000 for Momp)	Dianova, Hamburg, Germany
αRabbit-HRP	Goat antibodies specific against rabbit; IgG (H+L); conjugated to horseradish-peroxidase (1:1,000)	Dianova, Hamburg, Germany

[a] Abbreviations: Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Gm^r, gentamycin resistance. [b] V= A, C or G

Online Resource 1 Reverse transcription (RT-)PCR analysis of *L. pneumophila* Corby. Bacteria were grown in AYE medium and samples were taken during different growth phases and prepared for RT-PCR as well as Western blot analysis. For RT-PCR experiments whole-cell RNA was isolated and equal amounts (10 ng) were used for amplification. For all samples RT-PCR was performed at (a) 24 cycles, (b) 27 cycles or (c) 30 cycles, respectively. Results were carried out in at least two independent experiments. Abbreviations: E, exponential growth phase; LE, late exponential growth phase; PE, post exponential growth phase; S, stationary growth phase.

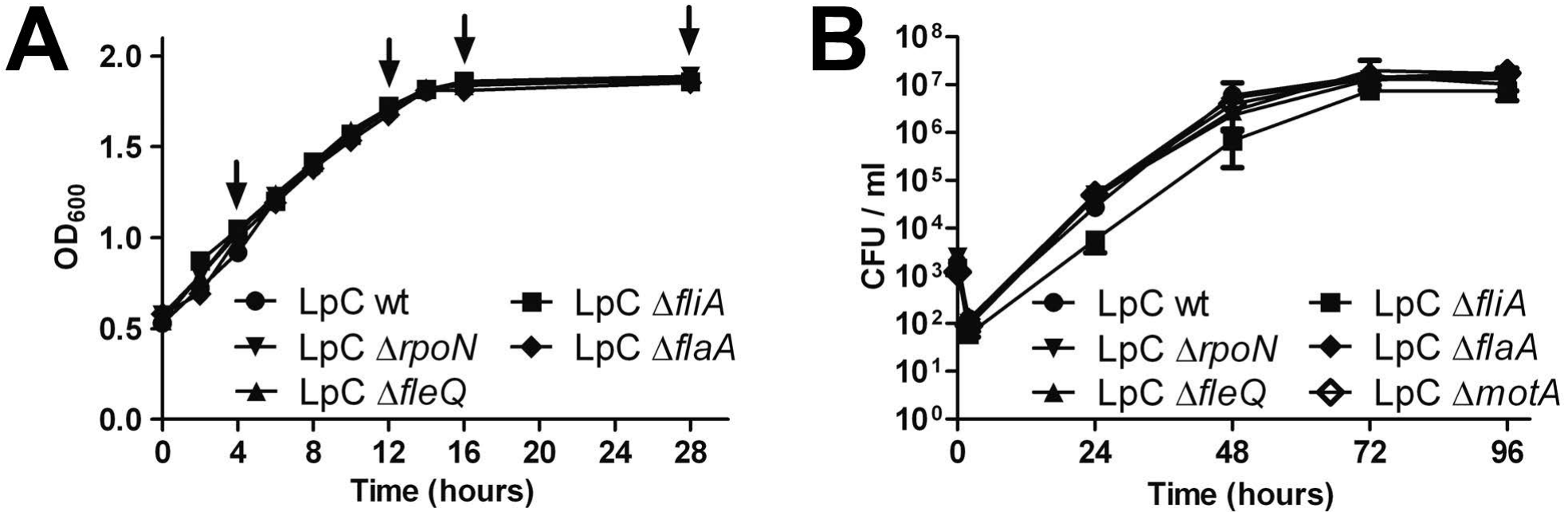
Online Resource 2 Determination of the transcriptional start site of *fliA* via 5'RACE experiments – raw data. RNA from late exponentially grown *L. pneumophila* Corby wild type (*Lp*C wt; a, b), *Lp*C Δ*fleQ* (a, c) and *Lp*C Δ*rpoN* (a, d) mutant strains was transcribed in cDNA, polyA-tailed and amplified. (a) All RNA samples contained a control RNA to verify the 5'RACE reaction via PCR. cDNA synthesis (K1; primers: neo2/rev and neo3/for; product: 157 bp), cDNA purification (K2; primers: neo2/rev and neo3/for; product: 157 bp) and d(A)-tailing reaction (K3; primers: neo2/rev and oligo d(T)-anchor; product: 293 bp) were verified with primers specific for the control RNA, respectively. The PCR product (R; primers: oligo d(T)-anchor and FliA_RACE_5R; product: ~ 500 bp) was sequenced using the anti-sense primer FliA_RACE_6R. The identified transcriptional start site is indicated (+1; b, c, d).

Fig. 3# Reverse transcription (RT-)PCR (a) and Western blot (b) analysis of *L. pneumophila* Corby wild-type and different mutant strains (Δ*rpoN*, Δ*fleQ*, Δ*fliA*, Δ*flaA*). Bacteria were grown in AYE medium, and samples were taken during different growth phases and prepared for RT-PCR as well as Western blot analysis. (a) For RT-PCR experiments whole-cell RNA was isolated and equal amounts (10 ng) were used for amplification. To analyze the rate of amplification semi-quantitatively, different numbers of cycles were performed (Online Resource 1). For the genes *rpoN*, *fleQ* and *fliA* 27 cycles are shown and for the gene *flaA* 24 cycles. (b) For Western blot analysis equal amounts (30 µg) of whole-cell lysates were analyzed by using antibodies against FleQ (53 kD), FliA (27 kD) and FlaA (48 kD). The major outer membrane protein Momp (25 kD) served as loading control. Abbreviations: E, exponential growth phase; LE, late exponential growth phase; PE, post exponential growth phase; S, stationary growth phase. (*) This band was identified to be FliA-specific by the analysis of the Δ*fliA* mutant strain with the αFliA antiserum by Western blotting.

Table 1 Strains, oligonucleotides, plasmids and antibodies used in this study

Name	Sequence or relevant characteristics[a]	Source or Reference
Strains		
Escherichia coli DH5α	K-12 DH5α F⁻ Φ80dΔ*lacZ*M15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 glnV44 thi-1 gyrA96 relA1*	(Hanahan 1983)
L. pneumophila (*Lp*)		
Corby	Virulent *L. pneumophila* serogroup 1, strain Corby	(Jepras et al. 1985)
Δ*rpoN* mutant	*Lp* Corby *rpoN*::Km^r	(Jacobi et al. 2004)
Δ*fleQ* mutant	*Lp* Corby *fleQ*::Km^r	(Jacobi et al. 2004)
Δ*fliA* mutant	*Lp* Corby *fliA*::Km^r	(Heuner et al. 2002)
Δ*flaA* mutant	*Lp* Corby *flaA*::Km^r	This study
Δ*fliD* mutant	*Lp* Corby *fliD*::Gm^r	This study
Δ*motA* mutant	*Lp* Corby *motA*::Km^r	(Dietrich 2000; Heuner and Albert-Weissenberger 2008)
Paris	Virulent *L. pneumophila* serogroup 1, strain Paris	(Cazalet et al. 2004)
Δ*rpoN* mutant	*Lp* Paris *rpoN*::Km^r	(Albert-Weissenberger et al. 2010)
Δ*fleQ* mutant	*Lp* Paris *fleQ*::Km^r	(Albert-Weissenberger et al. 2010)
Δ*fliA* mutant	*Lp* Paris *fliA*::Km^r	(Brüggemann et al. 2006)
Oligonucleotides		
PCR		
M13U	5' – GTAAAACGACGGCCAGT – 3'	Promega, Mannheim, Germany
M13R	5' – GGAAACAGCTATGACCATG – 3'	Promega, Mannheim, Germany
FliD_Mut_Test 1U	5' – AGCCAATTCTACACTATGAAATCAACC – 3'	This study
FliD_Mut_Test 2R	5' – GCTAATGGCTTTGCCAATGTGT – 3'	This study
FliD_Mut_Test 3U	5' – CGGACAAGATGCTACGTCTGC – 3'	This study
FliD_Mut_Test 4R	5' – CACCAAAGCAATTCACCGTCTT – 3'	This study
FlaA-F	5' – CATGATGCAAACATCGATCCA – 3'	This study
FlaA-R	5' – CTGCTACTTCTGTTCCTGTTG – 3'	This study
RT-PCR		
RpoN_RNA_1F	5' – GCGATATTGACCGAGTCATTGCT – 3'	This study
RpoN_RNA_2R	5' – CAGACTATTGTTTGCGCGTTGGAT – 3'	This study
FleQ_RNA_F	5' – GCGCGGATCGAAATATCGTTGATT – 3'	This study
FleQ_RNA_R	5' – GTTACCTGGCCAGCTGTATTCG – 3'	This study
FliA_RNA_F	5' – CAACAAACCCAGGAAGCTCTGG – 3'	This study
FliA_RNA_R	5' – GCGATGCGTTGCTTGACTTAGAAT – 3'	This study
FlaA_RNA_1F	5' – GCTAACGATGGTATCTCCCTATCA – 3'	This study
FlaA_RNA_2R	5' – CTGCTTTGGCATAGGCAGACG – 3'	This study
5'RACE		
neo2/rev	5' – GCTGCCTCGTCCTGCAGTTC – 3'	Roche, Mannheim, Germany
neo3/for	5' – GATTGCACGCAGGTTCTCCG – 3'	Roche, Mannheim, Germany
Oligo d(T)-anchor[b]	5' – GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV– 3'	Roche, Mannheim, Germany
FliA_RACE_4R	5' – GGCTGCTAATGGAATCTTGCAAC – 3'	This study
FliA_RACE_5R	5' – GACACACCCAACTCTTCAGCAA – 3'	This study
FliA_RACE_6R	5' – GCTCCAATATGCGCACAGCAT – 3'	This study
Plasmids		
pGEM-Δ*fliD*-gent	pGEM-fliD with 1.3-kb Km^r cassette disrupting internal *Eco*RI site at base 641 of 1,626-base FliD ORF; Ap^r, Gm^r	(Molofsky et al. 2005)
pKH3	pKS vector containing the cloned *flaA* gene disrupted by replacement of an internal 109-bp *Eco*RI-*Hind*III fragment with a Km^r cassette	(Dietrich et al. 2001)
Antibodies		
αMomp	MONOFLUO anti-*Legionella* Staining Reagent; mouse monoclonal antibodies for the epitope on the outer membrane protein Momp (1:100)	Biorad, Munich, Germany; (Helbig et al. 2007)
αFleQ	Rabbit polyclonal antibodies specific for *P. aeruginosa* FleQ (1:500)	Reuben Ramphal, Gainesville, Florida, USA; (Jacobi et al. 2004)
αFliA	Rabbit polyclonal antibodies specific for *L. pneumophila* FliA (1 :100)	This study
αFlaA	Rabbit polyclonal antibodies specific for *L. pneumophila* FlaA (1 :1,000)	(Jacobi et al. 2004)
αMouse-HRP	Goat antibodies specific against mouse; IgG (H+L); conjugated to horseradish-peroxidase (1:5,000 for Momp)	Dianova, Hamburg, Germany
αRabbit-HRP	Goat antibodies specific against rabbit; IgG (H+L); conjugated to horseradish-peroxidase (1:1,000)	Dianova, Hamburg, Germany

[a] Abbreviations: Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Gm^r, gentamycin resistance. [b] V= A, C or G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1.0
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0.0
OD600
0
4
8
12
16
20
24
28
Time (hours)
LpC wt
LpC ΔrpoN
LpC ΔfleQ
LpC ΔfliA
LpC ΔflaA
B
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU / ml
0
24
48
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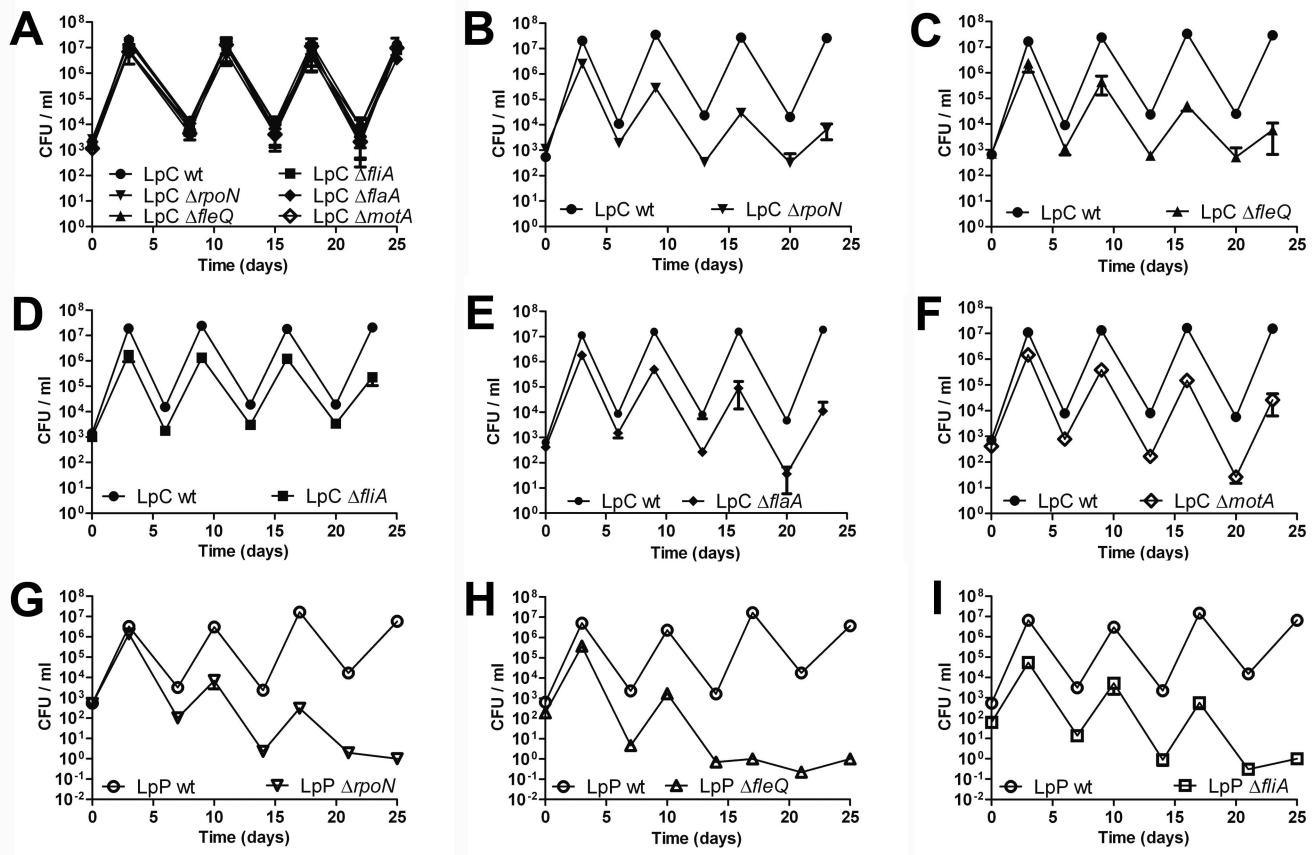
A
B
C
D
E
F
G
H
I
CFU / ml
Time (days)
LpC wt
LpC ΔrpoN
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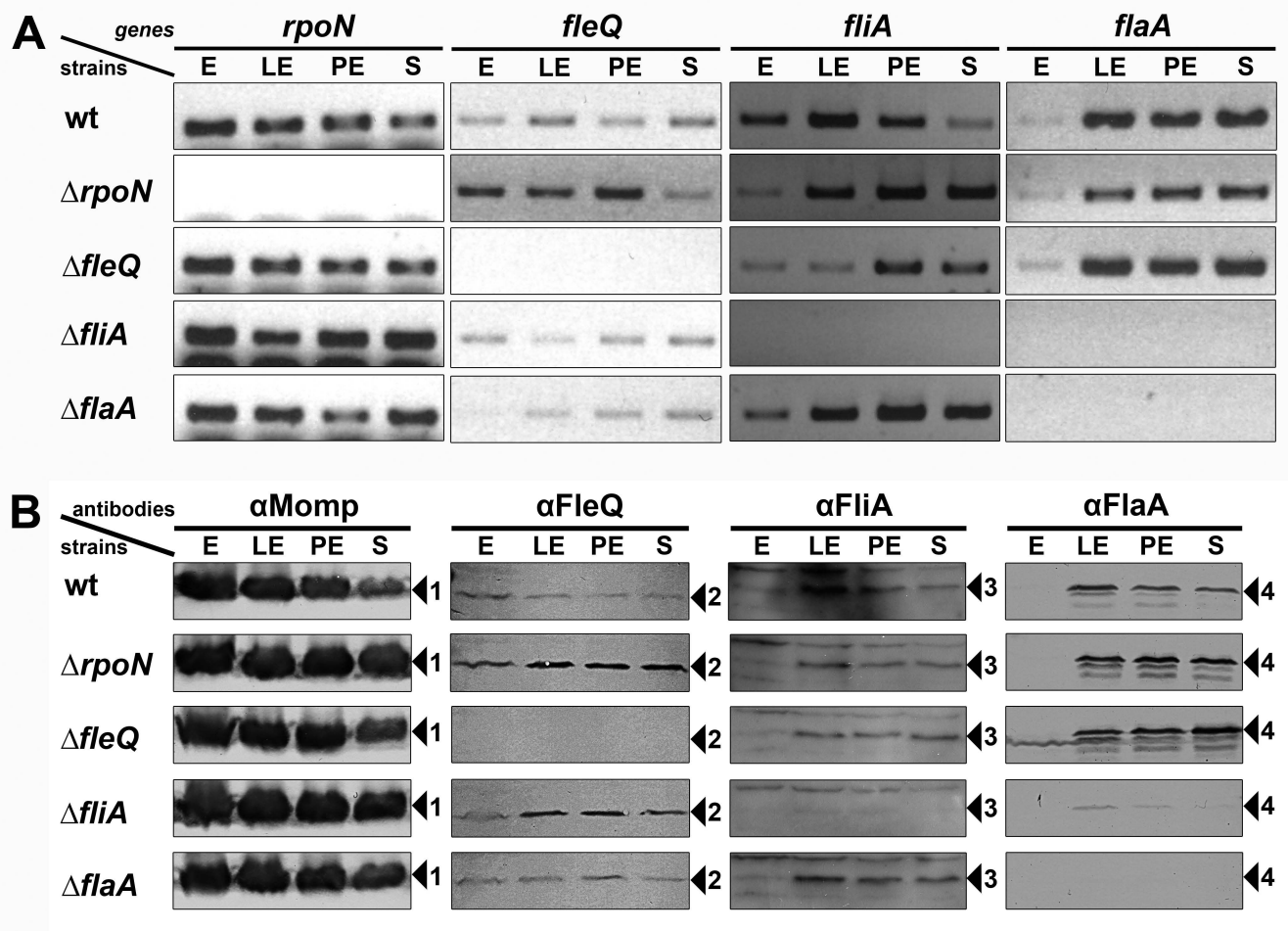
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antibodies
strains
αMomp
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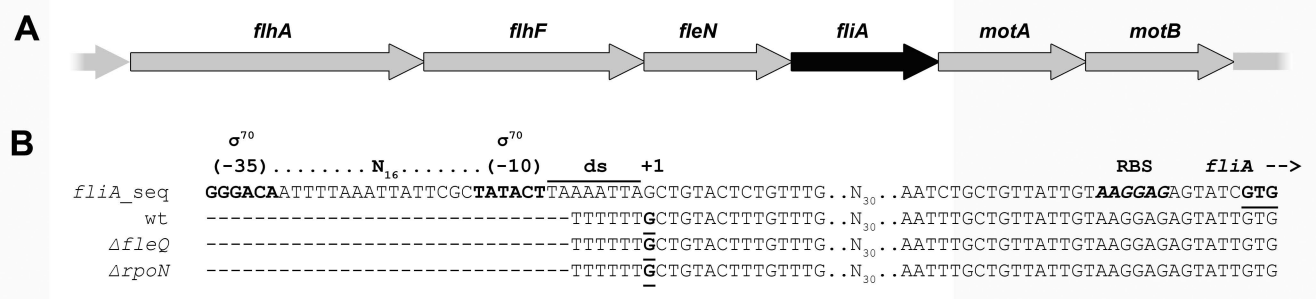
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flhF
fleN
fliA
motA
motB
B
σ70
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RBS
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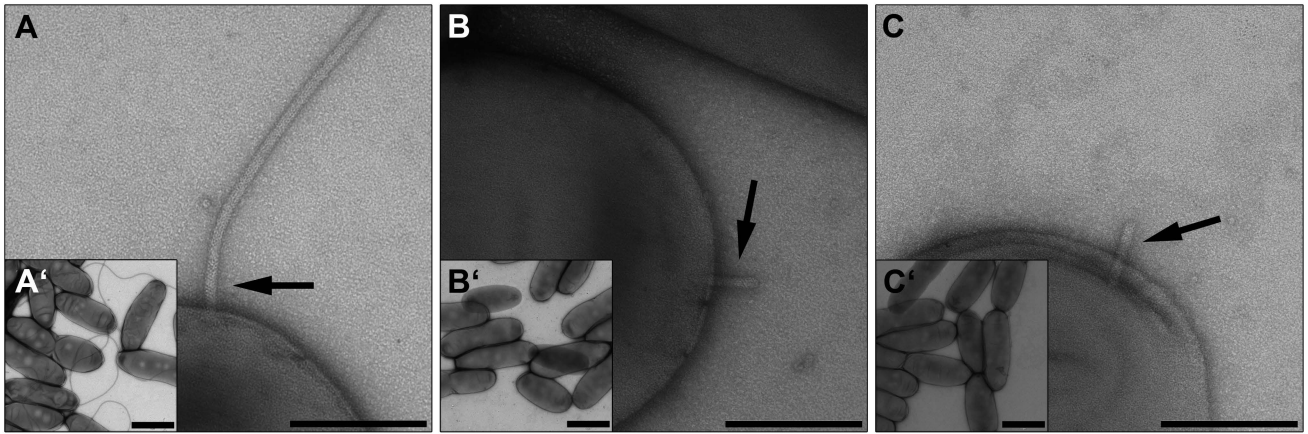
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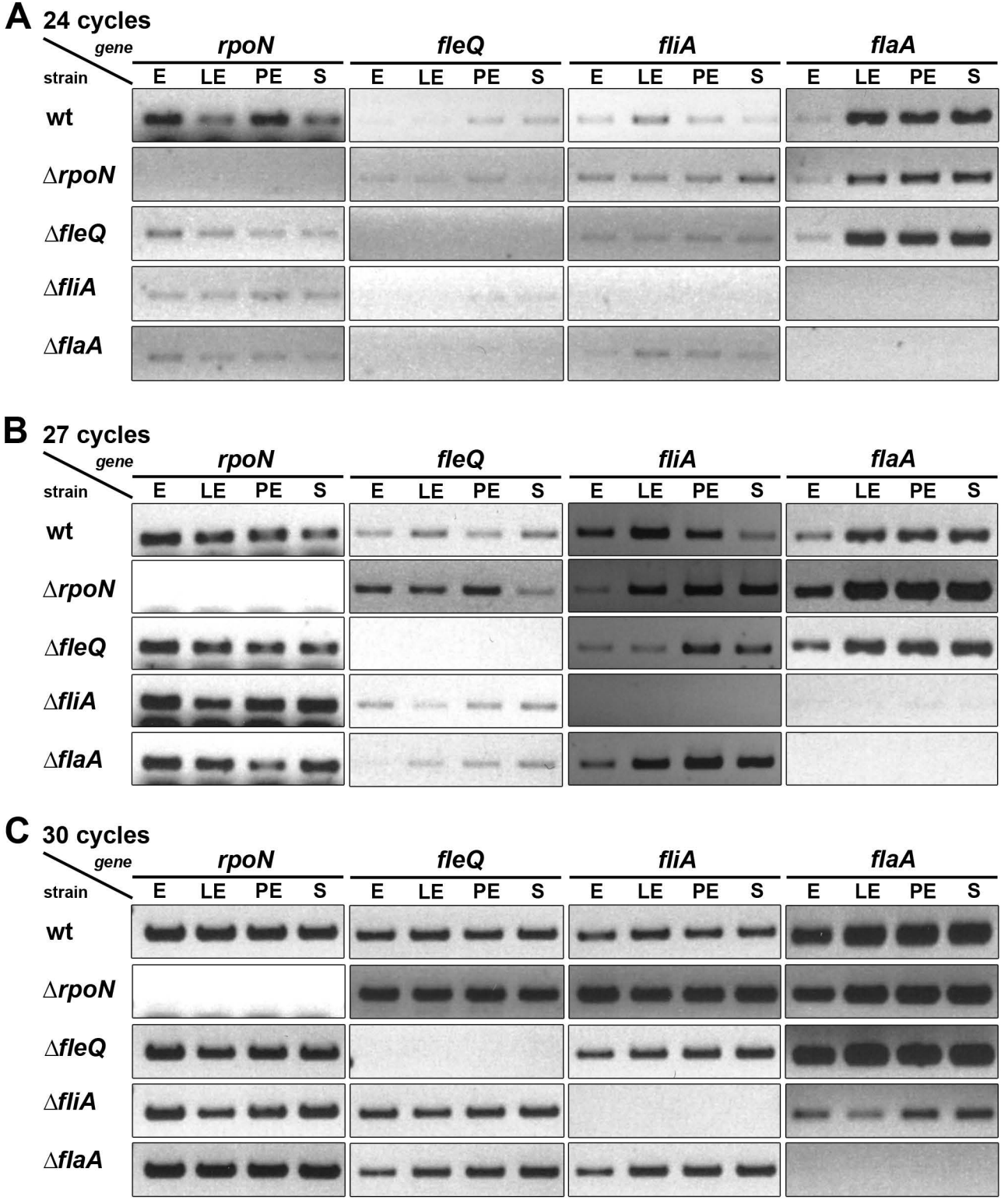
ΔfliA
ΔfliD
A
A‘
B
B‘
C
C‘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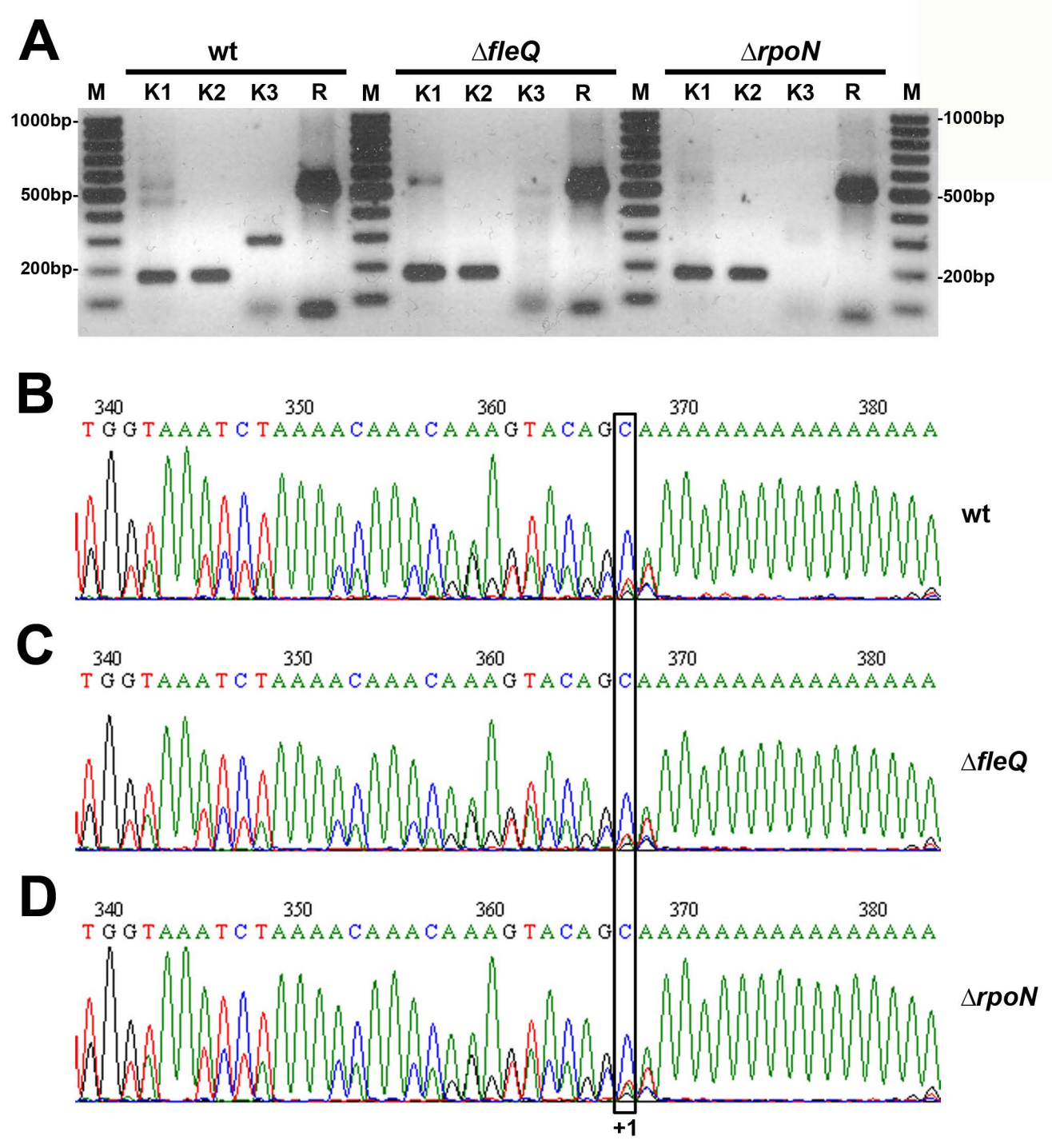
B
27 cycles
C
30 cycles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B
C
D
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360
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+1