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# Peptidyl-Prolyl-*cis*/*trans*-Isomerases Mip and PpiB of *Legionella pneumophila* Contribute to Surface Translocation, Growth at Suboptimal Temperature, and Infection

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**ABSTRACT** The gammaproteobacterium *Legionella pneumophila* is the causative agent of Legionnaires' disease, an atypical pneumonia that manifests itself with severe lung damage. *L. pneumophila*, a common inhabitant of freshwater environments, replicates in free-living amoebae and persists in biofilms in natural and man-made water systems. Its environmental versatility is reflected in its ability to survive and grow within a broad temperature range as well as its capability to colonize and infect a wide range of hosts, including protozoa and humans. Peptidyl-prolyl-*cis*/*trans*-isomerases (PPIases) are multifunctional proteins that are mainly involved in protein folding and secretion in bacteria. In *L. pneumophila* the surface-associated PPIase Mip was shown to facilitate the establishment of the intracellular infection cycle in its early stages. The cytoplasmic PpiB was shown to promote cold tolerance. Here, we set out to analyze the interrelationship of these two relevant PPIases in the context of environmental fitness and infection. We demonstrate that the PPIases Mip and PpiB are important for surfactant-dependent sliding motility and adaptation to suboptimal temperatures, features that contribute to the environmental fitness of *L. pneumophila*. Furthermore, they contribute to infection of the natural host *Acanthamoeba castellanii* as well as human macrophages and human explanted lung tissue. These effects were additive in the case of sliding motility or synergistic in the case of temperature tolerance and infection, as assessed by the behavior of the double mutant. Accordingly, we propose that Mip and PpiB are virulence modulators of *L. pneumophila* with compensatory action and pleiotropic effects.

**KEYWORDS** *Legionella pneumophila*, PpiB, human lung tissue explants, intracellular infection, macrophage infectivity potentiator, motility, peptidyl-prolyl *cis*/*trans* isomerase, sliding, temperature adaptation

The human pathogen *Legionella pneumophila* ubiquitously occurs in freshwater habitats, where it persists as a free-living bacterium and replicates intracellularly in amoebae residing mainly in biofilms (1–4). The transmission to humans occurs via man-made water systems that are colonized by *L. pneumophila* and its natural hosts. *Legionella*-containing aerosols that are generated by showers, cooling towers, and air-conditioning systems enable the entry of the bacteria into the human lung. Here, they replicate within human alveolar macrophages and destroy the lung tissue, causing Legionnaires' disease, a severe atypical pneumonia with mortality rates of up to 15% (5, 6).

Being considered an accidental human pathogen, *L. pneumophila* stands out with its environmental versatility and remarkably broad protozoan host spectrum that extends to simple metazoans like nematodes as well as human alveolar macrophages (7–9). The host spectrum can mainly be attributed to a type IVB secretion system (TIVBSS) with its more than 300 described and putative effectors (10–13). However, the contribution of factors other than the TIVBSS and its associated effectors on environmental fitness and infection processes has been only rudimentarily evaluated. Among those factors are the flagellum; pili; a type II secretion system (TIISS); several hydrolytic enzymes, including a chitinase; outer membrane vesicles; and peptidyl-proly-*cis/trans*-isomerases (PPIases) (14–20).

PPIases constitute a superfamily of proteins that is divided into three structural and inhibitor-based classes, (i) cyclophilins, (ii) parvulins, and (iii) FK506-binding proteins (FKBPs), which are inhibited by cyclosporine (CsA), juglone, and FK506 or rapamycin, respectively (21, 22). Nevertheless, all members are characterized by their ability to convert peptidyl-prolyl bonds from *cis* to *trans* or vice versa, as in these bonds no intrinsic preference for either of the isomers exists due to the unique side chain of proline residues (23, 24). By this, they primarily contribute to protein folding, stability, and activity. Furthermore, many PPIases have been shown to be associated with virulence in pathogenic microorganisms (25).

*L. pneumophila* possesses six PPIases, two cyclophilins (PpiA and PpiB), two parvulins (PpiD and SurA), and two FKBPs (trigger factor and Mip). Among these, the macrophage infectivity potentiator (Mip) is the best-characterized member (21). This outer membrane-associated protein, with a size of 25 kDa and a homodimeric structure, is important during the early stages of intracellular replication in U937 cells, explanted human alveolar macrophages, and environmental amoebae like *Acanthamoeba castellanii* (26–29). Mip-deficient strains are also attenuated in the guinea pig infection model, failing to spread to organs like the spleen, where a relationship between the PPIase activity and the outcome of the infection was demonstrated (30). In accordance with this, Mip binds via its PPIase domain to collagen IV in the extracellular matrix (ECM) and thereby contributes to transmigration of the bacteria through a barrier consisting of NCI-H292 epithelial cells and ECM (31, 32). Moreover, a secreted phospholipase C activity was shown to be dependent on Mip (33).

PpiB of *L. pneumophila* was shown to accumulate in culture supernatants and to contribute to growth at 17°C (34). Infection studies in *A. castellanii* showed that PpiB, previously also known as Lcy, has a weak effect on intracellular replication (35). Considering the impact of Mip on *Legionella* replication and the preliminary work on PpiB regarding the stress response as well as a possible implication in infection, we attempted to extend our knowledge relating to the involvement of the PPIases Mip and PpiB of *L. pneumophila* in extracellular fitness and virulence by using single- and double-knockout mutants.

## RESULTS

**The PPIases Mip and PpiB together facilitate surfactant formation and surface translocation of *L. pneumophila*.** *L. pneumophila* displays sliding motility through a flagellum- and pilus-independent surface translocation mechanism. It was shown that the type I secretion system (TISS) and the TIISS contribute to the secretion of surfactant, which promotes surface translocation (36). Since PPIases can also be involved in secretion processes in bacteria, we aimed at clarifying whether the PPIases of *L. pneumophila* also play a role in surfactant-mediated surface translocation (33, 37, 38). For this, an *mip*-negative strain (the Δ*mip* strain), a *ppiB*-negative strain (the Δ*ppiB* strain), and an *mip*- and *ppiB*-negative double mutant strain (the Δ*mip* Δ*ppiB* strain) were tested for their spreading phenotype on 0.5% (wt/vol) agar containing buffered charcoal yeast extract (BCYE) plates over 21 days at 30°C (Fig. 1A to E). Over the course of the experiment, all strains displayed comparable surface spreading with no significant differences. However, deletion of *mip* resulted in a reduction of the surfactant area by 60% (Fig. 1B and F). In the absence of *ppiB*, this area was reduced by 25% compared
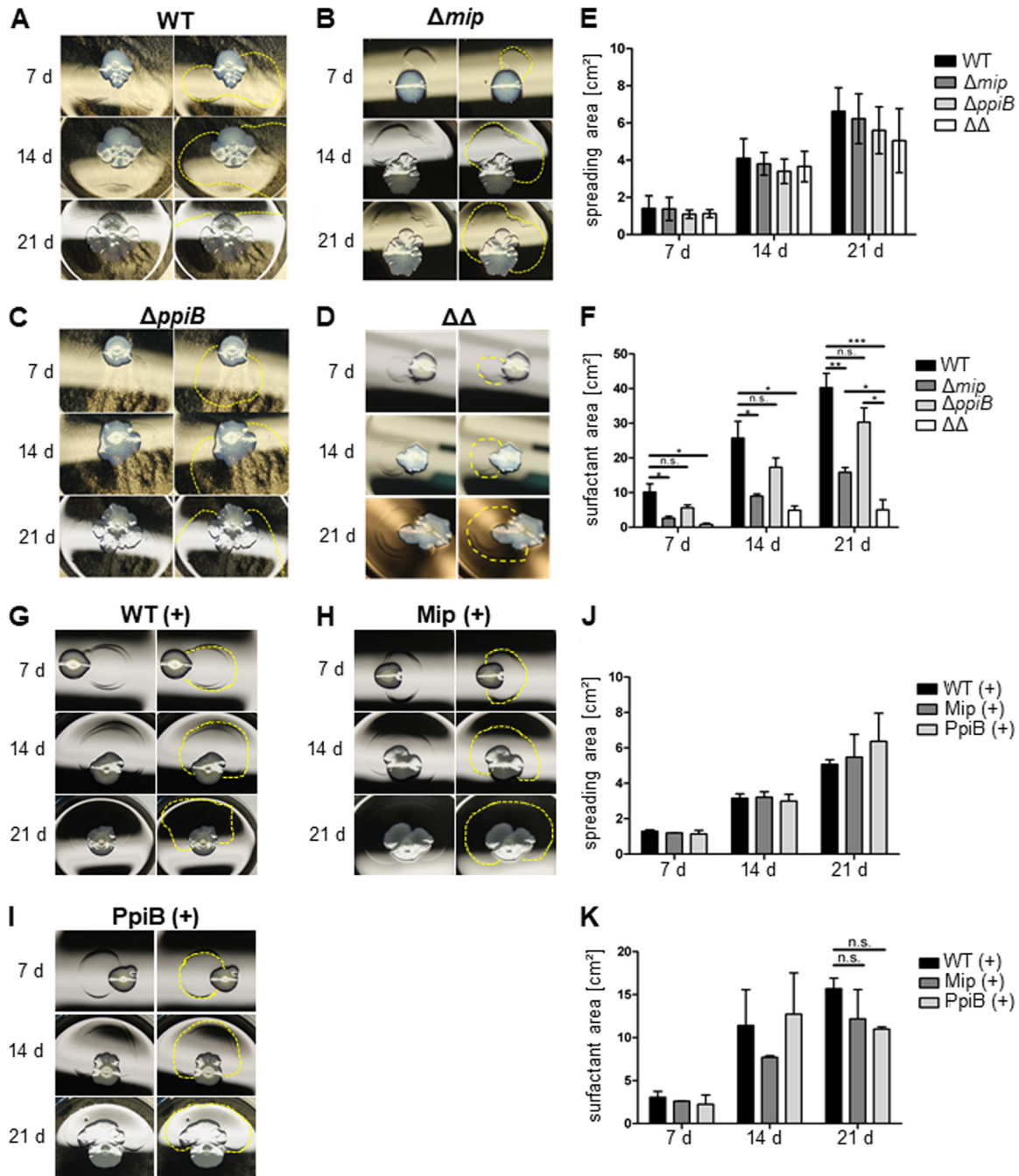
**FIG 1** The surface translocation of *L. pneumophila* depends on Mip and PpiB. (A to D) *L. pneumophila* wild-type (WT) (A), Δ*mip* (B), Δ*ppiB* (C), and Δ*mip* Δ*ppiB* (ΔΔ) (D) strains were grown on BCYE plates containing 0.5% agar at 30°C for 7, 14, and 21 days (d). For each time point and strain, a representative picture from three independent experiments performed in triplicate is presented on the left, while the boundaries of the surfactant film are highlighted on the right. (E) Quantification of the spreading area of the strains revealed no significant differences over 21 days. (F) In the case of the surfactant area, PPIase-negative Δ*mip* and Δ*mip* Δ*ppiB* strains showed a significant decrease in the secretion of surfactants. (G to I) The defect in the formation of a surfactant film was complemented by introducing the respective gene in the single-gene-knockout mutants. (J and K) This was confirmed by quantitative measurement of the spreading and surfactant film areas. Shown are the means and standard deviations from three experiments performed in duplicate. Statistical significance was calculated using an unpaired Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; n.s., not significant).

to that for the wild type; however, this difference was not statistically significant (Fig. 1C and F). Interestingly, deleting both PPIases resulted in a surfactant film 90% smaller than that of the wild type after 21 days (Fig. 1D and F). This phenotype was not dependent on different growth rates, as the surface spreading of all mutants was
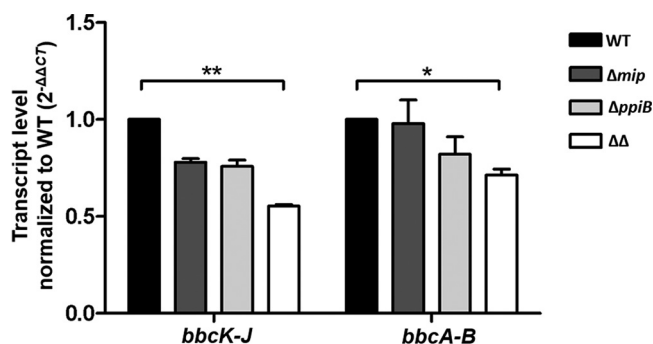
**FIG 2** Transcription of the *bbc* loci is affected by Mip and PpiB. Twenty nanograms of total RNA isolated from each strain grown on low-agar BCYE plates was subjected to qRT-PCR. As assessed by the transcript level normalized to that for the wild type, a significant decrease in the transcript level for both surfactant biosynthesis operons was measured in the Δ*mip* Δ*ppiB* mutant. Shown are the mean and standard deviation of three independent measurements from at least two separate RNA isolations. Statistical significance was calculated using an unpaired Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$).

comparable over the course of the experiment (Fig. 1E and F). Introduction of the respective genes into the *mip* or *ppiB* deletion mutants reverted the phenotype of surfactant film formation (Fig. 1G to I) and resulted in comparable spreading and surfactant film areas in all strains (Fig. 1J and K). In order to exclude the possibility of the involvement of the second annotated cyclophilin of *L. pneumophila*, PpiA, in sliding motility, we also created a *ppiA* single deletion mutant and a *ppiA ppiB* double deletion mutant. The Δ*ppiA* mutant was as effective as the wild type in forming a surfactant film, and the Δ*ppiA* Δ*ppiB* double mutant showed no significant difference from the Δ*ppiB* single mutant (see Fig. S1 in the supplemental material).

In *L. pneumophila*, the biosynthesis of surfactant depends on two separate operons, *bbcABCDEF* and *bbcGHIJK* (36). In order to evaluate whether the deletion of *mip* and/or *ppiB* influences the expression of these gene clusters, we performed quantitative reverse transcription-PCR (qRT-PCR), using primers targeting the overlapping regions of the first two genes in the respective operons, on RNA isolated from bacteria that were grown on 0.5% (wt/vol) agar containing BCYE medium. The comparison of the target mRNA concentration by the threshold cycle ($C_T$) method showed that in all PPIase mutants the transcript level for both operons was negatively affected. However, the levels of the *bbcABCDEF* and *bbcGHIJK* transcripts were significantly reduced by only approximately half in the Δ*mip* Δ*ppiB* mutant (Fig. 2).

**Mip and PpiB confer tolerance to suboptimal temperatures in *L. pneumophila*.** The cyclophilin PpiB of *L. pneumophila* was found to promote growth at low temperatures (34). In this work, we analyzed if the PPIase Mip also plays a role in growth at low temperatures. Moreover, we tested whether PpiB and Mip influence growth at high temperatures. For this, serial dilutions of each strain were applied onto BCYE agar plates and incubated at various temperatures. No growth difference was observed between the knockout strains and their isogenic wild type at 37°C or 30°C (Fig. 3A and B). However, at 17°C, both the Δ*mip* and Δ*ppiB* strains displayed a reduced growth capability compared to that of the wild type. While the single deletion mutants were affected in their growth to the same degree, the Δ*mip* Δ*ppiB* double mutant showed almost no growth at the highest bacterial density (Fig. 3C). Complementing the single deletion mutants with the respective gene reverted the phenotype to a level comparable to that for the wild type (Fig. 3C). At 44°C, deleting either of the PPIases caused a severe growth deficiency, which could again be complemented by the reintroduction of *mip* or *ppiB* (Fig. 3D). Similar to the findings for sliding motility, no substantial differences in growth at different temperatures, with the exception of that at 44°C, were observed between the Δ*ppiA* and the Δ*ppiA* Δ*ppiB* mutants. The mutant lacking both cyclophilins was also impaired in its growth at high temperatures (Fig. S2).

**Intracellular replication in the environmental host *A. castellanii* depends on the concerted action of PPIases Mip and PpiB.** In previous studies, decreased intracellular
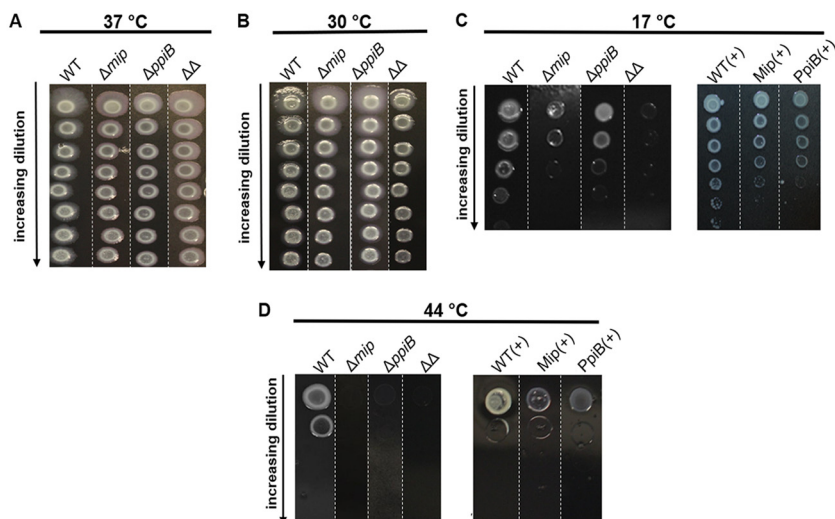
**FIG 3** Growth of different *L. pneumophila* PPIase-negative mutants at suboptimal temperatures. Bacteria grown in liquid culture were spotted in increasing 10-fold dilutions onto BCYE agar starting at 10⁹ bacteria/ml and grown at 37°C (A), 30°C (B), 17°C (C), or 44°C (D). Shown are representative pictures from three separate experiments performed in triplicate. Representative sections of bacterial plates from three separate experiments are shown and aligned for a better comparison of the strains. Single sections originating from different plates are separated by white dashed lines.

growth in *A. castellanii* was shown for the Mip-negative mutant strain (39, 40), while a *ppiB*-negative strain had a reduced rate of intracellular replication due to a decrease in invasion (35). In this study, we analyzed if the Δ*mip* Δ*ppiB* mutant strain shows an enhanced effect in intracellular replication compared to the single mutant strains. As a control, the Δ*dotA* type IV secretion system-deficient mutant strain was used (Fig. 4). We could confirm the reduced intracellular replication for the Δ*mip* strain (Fig. 4A). After 24 h of replication, the number of CFU of the Δ*ppiB* strain was reduced 5 times compared to that of the wild type and was 2.9 times higher than the number of CFU of the Δ*mip* strain. After 24 h the double-knockout strain showed a 14 times reduced number of CFU compared to that of the *mip* deletion strain, and after 48 h it showed a 200 times reduced number of CFU compared to that of the *mip* deletion strain (Fig. 4A). In its final outcome, the replication of the double mutant strain was comparable to that of the avirulent Δ*dotA*-negative mutant strain. Complementation of the strains in *trans* restored the intracellular replication ability (Fig. 4B). Deleting *ppiA* had no detrimental effect on the intracellular replication of *L. pneumophila* in *A. castellanii* (Fig. S3A).

**Mip and PpiB contribute to bacterial replication in HLTEs and THP-1 macrophages.** In order to evaluate the impact of Mip and PpiB in human lung tissue, we infected tumor-free human lung tissue explants (HLTEs) from five different donors with the wild-type, Δ*dotA*, Δ*mip*, Δ*ppiB*, or Δ*mip* Δ*ppiB* strain. The infected tissue was weighed, homogenized, and plated on BCYE medium at different time points. After 24 h, all mutant strains grew more slowly than the wild-type strain. This trend continued over the following 24 h, whereby the Δ*ppiB* mutant reached a higher number of CFU per gram of tissue than the Δ*mip* mutant and the Δ*mip* Δ*ppiB* mutant. The wild-type strain replicated at 48 h postinfection (p.i.), on average, 17.5-fold, whereas the Δ*mip* mutant replicated only 4-fold, the Δ*ppiB* mutant 5.7-fold, and the Δ*mip* Δ*ppiB* mutant 6.6-fold (Fig. 5A).

Alveolar macrophages constitute the main host cell type for *L. pneumophila* in human lungs (34). Accordingly, in order to verify whether the observed replication defect in HLTEs is due to the impairment in the infection of macrophages, we tested the PPIase mutants in the human macrophage-like cell line THP-1. The comparison of the total replication at 48 h p.i. showed that, although they were not as attenuated as the Δ*dotA* mutant, all PPIase mutants were impaired in their intracellular replication. The Δ*mip*
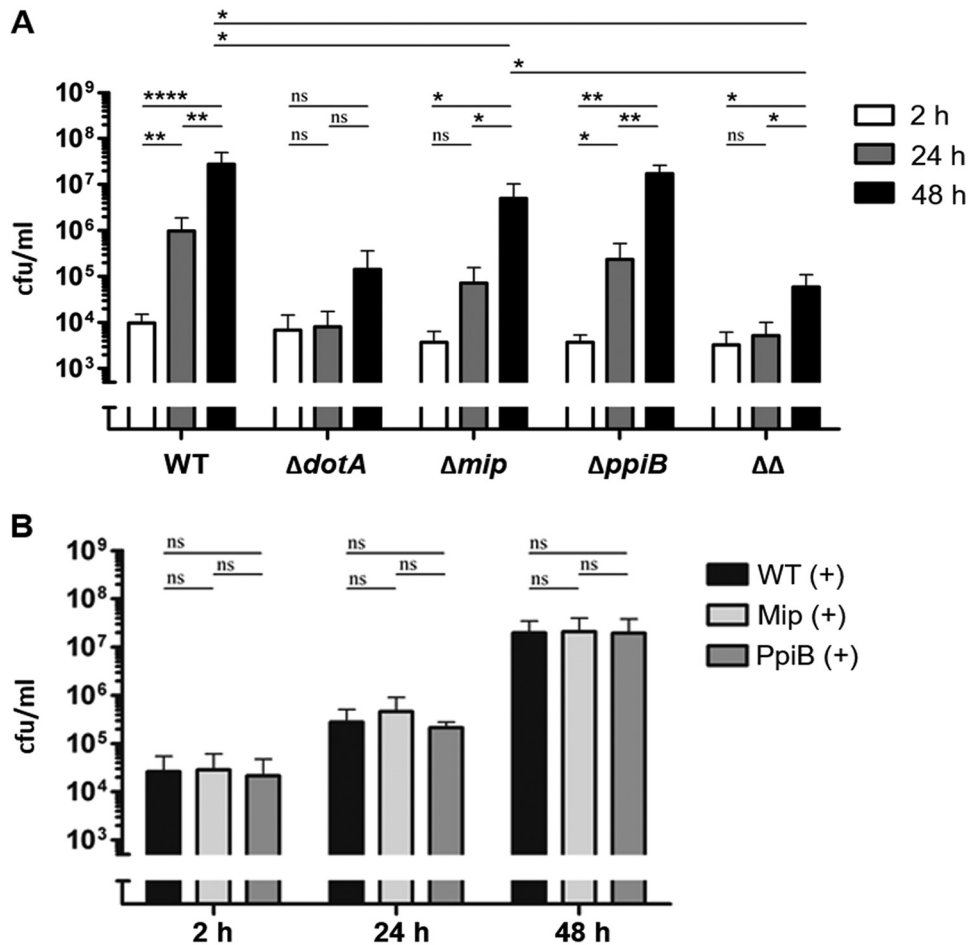
FIG 4 Replication of PPIase mutant strains in *A. castellanii*. *A. castellanii* was infected with different *L. pneumophila* mutant strains. After 2 h, 24 h, and 48 h, the amoebae were lysed and dilutions were plated out on BCYE agar plates. (A) Infection with the wild-type strain (WT), the Δ*dotA* mutant (Δ*dotA*), the *mip*-negative strain (Δ*mip*), the *ppiB*-negative strain (Δ*ppiB*), and the *mip*- and *ppiB*-negative double mutant strain (ΔΔ). The PPIase mutants showed decreased intracellular growth. (B) Complementing the single-knockout mutants with the respective gene restored the wild-type phenotype. Shown are the means and standard deviations from three experiments performed in triplicate. Statistical significance was calculated by an unpaired Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.0001$; n.s., not significant).

mutant replicated about 100 times less than the wild type, while the Δ*ppiB* mutant was about 200 times less replicative in the macrophages than the wild type. Interestingly, the strongest replication defect was observed in the Δ*mip* Δ*ppiB* mutant with, on average, a 400 times reduced final replication rate (Fig. 5B). The complementation of the mutant strains with the respective gene restored the growth in the initial 24 h (Fig. 5C). Also, in contrast to the deletion of *ppiB*, the deletion of *ppiA* had no effect on the intracellular replication of *L. pneumophila* in THP-1 cells (Fig. S3B).

## DISCUSSION

Initially, PPIases were postulated and subsequently identified to be enzymes that catalyze the *cis/trans* isomerization of peptidyl-prolyl bonds (23, 24, 41). Nowadays, it is known that the functions of PPIases are not restricted to this isomerization alone but also cover folding, transport, and the activity of proteins by means of chaperoning and protein-protein interactions (25). By this, they are connected to many physiological processes and diseases (42). Nevertheless, the decryption of the physiological role of PPIases is difficult due to their pleiotropic phenotypes and the absence of strong phenotypes in single deletion mutants of the respective PPIases (21, 43–45). The latter
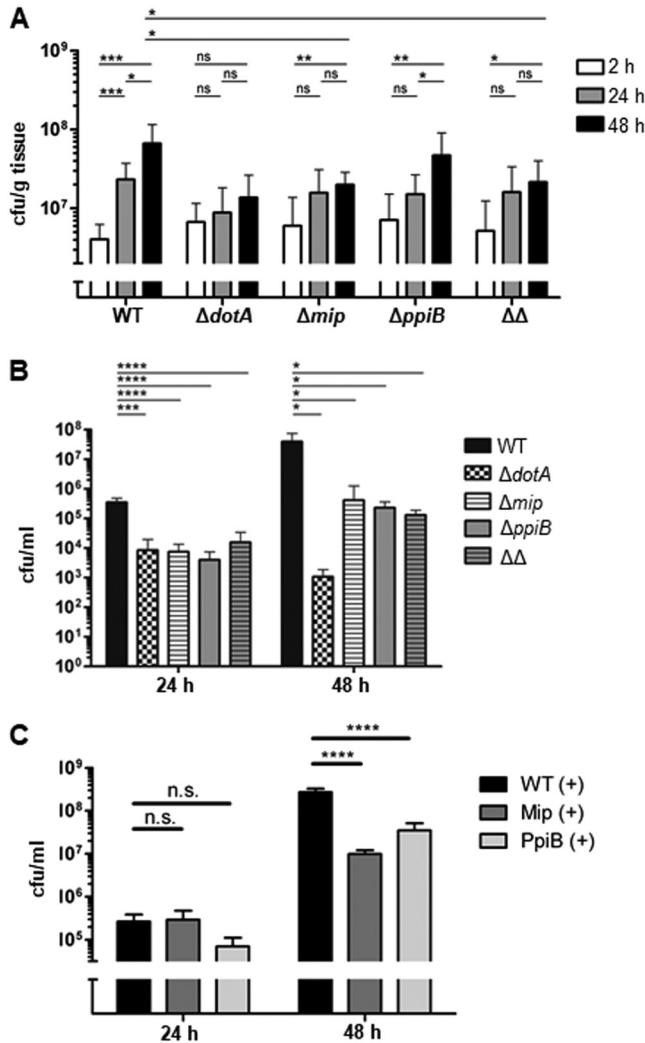
**FIG 5** Replication of PPIase mutant strains in human lung tissue explants (HLTEs) and THP-1 macrophages. (A) HLTEs were infected with the wild-type strain (WT) and the Δ*dotA*, Δ*mip*, Δ*ppiB*, and Δ*mip* Δ*ppiB* (ΔΔ) *L. pneumophila* mutants. Shown are the means and standard deviations from experiments performed in duplicate with tissue from five different donors. (B) Infection of THP-1 macrophages with wild-type, Δ*dotA*, Δ*mip*, Δ*ppiB*, and Δ*mip* Δ*ppiB* (ΔΔ) *L. pneumophila* strains. PPIase mutants showed decreased intracellular growth. Shown are the means and standard deviations from three experiments performed in triplicate. Statistical significance was calculated by an unpaired Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; n.s., not significant).

suggests that their activity is redundant with that of other PPIases and, hence, may be compensated for fully or partially by the activity of the others PPIases (42).

The Mip protein of *L. pneumophila* was the first microbial PPIase shown to promote virulence and as such is a prototypic PPIase (14, 29, 32). Studies about the contribution of the other five PPIases to the virulence and fitness of *L. pneumophila* have remained preliminary. Only the cyclophilin PpiB and, to a lesser extent, its homolog, PpiA, have been analyzed in the context of cold adaptation, as the former accumulates in culture supernatants of *L. pneumophila* isolates that are grown at 17°C (34). Accordingly, we aimed in our current study at expanding our understanding of the influence of PpiB on extra- and intracellular virulence-related attributes, including temperature tolerance, sliding motility, and infection of protozoan and human hosts. Also, we evaluated to what extent PpiB and Mip are redundant in their action and can compensate for the absence of the other.

We first focused on the extracellular virulence properties sliding motility and tem-

perature sensitivity. Motility is an important feature that contributes to the colonization and dissemination of bacteria in different habitats, and several PPIases were shown to be involved in bacterial motility. The parvulin PrsA2 contributes to the swimming motility of *Listeria monocytogenes*, as a PrsA2-deficient mutant displays reduced motility (46). A similar influence was observed for the Mip-like protein in *Burkholderia pseudomallei* during swarming motility (47). Recently, PpiB of *Escherichia coli* was shown to be a negative regulator of swarming and swimming motility as well as biofilm formation, and several cytosolic interaction partners that reverted the observed phenotypes to varying degrees were identified (48). However, for none of these bacteria are the molecular mechanisms by which the corresponding PPIase affects the degree of motility understood. In this study, we could show that the sliding motility of *L. pneumophila* is promoted by the FKBP Mip and the cyclophilin PpiB. Sliding motility is a special kind of bacterial movement that occurs on a secreted surfactant film where a reduced surface tension is utilized in order to facilitate passive spreading (49, 50). Hence, our studies show for the first time that PPIases influence flagellum-independent motility in *L. pneumophila*.

Deleting Mip or PpiB individually resulted in a significant decrease of the surfactant film size which was stronger than the reduction in colony size. Among the single mutants, the Δ*mip* strain produced a smaller surfactant film than the Δ*ppiB* strain. Interestingly, deletion of both PPIases resulted in the smallest surfactant film, which suggests that both PPIases contribute in an additive manner to the formation of the surfactant film. In *L. pneumophila*, TolC-dependent secretion and the TIISS were shown to act jointly, where the lipids forming the surfactant were secreted in a TolC-dependent manner, while the TIISS delivered the signal for secretion (36). Accordingly, it would be interesting for future studies to determine whether Mip or PpiB influences the formation of the surfactant film by affecting secretion systems or translocated factors. Interestingly, for the first time we were able to show that deleting *mip* and *ppiB* also has transcriptional consequences, as the transcript levels of the two main biosynthetic gene clusters were significantly reduced in the mutant lacking both genes. Until now, no link between bacterial PPIases and gene regulation has been reported, to the best of our knowledge. Here is the question: is the downregulation directly dependent on one or both of the PPIases, or is it a result of secondary effects due to other, PPIase-dependent but not yet identified regulators?

Next, we monitored the influence of Mip and PpiB on growth at suboptimal temperatures. The temperature optimum for *L. pneumophila* growth is 37°C, but the bacteria are versatile in this respect, since they can colonize freshwater habitats as well as technical water systems covering a broad temperature range (9, 51). At temperatures above 44°C, *L. pneumophila* forfeits its proliferative and respiratory capacity. At between 48.7 and 50°C, proliferation stops, but the metabolic activity still remains (52). In this study, we showed that both Mip and PpiB are important for the growth of *L. pneumophila* at 17 and 44°C. In the case of growth at 17°C, Mip and PpiB most likely compensate for the deficiency of each other, as single deletion mutants were affected to the same degree in their ability to grow. Only the deletion of both PPIases resulted in complete growth failure. This finding is supported by the findings of previous studies, where an accumulation of PpiB in the culture supernatants of *L. pneumophila* at 17°C was observed (34). At elevated temperatures, on the other hand, both PPIases seem to be equally important, as deleting either one of them diminished bacterial growth completely. The involvement of PPIases in growth at suboptimal temperatures is also known from other species. The PPIase PpiB from *Bacillus subtilis* works as a cold shock protein (53). On the contrary, PpiD of *Escherichia coli* plays a role under heat shock conditions. Additionally, the deletion of PpiD leads to an upregulation of the $\sigma^E$ stress response due to an accumulation of misfolded proteins in the periplasm (54). Furthermore, in previous studies with *E. coli*, a functional redundancy of the FKBP FkpA and the parvulin SurA was shown. These PPIases with chaperone activity play an important role in outer membrane protein biogenesis and compensate for each other in order to circumvent unfolded protein stress under heat shock (55).

**TABLE 1** Wild-type strains and mutant *L. pneumophila* strains used in this study

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| **Strains** | | |
| *L. pneumophila* Corby | Wild-type strain | 61 |
| *L. pneumophila* Corby Δ*dotA* | *dotA*::Tn*5* mutant of *L. pneumophila* Corby | Kindly provided by Antje Flieger, Robert-Koch-Institut, Wernigerode, Germany |
| *L. pneumophila* Corby Δ*mip* | *mip*::*nptI* mutant of *L. pneumophila* Corby | 40 |
| *L. pneumophila* Corby Δ*ppiB* | *ppiB*::*nptI* mutant of *L. pneumophila* Corby | This study |
| *L. pneumophila* Corby Δ*mip* Δ*ppiB* (ΔΔ) | *mip*::*nptI* and *ppiB*::*cat* mutant of *L. pneumophila* Corby | This study |
| *L. pneumophila* Corby Δ*ppiA* | *ppiA*::*aac1* mutant of *L. pneumophila* Corby | This study |
| *L. pneumophila* Corby Δ*ppiA* Δ*ppiB* | *ppiA*::*aac1* and *ppiB*::*nptI* mutant of *L. pneumophila* Corby | This study |
| **Complemented strains** | | |
| WT(+) | Wild-type strain carrying empty pBCKS plasmid | This study |
| Mip(+) | *mip*::*nptI* mutant of *L. pneumophila* Corby carrying pEWM103 | 40 |
| PpiB(+) | *ppiB*::*nptI* mutant of *L. pneumophila* Corby carrying pJRA1 *ppiB* cloned in pBCKS with SacI and KpnI | This study |
| pBCKS complementation vector | pUC *ori* Cm$^r$ (*cat*) | Stratagene (62) |

Previous studies already established the importance of Mip in the early phases of the intracellular infection cycle in amoebae as well as human macrophages and in a guinea pig infection model (29, 30, 39). Here, we show that out of the two PPIases, Mip seems to be more relevant for the infection of a wide range of hosts, including the complex human lung tissue, which is demonstrated here for the first time. PpiB on its own seems to have a minor role or might be fully compensated for by Mip in the context of the intracellular infection of amoebae. However, especially in the case of the natural host *A. castellanii*, a clear additive effect of both PPIases on infection efficiency could be observed, as the double-knockout mutant was severely impaired in its replication. Surfactant production is dispensable for infecting amoebae, human macrophages, or epithelial cells as well as A/J mice (36). Hence, attenuation of the PPIase mutants in infection models must be due to additional defects in protein folding or secretion. Thus, the fact that the replication levels of the Δ*mip* Δ*ppiB* mutant remained comparable to those of the Δ*dotA* TIVSS-deficient mutant raises the question of whether there might be an interconnection between these PPIases and the type IV secretion apparatus or the effectors that are secreted by it.

Taken together, we show for the first time that the PPIases Mip and PpiB together are important for extra- and intracellular virulence properties. They do so either in a synergistic manner, as in the case of sliding motility, or in a compensatory manner, as in the case of cold adaptation. This, furthermore, impacts the ability of *L. pneumophila* to infect its host cells and has implications for the course and outcome of the human lung infection.

## MATERIALS AND METHODS

**Bacterial strains and culture.** *L. pneumophila* Corby and all mutant strains (listed in Table 1) were cultured in buffered yeast extract broth (YEB) to the early stationary phase at 37°C and 200 rpm or on buffered charcoal yeast extract (BCYE) agar at 37°C for 3 days (30). When needed, 12.5 $\mu$g/ml chloramphenicol or 20 $\mu$g/ml kanamycin was used for selection.

**Construction of isogenic *L. pneumophila* knockout mutants.** For analysis of *L. pneumophila* PpiB, isogenic knockout mutants of the wild type and the Mip mutant strain were generated via homologous recombination as previously described (56). Up- and downstream flanking regions of *ppiB* with overhangs of 1 to 1.5 kb complementary to the 5′ and 3′ regions of the open reading frame of the respective resistance gene were amplified and merged together in a joining PCR. Naturally competent bacteria were transformed by adding 2 $\mu$g of the purified linear knockout construct to 1 ml of a bacterial suspension with an optical density at 600 nm (OD$_{600}$) of 0.8 to 1.0 and incubating at 30°C for 3 days. Depending on the selection marker, transformants were selected on BCYE agar containing 20 $\mu$g/ml kanamycin and 12.5 $\mu$g/ml chloramphenicol or 4 $\mu$g/ml gentamicin. The resulting clones were checked via PCR and sequencing.

**RNA isolation and qRT-PCR.** For comparing the expression levels of genes that are involved in surfactant production, the *L. pneumophila* Corby wild type and its isogenic mutants were grown on BCYE plates with 0.5% (wt/vol) agar for 3 weeks at 30°C. The RNA of the bacteria was isolated, using an RNeasy kit from Qiagen, by resuspending a loopful of bacteria in 350 $\mu$l RLT+ buffer (Qiagen) and proceeding by following the instructions in the manufacturer's manual. The expression levels of the *bbc* operons, which are responsible for the production of *Legionella* surfactant, were compared between the strains by

**TABLE 2** Oligonucleotides used in this study

| Name | Sequence | Reference or source |
|---|---|---|
| PpiB_F1 | CATGAGCTCCTGATGATTTGGCACCTGTTATG | This study |
| PpiB_R1 | AAGGTACCAGGAAGATCCCTCTTCCATACT | This study |
| PpiB_Cm_F | ATGGAGAAAAAAATCACTGGA | This study |
| PpiB_Cm_R | TTACGCCCCGCCCTG | This study |
| PpiB_UFF | ATTCCTGGCATTAAACGCGTG | This study |
| PpiB_DFR | CCTGCGACAAATAGTATTGCAG | This study |
| PpiB_UFR_Kan | GTTTCCCGTTGAATATGGCTCATCATGGATGTAGAAATTAAAACCATTATT | This study |
| PpiB_DFF_Kan | ATTTGATGCTCGATGAGTTTTTCTAAGAGGTTAGAGAACTGGCTTAG | This study |
| PpiB_UFR_Cm | TCCAGTGATTTTTTTTCTCCATCATGGATGTAGAAATTAAAACCATTATT | This study |
| PpiB_DFF_Cm | CAGGGCGGGGCGTAAGAGGTTAGAGAACTGGCTTAG | This study |
| PpiA_UFF | GGCAAAGGCGATAAGTCATACTC | This study |
| PpiA_UFR_Gm | CATCGTTGCTGCTGCGTAACATAATAAGCTCTCCATAGAATGGCTAAG | This study |
| PpiA_DFF_Gm | GACCCAAGTACCGCCACCTAAAAGATCAGTGCCTGTGTCAGATTAG | This study |
| PpiA_DFR | CGAAAAAGAGTTGGTTAATGAGCC | This study |
| PpiA_Gm_F | ATGTTACGCAGCAGCAACGATG | This study |
| PpiA_Gm_R | TTAGGTGGCGGTACTTGGGTC | This study |
| Lpc1693_rtF | CTGGCGAAGGCTCTCACTCA | 36 |
| Lpc1694_rtR | TCACGCAGCACAAATTCGCA | 36 |
| Lpc1699_rtF | CAGTCAGTGGTGGTCTGCCT | 36 |
| Lpc1700_rtR | TGTACGAGAGGGCTGCTTGG | 36 |
| gapA_rtF | CGCATCGCAAATCCGTCCAA | This study |
| gapA_rtR | GCGTGATCCAGCCAAACTGC | This study |

quantitative reverse transcription-PCR (qRT-PCR). For this, 20 ng total RNA of each strain was reverse transcribed and amplified in 40 cycles using a Luna Universal one-step reverse transcription-quantitative PCR kit (NEB) following the manufacturer's instructions. The primer sequences for the reverse transcription of parts of the *bbc* operons were published previously (36) (Table 2). Primers targeting *gapA* (the gene for glyceraldehyde 3-phosphate dehydrogenase) were used in order to confirm template uniformity between samples and in order to calculate the relative change in gene expression as the ratio of normalized target concentrations by the threshold cycle ($\Delta\Delta C_T$) method (57).

**Sliding motility.** Sliding motility assays with the *L. pneumophila* wild type and its isogenic mutant strains were performed as described previously (58). All strains were cultured to the early stationary phase ($OD_{600}$, 2.8 to 3), and 10 $\mu$l of the cultures was spotted on BCYE agar plates containing 0.5% agar and incubated at 30°C for 7, 14, and 21 days. To assess the impact of PPIases on the surface translocation of *L. pneumophila*, images were taken with a Canon 450D camera and an EFS 18- to 55-mm macro lens. The sizes of the surfactant film area and colony area in relation to the area of the petri dish were calculated using Adobe Photoshop CS5 extended (version 12.0.4).

**Growth temperature assay.** The growth of the *L. pneumophila* wild type and its isogenic PPIase mutants at different temperatures was monitored as previously described (34). Briefly, stationary-phase cultures were adjusted to $1 \times 10^9$ bacteria/ml and diluted to $10^8$, $10^7$, $10^6$, $10^5$ $10^4$, $10^3$, and $10^2$ bacteria/ml. An amount of 10 $\mu$l of all dilutions was dropped on a BCYE agar plate and incubated for 3 to 5 days at 17°C, 37°C, and 44°C. Growth on the agar plates was documented by taking pictures with a Canon 450D camera and an EFS 18- to 55-mm macro lens.

**Infection of *Acanthamoeba castellanii* with *L. pneumophila*.** *A. castellanii* (ATCC 30234) was grown and passaged every 3 to 4 days in peptone-yeast extract-glucose medium at 22°C and was used for infection as previously described, with slight modifications (40). To analyze the intracellular replication of the *L. pneumophila* mutant strains in *A. castellanii*, 5 ml of the amoeba was seeded into 25-cm² cell culture flasks (TPP) at a concentration of $5 \times 10^5$ cells/ml in amoeba buffer and infected at a multiplicity of infection (MOI) of 0.5. After 2, 24, and 48 h, the cells were detached by knocking the flask. A 200-$\mu$l aliquot was transferred from each flask into 1.5-ml tubes, and the cells were lysed mechanically by centrifuging at $20,000 \times g$ for 5 min, followed by vigorous vortexing for 15 s. Serial dilutions were prepared with water and plated on BCYE agar plates. Bacterial colonies were counted after cultivation for 4 days at 37°C, and the number of CFU per milliliter was plotted in relation to time.

**Infection of a THP-1 macrophage-like cell line with *L. pneumophila*.** To analyze the intracellular replication of *L. pneumophila* mutant strains in human macrophages, the acute monocytic leukemia cell line THP-1 (DSMZ ACC16) was used (59). The cells were adjusted to $5 \times 10^5$ cells/ml in cell culture medium (RPMI 1640, 2 mM L-glutamine, 10% fetal calf serum [FCS]) supplemented with 100 nM phorbol-12-myristate-13-acetate (PMA) for differentiation into macrophage-like cells. Two hundred microliters of this suspension was seeded into each well of a 96-well plate (TPP) and incubated at 37°C in 5% $CO_2$ for 48 h. Early-stationary-phase *L. pneumophila* wild-type and mutant strains were adjusted to $1 \times 10^6$ bacteria/ml in cell culture medium. Differentiated THP-1 cells were washed one time with prewarmed cell culture medium and infected at an MOI of 1. After 2, 24, and 48 h, the cells were lysed by adding Triton X-100 at a final concentration of 0.1% (vol/vol), and several dilutions were plated on BCYE agar plates and incubated at 37°C for 4 days. Bacterial colonies were counted, and the numbers of CFU per milliliter were plotted in relation to time.

**Infection of human tissue lung explants (HLTEs) with *L. pneumophila*.** Lung infections were performed as described previously (60). Briefly, tumor-free pulmonary tissue samples were obtained from surgery patients and infected with the *L. pneumophila* strains. For infection, the bacteria were adjusted to $10^7$ bacteria/ml in RPMI 1640 (Gibco, Darmstadt, Germany) with 10% fetal calf serum (FCS), 20 mM HEPES, and 1 mM sodium pyruvate. The samples were incubated at 37°C in 5% $CO_2$ for 2, 24, and 48 h. For determination of the number of CFU, duplicate samples from five donors were infected. At the indicated time points, samples were weighed and homogenized in deionized water. Dilutions were plated on BCYE agar and incubated at 37°C with 5% $CO_2$ for 4 days. The number of CFU per gram of tissue was calculated; means and standard deviations of the results for samples were compared by using an unpaired Student's *t* test.

**Ethics statement.** This study was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the Hannover Medical School (no. 2235-2014).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI .00939-17.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

## ACKNOWLEDGMENT

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