



The Tail-Specific Protease Is Important for *Legionella pneumophila* To Survive Thermal Stress in Water and inside Amoebae

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ABSTRACT *Legionella pneumophila* (*Lp*) is an inhabitant of natural and human-made water systems, where it replicates within amoebae and ciliates and survives within biofilms. When *Lp*-contaminated aerosols are breathed in, *Lp* can enter the lungs and may infect human alveolar macrophages, causing severe pneumonia known as Legionnaires' disease. *Lp* is often found in hot water distribution systems (HWDS), which are linked to nosocomial outbreaks. Heat treatment is used to disinfect HWDS and reduce the concentration of *Lp*. However, *Lp* is often able to recolonize these water systems, indicating an efficient heat shock response. Tail-specific proteases (Tsp) are typically periplasmic proteases implicated in degrading aberrant proteins in the periplasm and important for surviving thermal stress. In *Lp* Philadelphia-1, Tsp is encoded by the *lpg0499* gene. In this paper, we show that Tsp is important for surviving thermal stress in water and for optimal infection of amoeba when a shift in temperature occurs during intracellular growth. We also demonstrate that Tsp is expressed in the postexponential phase but repressed in the exponential phase and that the *cis*-encoded small regulatory RNA Lpr17 shows the opposite expression, suggesting that it represses translation of *tsp*. In addition, our results show that *tsp* is regulated by CpxR, a major regulator in *Lp*, in an Lpr17-independent manner. Deletion of CpxR also reduced the ability of *Lp* to survive heat shock. In conclusion, our study shows that Tsp is likely an important factor for the survival and growth of *Lp* in water systems.

IMPORTANCE *Lp* is a major cause of nosocomial and community-acquired pneumonia. *Lp* is found in water systems, including hot water distribution systems. Heat treatment is a method of disinfection often used to limit the presence of *Lp* in such systems; however, the benefit is usually short term, as *Lp* is able to quickly recolonize these systems. Presumably, *Lp* responds efficiently to thermal stress, but so far, not much is known about the genes involved. In this paper, we show that the Tsp and the two-component system CpxRA are required for resistance to thermal stress when *Lp* is free in water and when it is inside host cells. Our study identifies critical systems for the survival of *Lp* in its natural environment under thermal stress.

KEYWORDS *Legionella pneumophila*, tail-specific protease, small regulatory RNA, thermal stress, *cis*-encoded sRNA, Legionnaires' disease, amoeba, CpxR

Legionnaires' disease (LD) is a severe form of pneumonia in humans caused by the Gram-negative bacterium *Legionella pneumophila* (*Lp*) (1). *Lp* is often responsible for nosocomial and community-acquired pneumonia (1). In the environment, *Lp* can be found in natural and human-made aquatic environments, such as cooling towers and water distribution systems, where it can replicate within phagocytic protozoa (2–4). Notably, *Lp* replicates within *Vermamoeba vermiformis* (formerly *Hartmannella*

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vermiformis), a thermotolerant amoeba commonly found in both natural and human-made water systems (5, 6). *V. vermiformis* protects *Lp* from predation, competition, and various disinfection methods such as heat treatments, potentially contributing to nosocomial infections (3, 5, 6). In addition, intracellular growth of *Lp* within amoeba increases its pathogenicity and facilitates the establishment of infection in humans (3). The main virulence factor of *Lp* is the type IVb secretion system called Icm/Dot, which translocates more than 300 effectors inside the host cells (7). These effectors are responsible for stopping the maturation of the phagosome and creating a specialized vacuole where *Lp* can grow (7).

The preferred growth temperature of *Lp* is between 25°C and 42°C, though it has been found in water systems at temperatures below 20°C or above 60°C (8). Its ability to live in a wide range of temperatures allows *Lp* to colonize hot water distribution systems (HWDS) (9). The higher temperature found within HWDS also reduces the microbial diversity, therefore making it easier for *Lp* to compete in these environments (10, 11). A method used to limit the proliferation of *Lp* in water systems is to maintain the temperature at the outlet above 55°C (8, 9). When a system is heavily colonized by *Lp*, it can be treated by superheat and flush, also referred to as pasteurization, consisting of increasing the temperature at the heater to about 75°C in order to provide a temperature of at least 65°C at the outlets (12).

An increase in temperature, even by a few degrees, can cause proteins to unfold, which leads to protein aggregates that can be lethal, as these aggregates accumulate in the cells (13, 14). Protein misfolding and aggregation result in an imbalance of protein homeostasis, which triggers the heat shock response (14). The canonical heat shock response is composed of two heat shock regulons, the σ^H and σ^E heat shock regulons (14). This response results in the production of chaperones and proteases to refold or destroy misfolded proteins and aggregates (14). In *Lp*, the two-component system LetA/S is the only regulatory system known to be important for surviving thermal stress (15).

Protein degradation by proteases is an important cellular function, as it allows the removal of aberrant proteins, the regulation of intracellular protein concentration; produces active molecules from precursors such as the alpha-toxin from *Staphylococcus aureus* or the heat-stable toxin produced by enterotoxigenic *Escherichia coli* (ETEC); and allows the cell to recycle amino acids during starvation (16, 17). Carboxyl-terminal proteases (CTPs) are serine proteases conserved in most Gram-negative bacteria (18–20). They can also be found in *Archaea*, Gram-positive bacteria, eukaryotes, viruses, as well as in organelles such as chloroplasts (20–22). CTPs are mostly located in the periplasm, though some are located in the cytoplasm, while others are secreted in the extracellular environment (19). CTPs are involved in several different processes in Gram-negative bacteria. *E. coli*'s CTP, called tail-specific protease (Tsp) and sometimes Prc, was the first bacterial CTP characterized and is involved in regulating peptidoglycan assembly (19). The assembly of the peptidoglycan layer plays a role in the bacteria's size and shape (23). This important process is dependent on penicillin-binding proteins (PBP) (20, 24). Tsp recognizes a sequence of amino acids located on the C-terminal end of the precursor of PBP-3, resulting in its activation (25, 26). As a result, the *E. coli*'s *tsp* mutant is sensitive to various antibiotics and thermal and osmotic stress, and it displays a filamentous morphology (25, 27). Overproduction of Tsp is detrimental to cell growth (25). The susceptibility to thermal stress is dependent on osmolarity. These phenotypes are attributed to increased permeability of the outer membrane in the *tsp* mutant (25, 28). The *tsp* mutant and the wild type (WT) expressed similar levels of GroEL and DnaK, two heat shock proteins, when exposed to thermal stress in high-osmolarity buffer (25). However, when the strains were in low-osmolarity buffer and exposed to thermal stress, the *tsp* mutant showed decreased expression of DnaK and almost no expression of GroEL (25). *E. coli*'s Tsp also targets MepS, a peptidoglycan hydrolase which cleaves peptide cross-links between the glycan chains of the peptidoglycan layer (29, 30). Tsp is

also able to degrade misfolded proteins in the periplasm, suggesting a role in maintaining protein homeostasis in the periplasm (26, 31, 32).

Pseudomonas aeruginosa codes two CTPs, called Prc and CtpA (19, 33). Prc is a homologue of *E. coli*'s Tsp and has been shown to degrade a mutant form of the anti-sigma factor MucA, preventing development of mucoidy (33–35). CtpA is involved in the proper function of the type 3 secretion system (T3SS), required for cytotoxicity in cultured cells and virulence in the animal model of acute pneumonia (19, 33). The activity of CtpA is dependent on LcbA, an outer membrane lipoprotein with tetratricopeptide repeat (TPR) motifs (36). CtpA degrades four peptidoglycan hydrolases, including MepM and PA4404, belonging to the M23 peptidase family, and PA1198 and PA1199, belonging to the NlpC/P60 peptidase family (36).

The *lpg0499* gene in *Lp* encodes a CTP named Tsp, most homologous to the *P. aeruginosa* CtpA (37). The *tsp* gene is upregulated 54-fold in the postexponential (PE) phase compared to the exponential (E) phase (38). The *tsp* gene was also strongly upregulated during infection of *Acanthamoeba castellanii* and THP-1 (38, 39). *tsp* is regulated by the *Legionella* quorum-sensing system, an important activator of genes required in the transmissive phase (40). Transcriptomic analysis of a *letS* mutant in water revealed that *tsp* is downregulated in the mutant (15). In addition, the expression of *tsp* was downregulated in a *cpxR* mutant grown to the PE phase, suggesting that CpxR is also required for expression of *tsp* in the PE phase (41). CpxR regulates the expression of several Icm/Dot effectors and is required for growth in *A. castellanii* (41–44). A previous study showed that a *tsp* mutant in the *Lp* serogroup 1 strain 130b did not have a growth defect in *Acanthamoeba castellanii*, THP-1 macrophages, or low-salt chemically defined media at 42°C (37). A small regulatory RNA (sRNA) named Lpr17 (Lppnc0140 in strain Paris) is encoded complementary to *lpg0499* (45, 46). sRNAs are short RNA molecules involved in posttranscriptional regulation of genes required for virulence and response to various conditions such as sugar metabolism, iron homeostasis, and biofilm formation (47, 48). Base-pairing sRNAs are the most common type of sRNA, and they act by hybridizing to their target mRNA. Lpr17 is a *cis*-encoded sRNA. Such sRNAs pair perfectly with their target due to their being encoded on the complementary strand of their target (47). Lpr17 is therefore likely to control *tsp* expression.

In this paper, we have investigated the role of the Tsp of *Lp* in the resistance to thermal stress in water and during infection of amoeba. In addition, we have studied the regulatory function of the *cis*-encoded sRNA Lpr17 as well as confirmed the role of CpxR in the regulation of Tsp.

RESULTS

Tsp is important for *L. pneumophila* to survive thermal stress. Since tail-specific proteases have been implicated in managing thermal stress in other bacteria, the importance of Tsp for the survival of *Lp* after a heat shock at 55°C was tested (Fig. 1). The strains were suspended in the freshwater artificial medium Fraquil and incubated for 24 h at room temperature prior to the temperature stress. Incubation in Fraquil induces a specific transcriptomic program related to starvation and long-term survival in water (49, 50). After 15 min at 55°C, the CFU count of the Δ *tsp* mutant decreased by 10,000-fold, while the CFU count of the WT and the complemented strain decreased by only 10-fold. Thirty minutes after thermal stress, the CFU count of the Δ *tsp* mutant was 100 times lower than the CFU count of the WT and complemented strain; however, this was not statistically significant ($P > 0.05$).

Tsp is important for intracellular multiplication in *V. vermiformis* following a temperature shift. Given the susceptibility of the Δ *tsp* mutant to thermal stress, we hypothesized that the ability of the mutant to grow inside amoeba could be compromised if a temperature shift occurs, which is likely to happen in HWDS. The ability of the Δ *tsp* mutant to replicate within *V. vermiformis* was then investigated (Fig. 2). The infection was therefore carried out at 3 different temperatures in parallel as follows: room temperature for 5 days, 37°C for 5 days, and room temperature for 2 days followed by 37°C for 3 days (referred to in the manuscript as a temperature shift). The

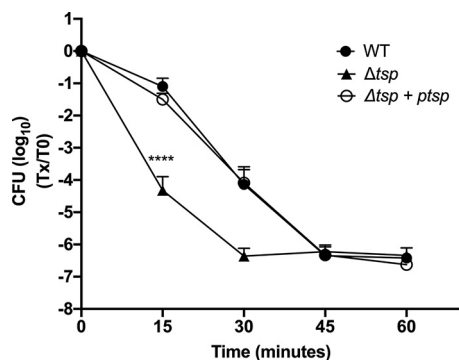


FIG 1 Tsp is required for *Lp* to survive a thermal stress in water. The WT, *tsp* mutant (Δtsp), and complemented strain ($\Delta tsp + ptsp$) were suspended in Fraquil for 24 h and then subjected to thermal stress at 55°C. The survival of the strains was measured by CFU counts every 15 min from 0 to 60 min. The data shown represent the average of three independent biological replicates with standard deviation. A two-way analysis of variance (ANOVA) with a Tukey correction for multiple comparison was used to determine statistical difference (****, $P < 0.0001$).

temperature shift would simulate the change in temperature encountered in HWDS, albeit to a lower degree, and would test the importance of Tsp for survival under these conditions. At room temperature, none of the strains tested were able to replicate intracellularly (Fig. 2A). At 37°C, all strains were able to replicate intracellularly as well as the WT, with the exception of the *dotA* mutant, the negative control (Fig. 2C), confirming what was previously reported (37). However, when the temperature was shifted from 25°C to 37°C 2 days after the start of the infection, the yield of intracellular bacteria of the Δtsp mutant at the end of the experiment was significantly lower than that of the WT and the complemented strain (Fig. 2B).

Lpr17 is expressed in the E phase. Next, we sought to investigate the regulation of Tsp. The small regulatory RNA Lpr17 is encoded antisense to the 5' end and promoter region of the *tsp* gene and the 3' end of *lpg0500*, which codes for a peptidase of the M23/M37 family (Fig. 3A). *cis*-Encoded sRNAs tend to regulate the gene encoded on the complementary strand (47). Therefore, the expression of the Lpr17 sRNA was analyzed by Northern blotting in the WT, *tsp* mutant (Δtsp), and complemented strain ($\Delta tsp + ptsp$) (Fig. 3B). To make the mutant, the *tsp* gene was replaced with a kanamycin resistance cassette, and, in the process, a section of the Lpr17 coding region, including its transcription start site (TSS), was also removed. The complementation in *trans* was done by cloning the full-length *tsp* with 441 bp upstream of the ATG to include the putative promoter on a plasmid. This fragment also contains the full-length *lpr17* gene. Lpr17 was only detected in the E phase by Northern blotting. The expression of Lpr17 is stronger in the complemented strain, probably caused by the copy number of the plasmid used for complementation. In the complemented strain, Lpr17 was also expressed in the PE phase, although much less than in the E phase.

***tsp* is transcribed in the E and PE phases.** Reverse transcriptase PCR (RT-PCR) was used to investigate when the *tsp* gene is transcribed and if the *tsp* and *lpg0500* genes are transcribed as part of a polycistronic mRNA (Fig. 3C). Amplification with a forward primer (499-R) and a reverse primer (499-F) within the *tsp* gene revealed that the gene is transcribed in both the E and PE phases. The 499-R primer, along with a forward primer within *lpg0500* (500-F), showed that the 2 genes are not expressed as polycistronic mRNA. Our RT-PCR results suggest that *tsp* is slightly more expressed in the PE phase than the E phase. Using RT-quantitative PCR (RT-qPCR), *tsp* is 4.3-fold more expressed in the PE phase than the E phase (Fig. 3D).

Tsp is expressed in the PE phase. In order to detect the expression of Tsp, the protein was tagged with a hexahistidine tag and cloned onto pXDC39, a derivative of pMMB207c lacking the *Ptac* promoter and *lacI^q* (51). This plasmid also encodes a full-length copy of *lpr17*. The expression of Tsp and Lpr17 was investigated by Western

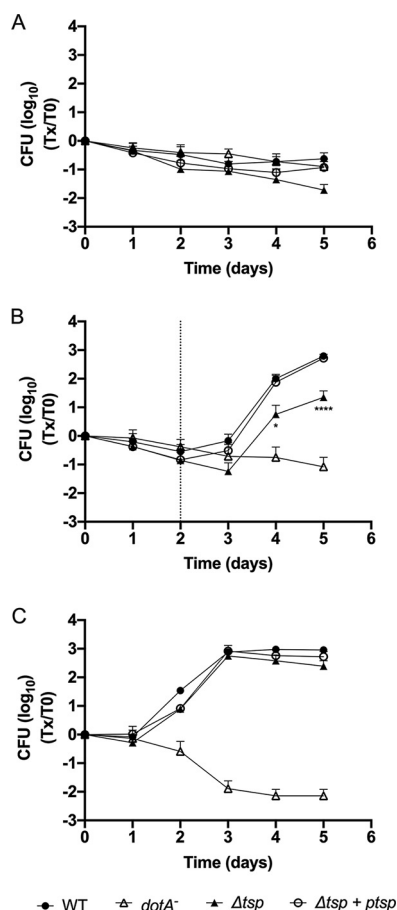


FIG 2 Tsp is required for intracellular multiplication in *V. vermiformis* during a temperature shift. Infection of the host cell *V. vermiformis* was carried out at room temperature for 5 days (A), at room temperature for 2 days and then at 37°C for 3 days (B), and at 37°C for 5 days (C). Host cells were infected with the WT, *tsp* mutant (Δ *tsp*), complemented strain (Δ *tsp* + *ptsp*), and the *dotA* mutant, a negative control, suspended in Fraquil. The dotted line in panel B represents the day on which the shift in temperature occurred. The data represent average and standard deviation of 6 biological replicates. A two-way analysis of variance (ANOVA) with a Tukey correction for multiple comparison was used to determine if the results were statistically different. The statistical significance shown compares the WT and *tsp* mutant (*, $P < 0.05$; ****, $P < 0.0001$).

blotting and Northern blotting, respectively, in the WT and the *tsp* mutant harboring the Tsp-His construct (*ptsp*-His) in the E and PE phases (Fig. 4). The WT and the *tsp* mutant containing an empty vector served as controls. As expected, the Lpr17 sRNA is more expressed in the E phase than the PE phase (Fig. 4A), similar to what was seen in the complemented strain (Fig. 3B). In contrast, the Tsp protein is detected only in the PE phase (Fig. 4B).

Lpr17 is repressed during thermal stress. Lpr17 is expressed in an opposite manner to Tsp: the sRNA is expressed in the E phase, while the protein is expressed only in the PE phase (Fig. 4). However, *tsp* seems to be transcribed in both the E and PE phases (Fig. 3C), and the relatively small increase in expression of the transcript between the E and PE phases is insufficient to explain this observation. This suggests that Lpr17 blocks expression of Tsp. Since Tsp is important for survival at 55°C, we hypothesize that Lpr17 should be repressed under this condition as well. Therefore, the expression of Lpr17 during thermal stress was analyzed by Northern blotting (Fig. 5). The strains were grown to the E phase in order to induce expression of Lpr17 and then subjected to thermal stress at 55°C. As seen in Fig. 5, Lpr17 sRNA was strongly repressed after 15 min at 55°C in the WT and complemented strains. This could be due to transcriptional

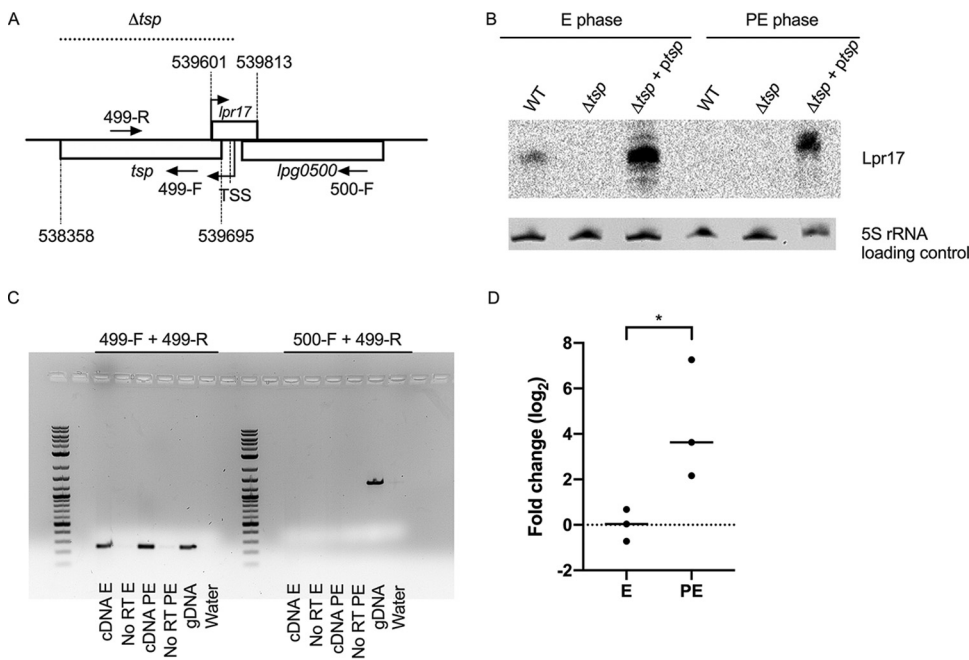


FIG 3 *cis*-Encoded Lpr17 sRNA is expressed in the E phase, while *tsp* is expressed in both the E and PE phases. (A) *tsp* is encoded downstream of *lpg0500*, and the Lpr17 sRNA is encoded on the complementary strand of *tsp* overlapping with its TSS and the promoter region. The coordinates of *tsp* and *lpr17* in the Philadelphia-1 genome are indicated. The dotted line indicates the portion of the genome that was replaced with a kanamycin resistance cassette in the Δtsp mutant. (B) Northern blotting was used to investigate the expression of Lpr17 in the WT, *tsp* mutant (Δtsp), and complemented strain ($\Delta tsp + ptsp$) grown to exponential phase (E) and postexponential phase (PE) in AYE broth. 5S rRNA was used as a loading control. (C) An RT-PCR was performed on cDNA from the WT strain grown to the E and PE phases in AYE broth to determine if *tsp* is encoded on a polycistronic operon with the upstream gene *lpg0500*. RNA from the E and PE phases that was not reverse transcribed (No RT) as well as water were used as a negative control; genomic DNA (gDNA) of the WT strain served as a positive control. (D) RT-qPCR was used to investigate the expression of *tsp* in the WT grown in AYE broth to the PE phase compared to the E phase. One microgram of RNA was used for reverse transcription. The C_t values of *tsp* were normalized against 16S rRNA. An unpaired *t* test was used to assess statistical significance (*, $P < 0.05$).

repression or increased sensitivity of the sRNA to RNases or both. Lpr17 is repressed whenever Tsp is produced or needed and supports the hypothesis that Lpr17 blocks translation of Tsp. Further experiments will be needed to confirm this possibility.

CpxR regulates *tsp* independently of Lpr17. A previous transcriptomic study has identified CpxR as a potential regulator of *tsp* in *Lp* (41) since the *tsp* gene was downregulated in the $\Delta cpxR$ mutant in the PE phase (41). In order to confirm that the downregulation observed also affects Tsp protein levels, the *ptsp-his* plasmid was electroporated in the $\Delta cpxR$ mutant, and the expression of Tsp in the $\Delta cpxR$ mutant was analyzed by Western blotting (Fig. 6A). In the PE phase, Tsp is not expressed in the absence of CpxR. Since the Lpr17 sRNA seems to negatively regulate the expression of Tsp, we hypothesized that the deletion of CpxR may cause overexpression of Lpr17, which would inhibit Tsp expression. The expression of Lpr17 was therefore analyzed by Northern blotting (Fig. 6B). The expression level of Lpr17 in the PE phase in the $\Delta cpxR$ mutant is similar to the WT, indicating that the regulation of Tsp by CpxR is Lpr17 independent.

The response regulator CpxR is important for surviving thermal stress. Since the *cpxR* mutant produces no Tsp in the PE phase, the ability of the *cpxR* mutant to survive thermal stress was investigated (Fig. 7). The *cpxR* mutation was complemented in *trans* by cloning the $\Delta cpxR$ gene under the control of a *Ptac* promoter in the vector pMMB207c, since *cpxR* is the fourth gene in a polycistronic mRNA. The *cpxR* mutant is unable to survive thermal stress compared to the WT and shows a much faster death than the wild type. Basal expression from the vector without addition of isopropyl- β -D-thiogalactopyranoside (IPTG) was enough to complement the mutation ($\Delta cpxR +$

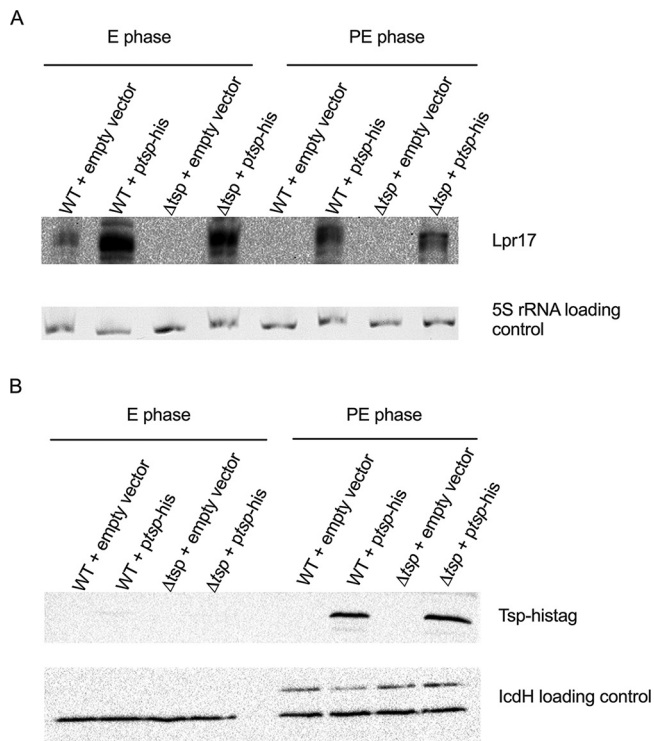


FIG 4 Tsp is expressed in the PE phase. Tsp was tagged with a hexahistidine tag and cloned in plasmid pXDC39 under its own promoter and transferred to the WT (WT + *ptsp*-His) and *tsp* mutant (Δ *tsp* + *ptsp*-His) strains. The strains were grown to the E and PE phases in AYE broth, and expression of the Lpr17 was analyzed by Northern blotting (A), while the expression of the Tsp was analyzed by Western blotting (B). The strains with the empty vector (WT + empty vector; Δ *tsp* + empty vector) served as negative controls. 5S rRNA and IcdH serve as the loading control for Northern blotting and Western blotting, respectively.

pcpxR). To ensure the complementation of the Δ *cpXR* mutant was due to the *cpXR* gene cloned, the *cpXR* mutant with the empty vector pMMB207c (Δ *cpXR* + pMMB207c) was also tested and showed a similar survival defect to the Δ *cpXR* mutant. The results were only significantly different after 15 min ($P=0.001$). The CFU counts for the three replicates at the later time points showed large variation, and the difference was not considered significantly different.

DISCUSSION

Similar to *tsp* mutants in other species, the *tsp* mutant of *Lp* is sensitive to thermal stress. The *tsp* mutant shows increased susceptibility to 55°C for 15 min (Fig. 1). The *tsp* mutant is also sensitive to changes in temperatures. During infection of *V. vermiformis*,

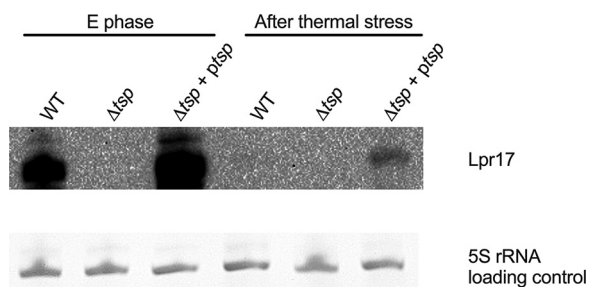


FIG 5 Lpr17 is repressed following thermal stress. The WT, *tsp* mutant (Δ *tsp*), and complemented strain (Δ *tsp* + *ptsp*) were grown to E phase in AYE broth and then subjected to heat shock at 55°C for 15 min. Samples for RNA extraction were taken before (E phase) and after thermal stress for Northern blot analysis.

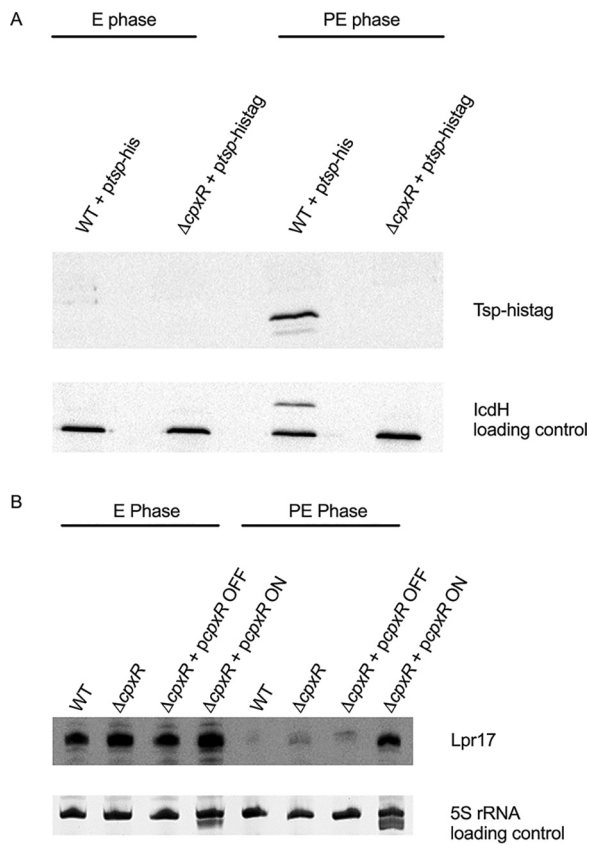


FIG 6 CpxR regulates Tsp in an Lpr17-independent manner. The WT and *cpxR* mutant Δ *cpxR* containing *ptsp*-His were grown to the E and PE phases and analyzed by Western blotting (A) and Northern blotting (B). 5S rRNA and IcdH were used as a loading control for Northern blotting and Western blotting, respectively.

shifting the temperature from 25°C to 37°C impacts the ability of the *tsp* mutant to reach a similar level to the WT at the end of the experiment (Fig. 2B). The *tsp* mutant is unable to reach the same yield achieved at 37°C without a temperature shift (Fig. 2C). The yield of the *tsp* mutant is similar to the WT when the temperature is maintained at

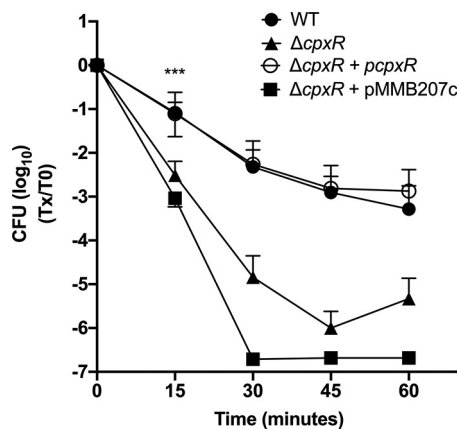


FIG 7 CpxR is required for *Lp* to survive a thermal stress. The WT, *cpxR* mutant (Δ *cpxR*), *cpxR* mutant with the empty vector (Δ *cpxR* + pMMB207c), and complemented strain (Δ *cpxR* + *pcpxR*) were suspended in Fraquil for 24h and then subjected to thermal stress at 55°C. The survival of the strains was measured by CFU counts every 15 min from 0 to 60 min. The data shown represent the average of three independent replicate with standard deviation. A two-way ANOVA with a Tukey correction for multiple comparison was used to determine if the results were significantly different. The statistical difference shown in Fig. 7 compares the WT and *tsp* mutant; ***, $P < 0.005$.

37°C throughout the infection (Fig. 2C). This phenotype could be the result of higher mortality of the *tsp* mutant within cells during the temperature shift, which would reduce the effective infection dose, or a reduced ability to grow intracellularly following the shift. Nevertheless, the ability of *Lp* to replicate within *V. vermiformis* despite shifts in temperature is likely important for its persistence in hot water distribution systems. Sporadic use of HWDS is likely to generate sections of lower temperature, such as in dead ends, that momentarily get warmer when the hot water flows through the system. In addition, increasing the temperature of the system is often used to disinfect hot water distribution systems (8). The amoeba cysts are able to survive treatments up to 80°C, and the ability of *Lp* to survive and replicate efficiently once the temperature decreases is important for recolonization of the water system (3).

Possibly, the absence of Tsp causes a disturbance in the cell membrane, resulting in increased sensitivity to thermal stress. This reasoning was previously suggested to explain decreased resistance to thermal stress of *tsp* mutants in *Borrelia burgdorferi*, *Escherichia coli*, and *Staphylococcus aureus* (20, 25, 52). Alternatively, accumulation of misfolded proteins in the *tsp* mutant could explain the inability to cope with thermal stress. It has previously been shown in other bacteria that Tsp degrades misfolded proteins that accumulate in the periplasm (26, 31, 32). Degradation of misfolded proteins increases the pool of amino acids available for *de novo* protein synthesis. Possibly, the accumulated misfolded proteins are not degraded in the absence of Tsp, leading to a decrease in the pool of amino acids (13, 14). This might explain the reduced yield of the *tsp* mutant during infection of amoeba following the temperature shift.

The *tsp* mutant is still able to replicate within amoeba to some extent following the temperature shift, but the yield, compared to the WT, is lower than when infection is carried out at a constant temperature of 37°C (Fig. 2). This suggests that in the absence of Tsp, other proteases are able to degrade misfolded proteins, thereby relieving the stress. The presence of an N-terminal secretion signal in Tsp was found using two bioinformatics software programs, SignalP and Phobius (53–55). This is consistent with *tsp* from other species that also have an N-terminal secretion signal required for transport to the periplasm (19). This suggests that the Tsp of *Lp* is located in the periplasm and could therefore degrade misfolded and aggregated proteins in the periplasm. The expression of alternate periplasmic proteases could explain the reduced yield compared to the WT observed following the temperature shift. In *E. coli*, the DegP periplasmic protease is important for surviving elevated temperatures and is responsible for degrading misfolded proteins and aggregated proteins in the periplasm (56–60). DegQ is a homolog of DegP that is also found in the periplasm and is responsible for degrading denatured proteins (61–63). In *Legionella*, the DegP homologue is required for surviving thermal stress but is not required for infection of amoeba (64). The infection was done at a constant temperature, and therefore, it is unknown if DegP plays a role during amoeba infection if a temperature change occurs (64). *Legionella* codes for a DegQ homologue; however, its role in surviving thermal stress has not been investigated (65, 66). In the absence of *tsp*, it is possible that these proteases also contribute to removing the aggregated and misfolded proteins at the temperature that occurs during amoeba infection. However, subjecting the strain to 55°C might be too big of a stress for the other proteases to compensate for the lack of *tsp*.

The CtpA of *P. aeruginosa* cleaves MepM, belonging to the M23 peptidase family. It is interesting to note that *tsp* is encoded downstream of *lpg0500*, which codes for an M23/M37 family peptidase. *Lp* codes for two other peptidases belonging to the M23/M37 family, *lpg0567* and *lpg0825*, the former being a homologue of MepM of *P. aeruginosa*. In addition, *Lp* codes for at least 10 TPR proteins, the same type of protein as the CtpA partner LcbA, suggesting one or some of these TPR proteins might be required for the activity of the Tsp of *Lp*, similar to what has been reported in *P. aeruginosa* (67).

cis-Encoded sRNAs, such as Lpr17, typically regulate genes coded on the complementary strand (47). The expression patterns of Lpr17 and Tsp are opposite from each other, where the sRNA is expressed only in the E phase (Fig. 3B), while the Tsp protein

TABLE 1 Strains, mutants, and plasmids used in this study

Strain, mutant, or plasmid	Relevant genotype	Reference or source
<i>Legionella pneumophila</i> Philadelphia-1 strains and mutants		
JR32	Philadelphia-1 derivative; Sm ^r ; r ⁻ ; m ⁺	74
KS79	JR32 $\Delta comR$	73
<i>dotA</i> mutant	JR32 <i>dotA::Tn903dlllacZ</i>	74
Δtsp (SPF365)	KS79 <i>tsp::Km</i> ; Km ^r	This study
$\Delta tsp + ptsp$ (SPF403)	Km ^r , Cm ^r	This study
KS79 + pXDC39 empty vector (SPF132)	Cm ^r	This study
KS79 + <i>ptsp</i> -His (SPF451)	Cm ^r	This study
$\Delta tsp + pXDC39$ empty vector (SPF469)	Km ^r , Cm ^r	This study
$\Delta tsp + ptsp$ -His (SPF452)	Km ^r , Cm ^r	This study
Plasmids		
pSF6	pGEMT-easy- <i>rrnB</i>	77
pXDC39	pMMB207c $\Delta Ptac$, Δlac^q , Cm ^r	Xavier Charpentier
<i>ptsp</i> (pSF113)	pXDC39 containing <i>tsp</i> ; Cm ^r	This study
<i>ptsp</i> -his (pSF129)	pXDC39 containing <i>tsp</i> -His; Cm ^r	This study

is expressed only in the PE phase (Fig. 4B). This pattern of expression suggests a negative regulation of Tsp by Lpr17. This is further supported by the complete repression of Lpr17 during thermal stress (Fig. 5). Furthermore, RT-PCR on cDNA extracted from the E and PE phases (Fig. 3C) shows the presence of the *tsp* mRNA transcript in both phases, whereas the protein is detected only in the PE phase. Taken together, our results suggest that the Lpr17 sRNA inhibits translation of the protein, possibly by blocking the binding of ribosome to the ribosome binding site (RBS) without initiating degradation of the transcript (Fig. 3A). This is reminiscent of several *cis*-encoded sRNAs that overlap the 5' untranslated region of their target gene (68). Determining the exact mechanism would require additional experiments that are beyond the scope of the present study.

In *E. coli*, the CpxR/A two-component system regulates genes involved in dealing with envelope stress and misfolded proteins in the periplasm (69). In *E. coli*, CpxR upregulates the DegP periplasmic protease (70–72). In *Lp*, CpxR does not regulate DegP and DegQ in the E and PE phases, but seems essential for expression of Tsp (41). Therefore, the inability of the *cpxR* mutant to tolerate thermal stress could rely on the absence of Tsp; however, complementation of *cpxR* defect by expressing *tsp* in *trans* was unsuccessful, suggesting that CpxR regulates other determinants of thermal stress resistance. It is unclear if CpxR directly binds to the promoter region or if the regulation of *tsp* is indirect. Additional experiments are required to investigate the exact mechanism.

In conclusion, we have demonstrated that Tsp is necessary for survival of thermal stress in water and during intracellular growth. Such situations are probably encountered by *Lp* in its normal environment. Therefore, Tsp is likely to be a critical genetic determinant for survival and growth in water systems. Our results show that *tsp* is likely regulated by a complex network consisting minimally of Lpr17 and CpxR. Finally, we have determined that CpxR is an important regulator of thermal stress tolerance in *Lp*.

MATERIALS AND METHODS

Bacterial strains and media. Table 1 describes the bacterial strains used in this study. The WT *Lp* strain used in this study is KS79, a $\Delta comR$ mutant of the JR32 strain, which allows the strain to be constitutively competent (73). JR32 is a Philadelphia-1 derivative that is salt sensitive, streptomycin resistant, and restriction negative (74). *Lp* strains were cultured on CYE agar [*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered charcoal yeast extract] supplemented with 0.25 mg·ml⁻¹ of L-cysteine and 0.4 mg·ml⁻¹ of ferric pyrophosphate (75). Strains were cultured in AYE broth, which is CYE lacking charcoal and agar. If required, media were supplemented with 25 μ g·ml⁻¹ of kanamycin and 5 μ g·ml⁻¹ of chloramphenicol (75).

TABLE 2 Primers used in this study

Primer	Sequence (5'→3')	Source
Lpg499-UF	ATAAACGGACTGTGCTAAACCAAGAGCTG	This study
Lpg499-UR	GTCTAGCTATCGCCATGTAAGCGATCCTCAGATGC	This study
Lpg499-DF	GCTGAAGATCAGTTGGGTTGACCAATTAGTCACTCC	This study
Lpg499-DR	TATCTATGCTCCGTTTTCCAGTCATTGACG	This study
Lpg499-Km-F	GCATCTGAGGAGATCGCTTACATGGCGATAGCTAGAC	This study
Lpg499-Km-R	GGAGTGACTAATTGGTCAACCCAAGTATCTTCAGC	This study
Lpg499-F1	TTCGAGCTCTAATTCAAAGCGGCAAACCGTTCAC	This study
Lpg499-R1	CGACTTAGATTATCTGTTAGCTAACGCCATTCCTTCC	This study
pXDC39-scr-R	AAACAGCCAAGCTTGCATGC	This study
499-F	ATGCTAACCGGCCTTGATCC	This study
499-R	CCGCGCATTAAATTAACAGC	This study
500-F	TGCAAGCTCAGCAGGAAATG	This study
Lpr17-50	TCCAACAAAACGAGCAAATCGC	This study
Lpr17-5I	TTGTCTTAAAGCTTAGCCGCTGGC	This study
Lpr17-30	TGGTTTCTTCAGCCGAAAACGC	This study
Lpr17-3I	ATAGCGATCTCCTCAGATGCAAGG	This study
5'RACE Adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA	This study
Poly(dT)	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN	This study
5'RACE-Outer	GCTGATGGCGATGAATGAACACTG	This study
5'RACE-Inner	CGCGGATCCGAACACTGCTGTTTCTGGCTTTGATG	This study
3'RACE-Outer	GCGAGCACAGAATTAATACGACT	This study
3'RACE-Inner	CGCGGATCCGAATTAATACGACTCACTATAGG	This study
Lpg499-his-F	ATCTCTAGAATCGCAAGTGTAGGCCATACGGTGG	This study
Lpg499-his-R	ATGCTGACGATTAATGGTGTGGTGTGCTGTTAGCTAACGCCATTCCTTCC	This study
499-qPCR-F	TCTGAAGCAGAGGCAGAACC	This study
499-qPCR-R	CAATGACAAGGCAGGTAAGC	This study

Deletion of *tsp* and complementation of the mutant. The *tsp* gene was replaced with a kanamycin resistance cassette by allelic exchange, as previously described, to construct the deletion mutant strain (76). PCR primers are described in Table 2. A 1-kb fragment upstream of *tsp* was amplified using primers Lpg499-UF and Lpg499-UR. A 1-kb fragment downstream of *tsp* was amplified using primers Lpg499-DF and Lpg499-DR. The kanamycin cassette was amplified from the pSF6 plasmid (77) using primers Lpg499-Km-F and Lpg499-Km-R. Each 1-kb fragment was purified using a gel extraction kit (Qiagen) and ligated by PCR using primers Lpg499-DR and Lpg499-UF to generate a 3-kb fragment that was purified by a gel extraction kit (Qiagen). The purified 3-kb fragment was introduced into the KS79 strain by natural transformation (78), and the recombinants were selected on CYE agar supplemented with kanamycin. PCR was used to confirm the allelic exchange, and the Δ *tsp* mutant strain was named SPF365.

The *tsp* mutation was complemented in *trans* by amplifying the *tsp* gene with its native promoter from the KS79 WT genomic DNA using primers Lpg499-F1 and Lpg499-R1. The Lpg499-F1 primer is located 441 nucleotides upstream of the ATG of *tsp*, and the amplicon includes both the *tsp* and *lpr17* genes. The amplicon was cloned in the pXDC39 plasmid vector using the restriction enzymes *Sac*I (New England Biolabs) and *Xba*I (New England Biolabs). The restriction digestion was done at 37°C for 1 h, column purified (Qiagen), and ligated for 2 h at room temperature using T4 DNA ligase (New England Biolabs). The ligation product was transformed into *E. coli* DH5 α , and the transformants were selected on LB agar containing 25 μ g/ml of chloramphenicol. Cloning was confirmed by PCR using primers Lpg499-F1 and pXDC39-scr-R. The recombinant plasmid (*pts*p) was extracted and electroporated into the Δ *tsp* mutant strain. The transformants were selected on CYE agar containing 5 μ g·ml⁻¹ of chloramphenicol and then patched on CYE agar containing 25 μ g·ml⁻¹ of kanamycin and 5 μ g·ml⁻¹ of chloramphenicol. Screening was done by PCR using primers Lpg499-F1 and pXDC39-scr-R, and the resulting strain was called SPF403.

Thermal stress. *Lp* strains were grown on CYE agar for 3 days at 37°C. A suspension was prepared from a few colonies in Fraquil, an artificial freshwater medium (79, 80), at an optical density at 600 nm (OD₆₀₀) of 1.0. The suspension was washed three times with Fraquil to remove any trace of nutrients. The strains were incubated at 25°C for 24 h in 25-cm² cell culture flasks (Sarstedt). The strains were then diluted 1:10 in Fraquil, and 1 ml was distributed in 13-ml tubes (Sarstedt) and incubated in a 55°C water bath. Individual tubes were used for each time point. Tubes were removed from the water bath for CFU counts on CYE agar to determine the survival of the strains. CFU counts were done at 0, 15, 30, 45, and 60 min.

***Vermamoeba vermiformis* culture and infection.** *V. vermiformis* cells were grown at room temperature in 75-cm² cell culture flasks (Sarstedt) in modified PYNFH medium (ATCC medium 1034) and passaged when confluence was reached. Modified PYNFH contains, per liter, 10 g of peptone, 10 g of yeast extract, 1 g of yeast nucleic acid, 15 mg of folic acid, 1 mg of hemin, 100 ml of heat-inactivated fetal bovine serum, and 20 ml of buffer solution (18.1 g of KH₂PO₄ and 25 g of Na₂HPO₄ per liter). The amoebas were centrifuged at 200 × *g* for 10 min, the supernatant was discarded, and cells were resuspended in

fresh modified PYNFH medium and incubated for 3 days prior to infection. On the day the infection was to be carried out, the cells were centrifuged at $200 \times g$ for 10 min and resuspended at a concentration of 5.0×10^5 cells/ml in modified PYNFH lacking fetal bovine serum (FBS) and the buffer solution. *Legionella* is able to grow in modified PYNFH containing FBS and the buffer solution; therefore, it was omitted during the infection to ensure that the growth observed is due to intracellular multiplication. One milliliter of cells was seeded in each well of the 24-well plate (Sarstedt).

Bacterial strains were grown on CYE agar with antibiotics for 3 days at 37°C. On the day of the infection, the bacteria were suspended in Fraquil at an OD₆₀₀ of 0.1 and then diluted 1:10 in Fraquil. Five microliters of the 1:10 dilution were added to each well in triplicate in order to have a starting multiplicity of infection (MOI) of 0.1. The infection was carried out at room temperature for 5 days, at 37°C for 5 days, at 25°C for 2 days, and then at 37°C for an additional 3 days. CFU counts were performed on a daily basis.

RNA extraction. TRIzol (Thermo Fisher Scientific) was used for RNA extraction according to the manufacturer's protocol. Briefly, 200 μ l of chloroform (Thermo Fisher Scientific) was added to the bacterial pellet suspended in TRIzol, shaken, and incubated for 3 min at room temperature. The sample was added to a Phase Lock Gel Heavy 2-ml tube (VWR) and centrifuged at $12,000 \times g$ for 15 min at room temperature. Approximately 600 μ l of the supernatant was transferred to a microtube containing 600 μ l of 100% isopropanol (Thermo Fisher Scientific) and 5 μ g of glycogen (Thermo Fisher Scientific). Following incubation at room temperature for 10 min, the tubes were centrifuged at $17,000 \times g$ for 10 min at 4°C. The supernatant was removed, and 1 ml of 75% ice-cold ethanol (Greenfield Global) was added to the RNA pellet. The tubes were centrifuged at $17,000 \times g$ for 10 min at 4°C, the supernatant was removed, and the pellet was air-dried and resuspended in nuclease-free water (Thermo Fisher Scientific). The RNA was quantified using a UV spectrophotometer (Thermo Fisher Scientific).

Northern blotting. The expression of the Lpr17 sRNA was examined by Northern blotting as previously described (15). The strains were grown on CYE agar with required antibiotics for 3 days at 37°C, inoculated in AYE broth with required antibiotics, and grown at 37°C with shaking at 250 rpm. Aliquots of 10 ml of exponential-phase culture (OD₆₀₀, 0.5 to 1.0) and 5 ml of PE phase culture (OD₆₀₀ > 3) were centrifuged at $4,000 \times g$ for 10 min, the supernatant was removed, and the bacterial pellet was resuspended in 1 ml of TRIzol reagent. RNA extraction was carried out as mentioned above. Five micrograms of RNA were loaded on a 6% Tris-borate-EDTA-urea polyacrylamide gel, and the samples were migrated at 180 mV. The RNA was transferred to a positively charged nylon membrane (Thermo Fisher Scientific) using a semidry gel blotting system (Bio-Rad) for 20 min at 200 mA. The membrane was prehybridized in ULTRAhyb-Oligo hybridization buffer (Thermo Fisher Scientific) for 1 h at 37°C in a rotating chamber. We added 5 nM 5' biotinylated Lpr10 probe (Integrated DNA Technologies) to the prehybridization buffer, and the membrane was incubated overnight at 37°C in the rotating chamber. The membrane was twice washed with $2 \times$ SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.5% SDS (Bio-Rad) for 30 min at 37°C. The probes were detected with the chemiluminescent nucleic acid detection module (Thermo Fisher Scientific).

RT-PCR. RNA extracted from the KS79 WT strain grown to the E and PE phases was treated with DNase (Thermo Fisher Scientific). One microgram of DNase treated RNA was reverse transcribed with Protoscript II (New England Biolabs) and a no-reverse transcriptase control (no RT) was included by replacing the reverse transcriptase by nuclease-free water. The PCR was performed on cDNA, no-RT control, WT gDNA, and a no-template control (water) using primers described in Table 2. The amplicon was analyzed on a 1% agarose gel.

Quantitative PCR. RNA was extracted and cDNA synthesized as described above. Following cDNA synthesis, qPCR was done with primers 499-qPCR-F and 499-qPCR-R. qPCR was performed using iTaq universal SYBR green supermix (Bio-Rad) according to the manufacturer's protocol. The efficiency of the primer pairs was determined using dilution series of gDNA. Threshold cycle (C_t) values were normalized to the 16S rRNA as described previously (81).

Cloning of Tsp with hexahistidine tag. The hexahistidine tag was added to *tsp* by PCR. *tsp*, along with its native promoter, was amplified using primers Lpg499-his-F and Lpg499-his-R, the latter containing a hexahistidine tag. The amplicon was cloned in the pXDC39 plasmid vector using XbaI (New England Biolabs) and PstI (New England Biolabs). The restriction digestion was carried out at 37°C for 1 h, followed by column purification (Qiagen) and ligation at room temperature for 2 h using T4 DNA ligase (New England Biolabs). The ligation product was transformed into *E. coli* DH5 α , and the transformants were selected on LB agar supplemented with 25 μ g \cdot ml⁻¹ of chloramphenicol. The cloning was confirmed by PCR using primers Lpg499-his-F and pXDC39-scr-R. The plasmid (*ptsp*-his) was extracted and electroporated into KS79, and the *tsp* mutant and transformants were selected on CYE containing 5 μ g \cdot ml⁻¹ of chloramphenicol. The KS79 *ptsp*-His transformants were patched on CYE containing 5 μ g \cdot ml⁻¹ of chloramphenicol, while the *tsp* mutant *ptsp*-His transformants were patched on CYE containing 25 μ g \cdot ml⁻¹ of kanamycin and 5 μ g \cdot ml⁻¹ of chloramphenicol. Screening was done by PCR using primers Lpg499-his-F and pXDC39-scr-R. The pXDC39 empty vector was also electroporated in the KS79 WT and the *tsp* mutant, which serve as controls.

Western blotting. Strains were grown to the E and PE phases in AYE broth. Aliquots of 1.5 ml were centrifuged at $17,000 \times g$ for 3 min, the supernatant was decanted, and the cell pellet was resuspended in 200 μ l of 1 \times sample buffer (10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.002% bromophenol blue, and 5% β -mercaptoethanol). The samples were boiled for 5 min and sonicated for 15 min in an ice-cold water bath using ultrasonic cleaner (Cole-Palmer). Samples were then centrifuged for 15 min at $17,000 \times g$ at 4°C. The supernatant was transferred to a new tube and stored at -20°C until it was ready to use. The protein samples were standardized by adjusting the final OD₆₀₀ to 0.5 by diluting the

samples with 1× sample buffer. We loaded 15 μl of the standardized protein sample on a 12.5% polyacrylamide gel, and samples were migrated at 100 V. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 16 V for 24 h. The membrane was blocked for 30 min with a 5% milk protein solution. Tsp-His was detected with the anti-6× His tag antibody (Thermo Fisher Scientific), which is already conjugated with horseradish peroxidase (HRP) and does not require a secondary antibody for detection. The blot was incubated with this antibody at room temperature for the following 2 h. The IcdH antibody (Sigma) was used as a loading control. In this case, the blot was incubated with the IcdH primary antibody at room temperature for 2 h and with the secondary antibody (anti-rabbit HRP; Sigma) at room temperature for 30 min. Antibodies were detected using ECL Prime Western blotting detection reagents (GE Healthcare).

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