Copper resistance in *Legionella pneumophila*: role of genetic factors and host cells.

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Abstract

Copper is frequently found in drinking water due to its presence in the natural environment and the widespread usage of copper pipes. This toxic metal has a well-known antimicrobial activity, an activity harnessed in copper-silver ionization (CSI) to eliminate the opportunistic pathogen *Legionella pneumophila* from engineered water systems. Despite utilizing the antimicrobial properties of copper in *Legionella* control, little is known about how copper containing environments affect *L. pneumophila* populations. The goal of this study is to understand how *L. pneumophila* responds to copper within a hot water distribution system (HWDS) environment. To answer this question, different sequence types and regulatory mutants were exposed to copper to compare their survival. *L. pneumophila* isolates of 4 sequence types from 3 different HWDSs exhibited a wide diversity of phenotypes after copper stress. The Δ*letA* and Δ*letS* mutants were sensitive to copper, indicating that the LetAS two component system is important for copper resistance. Additionaly, transmissive phase cultures were more resistant to copper than replicative phase cultures. Therefore, the regulation of entry into transmissive phase by the LetAS system is essential for *L. pneumophila*'s ability to survive copper stress. In a water system, *L. pneumophila* replicates within eukaryotic hosts. When cocultured with the host ciliate *Tetrahymena pyriformis*, *L. pneumophila* was more resistant to copper than when the bacteria were in a monoculture. No difference in *L. pneumophila* replication inside of hosts in cocultures with or without copper was observed. This result confirms that the presence of host cells protects *L. pneumophila* from copper stress. Therefore, presence of host cells in water system may limit the efficacy of copper-based control strategies.

Keywords: *Legionella pneumophila*, *Tetrahymena pyriformis,* copper, transmissive phase, LetAS, engineered water systems.

1. Introduction

Legionella pneumophila is the causative agent of Legionnaires' Disease, a severe form of pneumonia, and of Pontiac fever, a more flu-like illness (Cunha et al., 2016). Cases of Legionnaires' Disease are increasing worldwide each year, making it a significant public health concern (Yu, Kamali, and Vugia, 2019). It is of particular concern as a nosocomial infection, as immunocompromised populations, the elderly, and people with comorbidities are at heightened risk of infection (Marston et al., 1994). In Canada, cases of legionellosis rose by 326% between 2010 and 2019, increasing from 140 cases per year to 655 cases per year (Government of Canada, 2021). However, the actual number of infections is likely much higher, as cases of Legionnaires' Disease frequently go underreported, with an average of 2.8 illnesses, 2.5 hospitalizations, and 2.5 deaths for every reported Legionnaires' Disease case, hospitalization, and death reported in Canada (McMullen et al., 2024). In the United States, a 2024 CDC report found *Legionella* infections were the leading cause of drinking water outbreaks and were responsible for 97% of hospitalizations and 98% of all deaths associated with waterborne pathogens between 2015 and 2020 (Kunz, 2024). *L. pneumophila* is found in freshwater environments and engineered water systems (EWSs), such as hot water distribution systems (HWDS) and cooling towers, where it is an obligate intracellular pathogen of protozoans, such as amoebas and ciliates (Fields et al., 1984; Rowbotham, 1980). *L. pneumophila* is transmitted to humans when water containing the bacteria is aerosolized from water distribution systems and inhaled (Cunha et al., 2016). Once inside of the lung, *L. pneumophila* is phagocytosed by alveolar macrophages, a process enhanced by the bacteria's Icm/Dot Type IVb Secretion system to invade alveolar macrophages, causing infection and tissue damage (Escoll et al., 2014).

L. pneumophila has a biphasic life cycle, with an intracellular and extracellular phase (Byrne and Swanson, 1998; Molofsky and Swanson, 2004). The intracellular phase of *Legionella'*s life cycle is called replicative phase. During this phase the bacteria replicate to high levels within the host cell and are less resistant to stressors (Molofsky and Swanson, 2004). Once it has depleted its host of nutrients, *L. pneumophila* causes the lysis of the host cell and is released to the extracellular environment of a water system (Byrne and Swanson, 1998). This extracellular phase is known as the transmissive phase, during which *L. pneumophila* cells are more infectious and stress resistant, but do not replicate. The switch between replicative and transmissive phase is mediated primarily by the LetAS two component system (Hammer and Swanson, 2002). When *L. pneumophila* begins to deplete its host of nutrients and enter stationary phase, LetS, the sensor kinase, autophosphorylates (Rasis and Segal, 2009). It then phosphorylates the response regulator LetA which then activates the expression of RsmY and RsmZ, two non-coding small RNAs. RsmYZ bind to the post-transcriptional repressor CsrA (Rasis and Segal, 2009). Binding of CsrA by RsmY and RsmZ relieves its repression of the translation of mRNA, allowing transcription of genes involved in motility, virulence, and stress tolerance, including genes responsible for resistance to heat shock, oxidative stress, and acid stress (Molofsky and Swanson, 1999; Mendis et al., 2018).

Several treatment methods are used by water system operators to eliminate *L. pneumophila*. One such method is copper-silver ionization (CSI) (Liu et al., 1994). Both copper and silver are antimicrobial metals, and together have a synergistic effect (Lin, Stout, and Victor, 1996). For CSI, a copper anode and silver electrode are installed into a water system and periodically electrified to release metal ions into the water and eliminate any bacteria that may be present (LeChevallier, 2023). This treatment method is more effective at penetrating biofilms than

chlorine and is less damaging to the water system as the metal ions do not cause corrosion. In addition to CSI, *L. pneumophila* can encounter copper from copper pipes, which are widely used in water distribution systems and have been found to select for chlorine-resistant bacteria (Khan et al., 2019). More generally, solid copper has been shown to select for copper resistant *Pseudomonas fluorescens* in an adaptive laboratory evolution study (Xu et al., 2022).

Copper has a well-characterized antimicrobial activity due to the high reactivity of Cu^{+} ions. When there is an excess of Cu^{+} ions inside of the cell, they will displace other metal ions from the core of metalloenzymes, rendering the enzymes non-functional (Giachino and Waldron, 2020). This mismetallation affects a wide variety of enzymes, disrupting cellular metabolic pathways and eventually leading to cell death. Copper ions also damage the cell membrane, increasing its permeability, though the precise mechanism of this damage remains unknown (Giachino and Waldron, 2020). Perhaps most famously, in aerobic conditions Cu^{+} ions react with oxygen to form toxic radical oxygen species (ROSs) (Solioz, 2018). These ROSs cause oxidative damage to the cell, disrupting redox potential and damaging the DNA. Despite initial success in using CSI in their HWDS to reduce the number of Legionnaires' Disease cases to zero, some users have reported the reemergence of Legionnaires' Disease (Demirjian et al., 2015). Investigation showed that *L. pneumophila* isolated from these water distribution systems remained viable when exposed to both copper and silver (Demirjian et al., 2015).

Currently, the only characterized copper resistance gene in *L. pneumophila* is *copA* (Kim et al., 2009; Trigui et al., 2013). CopA is a P-type ATPase pump, coupling the transport of Cu^{+} ions from the cytoplasm to the periplasm with ATP hydrolysis (Kim et al., 2009). Inside of the periplasm, Cu^{+} ions are converted into more stable Cu^{2+} ions by a multicopper oxidase, such as *Escherichia coli*'s CueO. Multiple putative multicopper oxidases have been identified in *L.*

pneumophila such as *mcoL* (Huston et al., 2008). CopA is an example of the acquisition of resistance genes by pathogens through horizontal gene transfer, as *copA* is encoded on the pLP100 mobile genetic element (Trigui et al., 2013). Copper resistance has also been observed to emerge through adaptation to a copper-containing microenvironment via the accumulation of point mutations. Bédard et al. isolated four isolates of *L. pneumophila* strain ST1427 from HWDS in a building with an ongoing Legionnaires' Disease outbreak (Bédard et al., 2021). Two of the isolates were sensitive to copper, while the other two, from a biofilm in a copper pipe within the same system, were tolerant to copper. The tolerant and sensitive isolates differed by only 29 single nucleotide polymorphisms (SNPs), indicating that the *L. pneumophila* isolates had adapted to their local microenvironment through the accumulation of point mutations. Of the 29 SNPs identified, only 1 occurred near a known copper resistance gene (*copA)*, suggesting the presence of genes with a previously unknown role in *L. pneumophila*'s response to copper stress.

The aim of this study is to deepen our understanding of how *L. pneumophila* survives copper stress. We examined environmental and clinical isolates from different HWDSs to determine the phenotypic diversity of resistance to copper stress in *L. pneumophila.* Then we examined the genes that may be involved in copper resistance, hypothesizing that *L. pneumophila*'s general stress response played a role in copper resistance. And finally, we investigated the ability of host cells to protect *L. pneumophila* from the toxic effects of copper.

2. Materials and Methods

2.1 Culturing L. pneumophila

L. pneumophila strains (Table 1) were kept as frozen stocks at -80ºC. Strains were grown on CYE (ACES-buffered charcoal yeast extract) agar plates adjusted to pH 6.9 with 10 mM KOH

and containing 0.25g/ L-cysteine and 0.4g/L ferric pyrophosphate supplements at 37ºC for 3 days (Feeley et al., 1979). When needed, media was supplemented with 5 mg/mL chloramphenicol, 25 mg/mL kanamycin sulfate, 15 µg/mL gentamicin, or 0.1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG). Individual colonies were grown in 1 mL of AYE broth (CYE lacking charcoal or agar) at 37ºC with shaking. IPTG was added to cultures and suspensions of SPF39 and SPF312, at a final concentration of 0.1 mM to induce expression of LetA and LetS, respectively.

Table 2: *L. pneumophila* strains and isolates used in this study

Isolates ID	**Description**	**SBT**	**Source[1]**	**Isolation Date**	**Ancestor**
Philadelphia-1		ST36	ATCC 33152	-	-
KS79	JR32 Δ*comR*	-	(de Felipe et al., 2008)	-	JR32
SPF186	Δ*copA*:GT^R	-	(Kim et al., 2009)	-	KS79
SPF605	lpg0563(T1) *phaP* Δ*1nt39* :Kn^R	-	(Liang, Cameron, and Faucher, 2023)	-	KS79
SPF159	Δ*cpxR*:Kn^R	-	(Tanner et al., 2016)	-	KS79
ΔletS	Δ*letS*: Kn^R	-	(Hovel-Miner et al., 2009)	-	KS79
SPF39	Δ*letS*+pSF21:CM^R	-	(Hovel-Miner et al., 2009)	-	ΔletS
ΔletA	Δ*letA*:Kn^R	-	(Gal-Mor and Segal, 2003)	-	KS79
SPF312	Δ*letA*+pMMB207-*letA*:CM^R	-	(Lynch et al., 2003)	-	ΔletA
SPF544	Environment	ST378	A (Najeeb et al., 2024, submitted)	2021	-
SPF546	Environment	ST2859	C (Matthews et al., 2022)	2021	-
SPF547	Environment	ST2859	C (Mathhews et al., 2022)	2021	-
SPF597	Environmental (Taken after 1 round of heat disinfection)	ST378	A (Najeeb et al., 2024, submitted)	2021	-
SPF599	Environment	ST378	A (Najeeb et al., 2024, submitted)	2021	-

SPF600	Environment	ST378	A (Najeeb et al., 2024, submitted)	2021	-
SPF601	Environment	ST378	A (Najeeb et al., 2024, submitted)	2021	-
SPF634	Environment	Similar to ST378	A (Najeeb et al., 2024, submitted)	2015	-
SPF635	Environment (Hot water tap)	Similar to ST378	B (Najeeb et al., 2024, submitted)	2015	-
SPF652	Environment	ST378	A (Najeeb et al., 2024, submitted)	2012	-
SPF661	Environment	ST378	A (Najeeb et al., 2024, submitted)	2013	-
SPF727	Environment	ST2859	C (Matthews et al., 2022)	2020	-
SPF728	Environment	ST2859	C (Matthews et al., 2022)	2020	-
SPF729	Environment	ST1427	C (Matthews et al., 2022)	2020	-

1) For isolates SPF544-SPF729, the site of isolation is indicated by letters: Site A, B and C.

2.2 Copper exposure and survival tests

Overnight cultures were pelleted at 5000×*g* for 5 minutes, then washed three times in Fraquil, a defined low-nutrient medium that simulates freshwater (Morel at al., 1975) and suspended in Fraquil. Cell concentration was determined by measuring the OD_{600} with a spectrophotometer. Bacterial suspensions were diluted to a final OD_{600} of 0.1, equivalent to about $1x10^8$ cells/mL. The diluted suspensions were incubated overnight at room temperature. In a 24 well plate, 10 µL of 8 mM $CuCl_2$ was added to each well for a final concentration of 0.08 mM $CuCl_2$. For the control conditions, 10 µL of Fraquil was added to each well. 990 µL of the bacterial suspensions were added to each well and left at room temperature for 4 hours. After the 4-hour incubation period, bacterial cultures were diluted and plated onto CYE. A CFU count was used to determine survival.

2.3 Comparison of transmissive and replicative phase

148 For a culture of E phase cells, a Philadelphia-1 culture was grown in 1 mL AYE media and
149 shaken at 37ºC for 12 hours. To generate a PE phase culture of *L. pneumophila,* and overnight
150 culture of the *L. pneumophila* strain Philadelphia-1 was grown in 1 mL AYE media and shaken
151 at 37C for 48 hours. Both cultures were pelleted at 8,000xg for 3 minutes and washed and
152 resuspended in 2 mL Fraquil 3 times. Bacterial suspensions were diluted to an OD_{600} of 0.1,
153 equivalent to $1x10^8$ cells/mL, then left at room temperature overnight. 10 µL of Fraquil, 8mM
154 $CuCl_2$, or 16 mM $CuCl_2$ were added to wells in a 24 well plate. 990 µL of transmissive phase
155 suspension or replicative phase suspension were added to each well. CFU counts of the bacterial
156 suspensions with copper were taken at 0, 1, 4, and 8 hours to determine bacterial reduction over
157 time.
158
159 *2.4 Host cell maintenance and coculture*
160 *T. pyriformis* was stored at -80ºC. To start cell cultures from these frozen stocks, stocks were
161 thawed at 37ºC with SPP+ 8% sucrose (proteose peptone, dextrose, yeast extract, FeCl, water,
162 and sucrose), then grown in PYNFH+ 4% sucrose+10% FBS overnight (ATCC medium 1034
163 with sucrose and heat inactivated FBS). Cultures were then transferred to ATCC 357 media
164 (protease peptone, tryptone, and potassium phosphate) for maintenance. 3 mL *T. pyriformis*
165 cultures in 20 mL ATCC 257 media were passaged weekly. Prior to infection, 3 mL of *T.*
166 *pyriformis* culture was passaged in 20 mL of complete modified PYNFH media (ATCC medium
167 1034) with FBS for 3 days. For infection, *T. pyriformis* cultures were pelleted at 600×*g* for 5
168 minutes, then washed with Fraquil. A sample of the cells were stained with 0.4% Trypsin blue
169 and counted with a hemocytometer. The washed *T. pyriformis* cells were diluted to $5x10^5$
170 cells/mL in Fraquil. *L. pneumophila* strains were resuspended in Fraquil and diluted to an OD_{600}

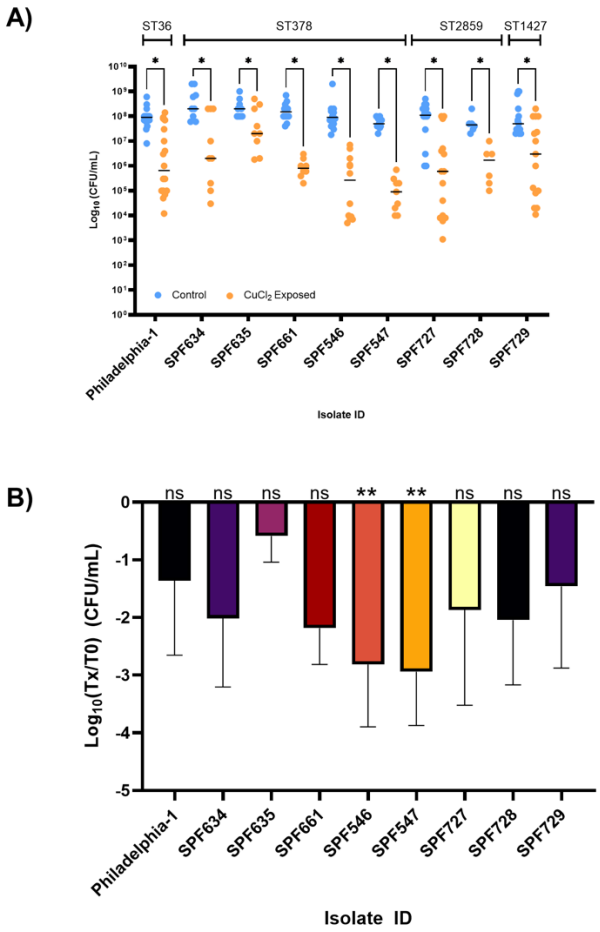
of 0.1 ($1x10^8$ cells/mL). To set up cocultures, 1 mL of diluted *T. pyriformis* cells was added to each well in a 24 well plate. 5 µL of *L. pneumophila* suspensions were added to each well, for a final concentration of $1x10^5$ cells/mL. For the copper exposed condition, 10 uL of 8 mM $CuCl_2$ was added. For the control condition, the cocultures were diluted with 10 uL of Fraquil. CFU counts of each infection were taken every 24 hours. A coculture of *T. pyriformis* and the *L. pneumophila* ΔdotA mutant was used as an infection control.

3. Results

3.1 Resistance of L. pneumophila *isolates to copper*

A variety of clinical and environmental isolates of *L. pneumophila* of different sequence types (ST) from 3 different HWDSs were tested for survival in the artificial freshwater Fraquil in the presence of copper (Fig. 1; Morel et al., 1975). The isolates were chosen because of their previous exposure to high levels of copper. Except for SPF635, all ST378 isolates were taken from a HWDS in site A, a healthcare facility built in 1971 (Table 2; Bédard et al., 2019). This HWDS utilizes copper pipes for all plumbing (Bédard et al, 2015). Because of the copper pipes, site A's HWDS had high levels of copper, with an average of 478 µg/L and a maximum of 743 µg/L (Bédard et al., 2019). Site B, where SPF635 was isolated from, is a healthcare facility built in 1903. Site C, where the ST2859 isolates were taken, is a recreational centre built in 1976. The HWDS of site C also utilized copper pipes and had high levels of copper, with an average of 2764 µg/L. Because these sites had high concentrations of copper in the HWDS, we wanted to determine if this previous exposure to copper selected for copper resistance in these strains compared to the lab strain *L. pneumophila* Philadelphia-1. A bioinformatics analysis of the ST378 isolates showed that these isolates have 2 copies of the P-type ATPase copper efflux

194 pump *copA* on their chromosome, while *Philadelphia-1* only has1 copy (Najeeb et al., 2024,
195 submitted). All isolates tested were significantly affected by $CuCl_2$ exposure but were not
196 significantly different from the wildtype (Fig. 1A). Only two isolates, SPF546 and SPF547 were
197 significantly different from the Philadelphia-1 type strain (P=0.0036 and P=0.0042, respectively)
198 (Fig. 1B). Overall, the survivability to copper seems to be variable amongst the strains tested,
199 with no clear correlation with the source of isolation and the concentration of copper in the water
200 system.
201



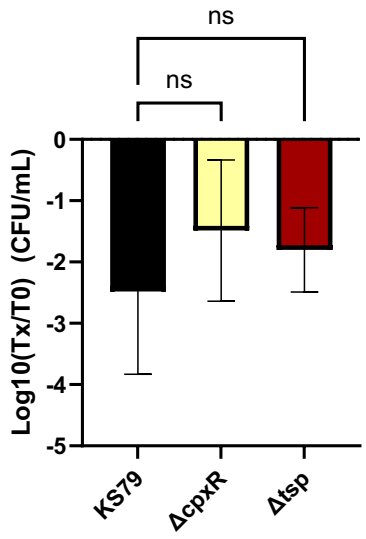
202
203 **Figure 1:** Effect of copper on environmental *L. pneumophila* isolates. Strains were grown in overnight broth culture,
204 then washed and resuspended in Fraquil to a final concentration of 10^8 cells/mL. Suspensions were incubated

overnight at room temperature, then exposed to 0.08 mM $CuCl_2$. Control suspensions were not exposed to copper. After incubation at room temperature for 4 hours, survival was determined using viable cell counts on CYE agar medium. A) Raw CFU count data. Significant effect of copper on survival of each strain was determined using paired, non-parametric t tests, $P < 0.05$ indicated with *, ns represents a non-significant result. Data shown are the values of individual replicates with the black line indicating the average. B) Ratio of $CuCl_2$:Control CFU counts. Data was analyzed with an uncorrected ordinary one-way ANOVA, then analyzed with multiple comparisons. Significant comparisons ($P < 0.05$) indicated with an * and non-significant comparisons indicated with ns.

Sites A, B, and C all had high concentrations of copper within their HWDSs. When environmental isolates of *L. pneumophila* taken from these systems were exposed to copper, a large diversity in the isolates' ability to survive copper stress was observed (Fig. 1A). A variety of responses to copper stress was observed in the ST2859 isolates, with SPF546 and SPF547 less resistant to copper than the other isolates. In general, there appears to be a wide diversity in susceptibility to copper stress among *L. pneumophila* strains, even among different isolates of the same sequence type taken from the same HWDS. This diversity could be attributable to differences in microenvironments within the water system. A HWDS is not uniform environment; there are dead end pipes, differing flow rates, and different water compositions depending on pipe material. Bédard et al. (2021) found differences in pipe microenvironment to play a large role in copper resistance in *L. pneumophila* (Bédard et al., 2021). Isolates taken from a biofilm in a copper pipe were copper resistant, while isolates from the water were sensitive. The resistant isolates had adapted to their copper-rich microenvironment within the biofilm. Because they lived in an environment that had a lower concentration of copper, the water isolates had not adapted to survive copper stress. It is possible that within site A and within site B, differences in flow rate or the presence of dead-end pipes created microenvironments that had

229 locally higher or lower concentrations of copper, resulting in isolates of *L. pneumophila* with a
230 wide diversity in survival in the presence of copper.
231
232 *3.2 CpxRA is unnecessary for copper resistance in* L. pneumophila.
233 To understand what genes may be involved in adaptation to a copper-containing
234 microenvironment, the survival of *L. pneumophila* mutants lacking stress regulators in the
235 presence of copper was tested. It was expected that a *ΔcpxR* mutant would be significantly more
236 susceptible to copper than the wild type, as in other Gram-negative species, such as *E. coli,* the
237 CpxRA two component system responds to copper stress, specifically to the damage to the cell
238 envelope caused by copper ions (Yamamoto and Ishihama, 2006). One of the proteins regulated
239 by CpxRA is the tail specific protease (Tsp) (Saoud, Mani, and Faucher, 2021). In other bacteria,
240 Tsp modulates peptidoglycan synthesis, and in *L. pneumophila* has been shown to be important
241 for surviving thermal stress (Lawrence et al., 2014; Singh et al., 2015; Saoud, Mani, and
242 Faucher, 2021). We hypothesized that misregulation of membrane homeostasis affects copper
243 sensitivity in *L. pneumophila*. The susceptibility to copper of Δ*cpxR*, a mutant in the CpxRA two
244 component system responsible for the envelope stress response, and Δ*tsp*, which lacks *L.*
245 *pneumophila*'s tail specific protein, was tested. In other Gram-negative species, the CpxRA two
246 component system responds to envelope damage induced by copper. As such, we expected that
247 the ΔcpxR mutant would show increased sensitivity to copper. However, survival of the ΔcpxR
248 mutant was not significantly different from the WT, indicating that the CpxRA system has no
249 role in copper resistance in *L. pneumophila* at least in the conditions tested (Fig. 2). Since copper
250 ions also cause the misfolding of proteins, the ability of a Δtsp mutant to survive in copper was
251 tested. Like Δ*cpxR*, the Δ*tsp* mutant showed no significant difference in survival after exposure

252 to copper from the WT, indicating Tsp does not play a role in *L. pneumophila*'s response to
253 copper stress (Fig. 2).
254



255
256 **Figure 2:** Effect of copper on *L. pneumophila* mutants in membrane stress response regulators. Strains were grown
257 in overnight broth culture, then washed and resuspended in Fraquil to a final concentration of 10^8 cells/mL.
258 Suspensions were incubated overnight at room temperature, then exposed to 0.08 mM $CuCl_2$. Control suspensions
259 were not exposed to copper. After incubation at room temperature for 4 hours, survival determined using viable cell
260 counts on CYE agar medium. Data shown represent average and standard deviation of 3 biological replicates.
261 Statistically significant difference from the WT (KS79) determined with one-way ANOVA and multiple
262 comparisons, * indicates $P < 0.05$.
263

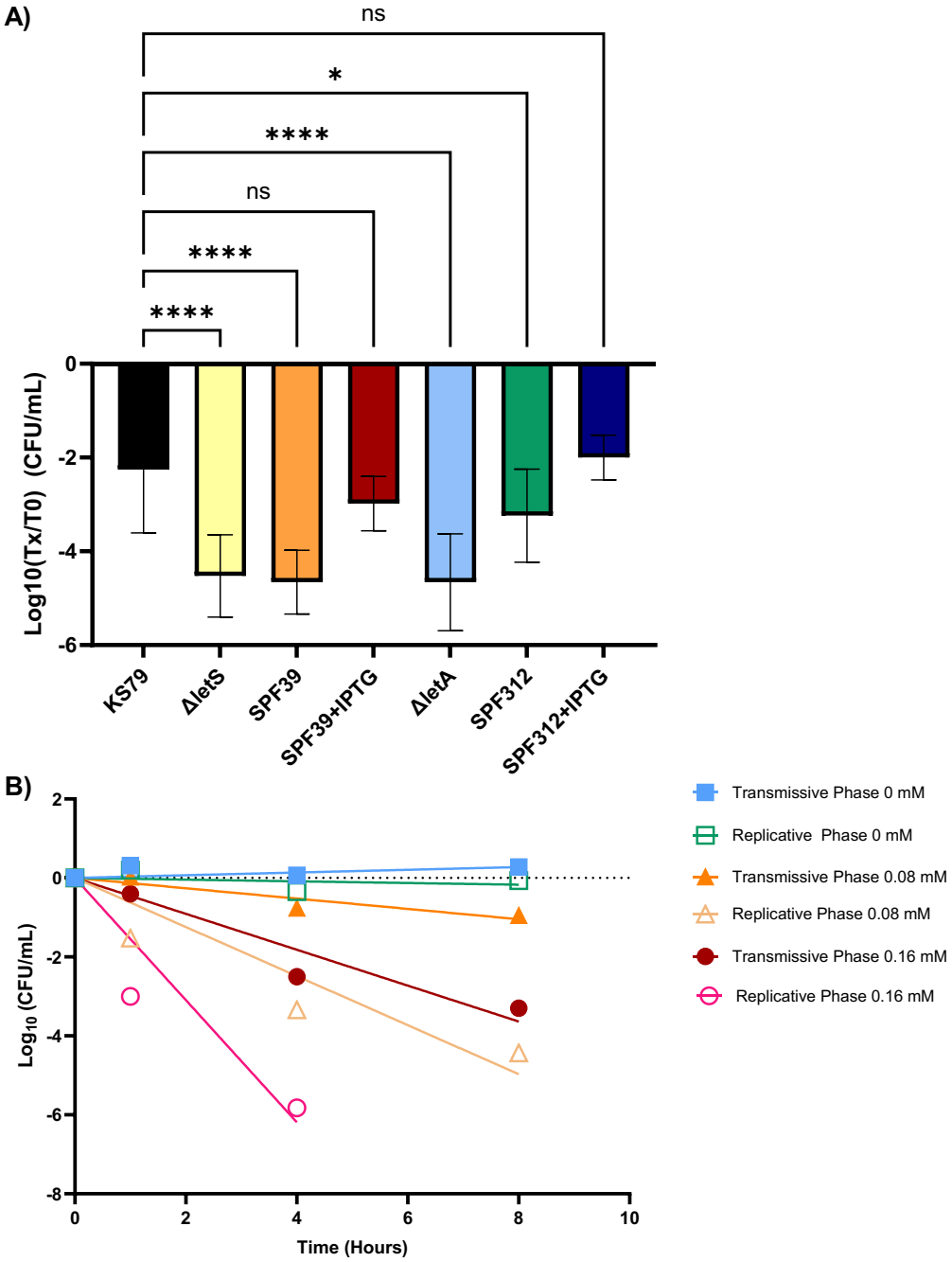
264 *3.3 LetA/S two component system has a role in copper resistance*
265

266 The biphasic lifestyle of *L. pneumophila* is important for stress resistance (Bachman and
267 Swanson, 2001; Lynch et al., 2003). The effect of mutation in *letA* and *letS* on copper resistance
268 was therefore investigated. When analyzed with one-way ANOVA and Tukey's multiple

comparisons, survival of *ΔletA* and *ΔletS* were significantly different ($P<0.0001$) from the wildtype KS79 (Fig. 3A). Complementation study was then carried out to confirm the role of these genes. The susceptibility of the merodiploid strains, SPF39 and SPF312, carrying a *letS* and a *letA* genes driven by the *Ptac* promoter, respectively, were tested. When uninduced, SPF312 was a bit more resistant to copper than the Δ*letA* mutant ($P<0.0001$), but SPF39 was no different than the Δ*letS* mutant ($P = 0.044$) (Fig. 3A). When induced with 0.01 mM IPTG and compared with Tukey's multiple comparisons, both SPF39 and SPF312 were significantly more resistant than the corresponding Δ*letS* and Δ*letA* mutant strains ($P=0.0014$ and $P<0.0001$, respectively) and return to wild-type levels (Fig. 2C). These results indicate that the LetAS two-component system plays a significant role in copper resistance in *L. pneumophila.*

Two possible options could explain the increased sensitivity of the Δ*letA* and Δ*letS* mutants to the effect of $CuCl_2$: A) the mutants were unable to activate expression of copper resistance genes, or B) as the LetAS system regulates entry into the transmissive phase of *L. pneumophila'* s life cycle, the Δ*letA* and Δ*letS* strains were unable to enter transmissive phase, which is more stress tolerant, and were therefore more sensitive to the effect of copper. A previous study by Mendis et al. (2018) examined the genes activated by LetA, but none of these genes were known to have a role in copper resistance (Mendis et al., 2018). The increased copper sensitivity in the Δ*letA* and Δ*letS* mutants is likely due to an inability to switch from replicative phase to transmissive phase. Therefore, the effect of copper on transmissive phase and a replicative phase culture of *L. pneumophila* was compared. *L. pneumophila* Philadelphia-1 cultures in transmissive phase and replicative phase were exposed to 0, 0.08 and 0.16 mM $CuCl_2$. CFU counts were taken at 0, 1, 4, and 8 hours to compare survival. The ratio against the baseline values of 0 mM $CuCl_2$ was calculated, transformed, and a line of best fit was graphed. At 0.08 mM $CuCl_2$, the

292 transmissive phase suspension had a slope of -0.2572, while the replicative phase suspension had
293 a slope of -0.4114 (Fig. 3B; Table 1; Supplementary Figure 1). When exposed to 0.16 mM
294 $CuCl_2$, the transmissive phase suspension had a slope of -0.4246, while the replicative phase
295 suspension had a slope of -0.6109 (Fig. 3B; Table 1; Supplementary Figure 1). At all
296 concentrations tested, the slope of the transmissive phase suspension was steeper than the slope
297 of the replicative phase suspension, indicating the transmissive phase suspension survived better
298 in the presence of copper (Fig. 3B; Table 1; Supplementary Figure 1). This confirms *L.*
299 *pneumophila* is more resistant to copper when it is in transmissive than when it is in replicative
300 phase. Taken together, these results indicated that LetAS regulation of entry into transmissive
301 phase is important for copper resistance in *L. pneumophila.*



302

303 **Figure 3:** The LetAS regulator is required for copper resistance. A) Survival ratios of *L. pneumophila* after exposure
304 to 8 mM $CuCl_2$ for 4 hours. Strains were grown in overnight broth culture, then washed and resuspended in Fraquil
305 to a final concentration of 10^8 cells/mL. Suspensions were incubated overnight at room temperature, then exposed to
306 0.08 mM $CuCl_2$. Control suspensions were not exposed to copper. After incubation at room temperature for 4 hours,
307 survival determined using viable cell counts on CYE agar medium. Expression of LetS and LetA in SPF39 (ΔletS +

pletS) and SPF312 (ΔletA + pletA), respectively, was induced with 0.1 mM IPTG. Data shown represent the average and standard deviation of 3 biological replicates. Statistically significant difference from the WT (KS79) was determined with one-way ANOVA and multiple comparisons, * indicates $P < 0.05$. B) Reduction in cell concentration over time of *L. pneumophila* Philadelphia-1 in transmissive phase and replicative phase. The transmissive phase culture was grown in a shaker at 37ºC for 48 hours, while the replicative phase culture was grown in a shaker 37ºC for 12 hours to reach replicative phase. Both cultures were washed and resuspended in Fraquil to a final concentration of 10^8 cells/mL and incubated at room temperature for 24 hours, then exposed to 0 mM, 0.08 mM, and 0.16 mM $CuCl_2$. CFU counts were taken at 0, 1, 4, and 8 hours. Ration against the T0 was calculated, transformed, and fitted with linear regression. Figure shows one representative replicate. See supplemental data for individual replicate data.

Table 1: Average slope and standard deviation of transmissive and replicative phase suspensions of four biological replicates after exposure to $CuCl_2$.

	Transmissive 0 mM	**Replicative 0 mM**	**Transmissive 0.08 mM**	**Replicative 0.08 mM**	**Transmissive 0.16 mM**	**Replicative 0.16 mM**
Slope	-0.05657	-0.02242	-0.2572	-0.4114	-0.4246	-0.6109
Standard Deviation	0.009482	0.01331	0.026	0.03583	0.03587	0.05994

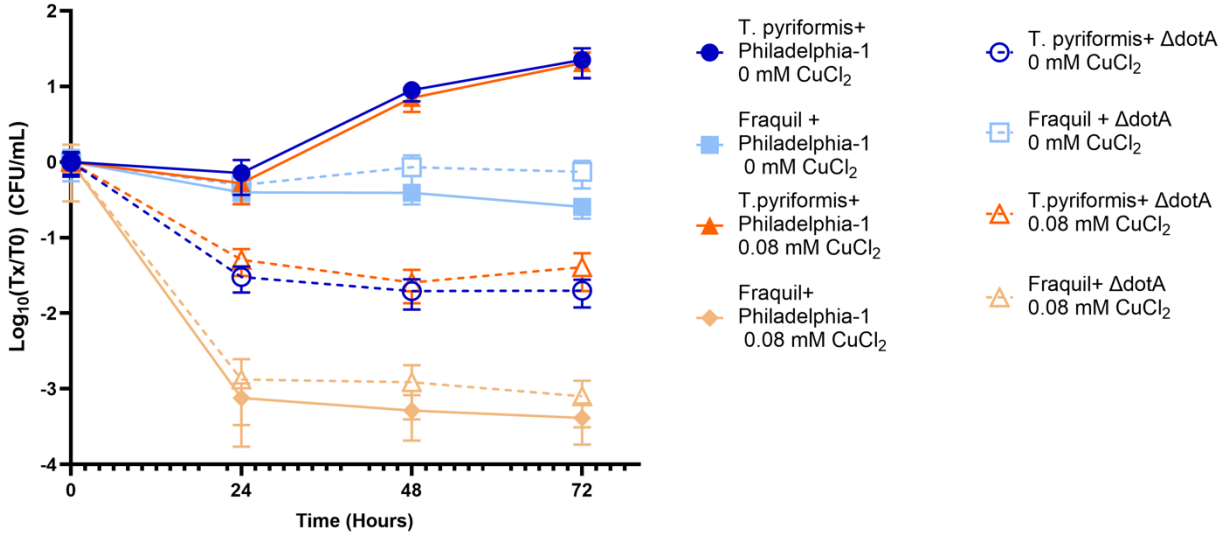
The results of this study are consistent with Lynch et al. (2003), who found that *letA* mutants were defective in the stationary phase stress response, and were sensitive to oxidative stress (Lynch et al., 2003). As copper reacts with oxygen to form toxic oxygen radicals, it is possible the Δ*letA* and Δ*letS* mutants are more sensitive to the oxidative stress caused by copper phase (Lynch et al., 2003; Hammer, Tateda, and Swanson, 2002). This would also explain the increased sensitivity of *L. pneumophila* in replicative phase to copper stress compared to *L. pneumophila* in transmissive phase. The LetAS system does not directly regulate known copper

329 resistance genes in *L. pneumophila* (Mendis et al., 2018). However, LetAS does regulate the
330 expression of genes involved in oxidative stress resistance, namely *sodB*, which encodes a
331 superoxide dismutase, and *ohr*, which encodes an organic hydroperoxide resistance protein
332 (Mendis et al., 2018). Both of these genes are involved in dismantling oxygen radicals to protect
333 *L. pneumophila* from oxidative stress (Broxton and Culotta, 2016; Brown, 2019). When the
334 ability of transmissive phase and replicative phase cultures to survive in the presence of copper
335 was compared, replicative phase cultures were more sensitive to copper stress than transmissive
336 phase cultures at both concentrations tested (Fig. 3B). Based on this result, the most likely
337 explanation for the sensitivity towards copper stress observed in the *ΔletA* and *ΔletS* mutants is
338 that without a functioning LetAS system, the mutant cells were stuck in replicative phase and
339 unable to enter the more stress tolerant transmissive phase, increasing their sensitivity to copper.
340 This result is consistent with the findings of Sahr et al. (2017). In their study, Sahr et al found
341 that the Δ*letA* mutant had a defect in entering an *Acanthamoeba castellanii* cell, but was unable
342 to replicate inside of the host, indicating that without LetA the mutant was halted in replicative
343 phase (Sahr et al., 2017). The increased copper sensitivity of the *ΔletA* and *ΔletS* mutants is also
344 consistent with the findings of Hammer, Tateda, and Swanson (2002), who found that *letA* and
345 *letS* mutants were unable to express transmissive phase traits (Hammer, Tateda, and Swanson,
346 2002). These mutants were non-motile, non-cytotoxic, and had poor efficiency for the infection
347 of macrophages (Hammer, Tateda, and Swanson, 2002) The LetAS system influences copper
348 sensitivity, possibly via control of oxidative stress resistance genes and the activation of other
349 general stress responses.
350
351

3.4 Presence of host cells protect L. pneumophila *from the effects of copper*

Next, we investigated the effect of intracellular growth on the susceptibility to copper. Cocultures of *L. pneumophila* and the ciliate species *Tetrahymena pyriformis* were set up in Fraquil with an MOI of 1 (Fig. 4). Control cocultures received 10 µL of Fraquil while treated coculture received 0.08 mM $CuCl_2$. In parallel, the survival of *L. pneumophila* without host cells was also tested. A CFU count was taken every 24 hours to quantify *L. pneumophila* replication, and the CFU counts were compared to the WT using an unpaired, one-tailed student's t-test.

Without host cells, the number of cells of *L. pneumophila* strains Philadelphia-1 and *ΔdotA* rapidly decreased in the presence of copper (Fig. 4, light orange solid dotted lines), but stayed relatively constant without copper (light blue solid and dotted lines), as expected. When cocultured with *T. pyriformis* Philadelphia-1 showed good replication in the presence of 0.08 mM $CuCl_2$, similar to growth observed in coculture without $CuCl_2$. Replication inside of host cells in the presence of 0.08 mM $CuCl_2$ was not significantly different from replication without $CuCl_2$ ($P=0.4423$) when analyzed with an unpaired, one-tailed t-test. When cocultured with *T. pyriformis, ΔdotA* showed a slight decrease over time in both the copper exposed and the control condition (Fig. 4, compare dark orange dotted line with dark blue dotted line); but survive much better that without host cells (Fig. 4, orange dotted line). Taken together, our results show that *L. pneumophila* can efficiently grow within *T. pyriformis* in the presence of copper and that the presence of host cell somewhat protects *L. pneumophila*.



373

374 **Figure 4:** Effect of copper on *L. pneumophila* in host cells. 10 uL of 8 mM $CuCl_2$ was added directly to cocultures of
375 *L. pneumophila* and *T. pyriformis.* The *L. pneumophila* strain ΔdotA was used as an infection control. Growth of *L.*
376 *pneumophila* strains Philadelphia-1 and ΔdotA in the presence of copper in Fraquil and in coculture with *T. pyriformis*.
377 MOI of 1. Samples were taken every 24 hours and plated on CYE agar plates for a CFU count to determine cell
378 survival over time. ΔdotA control for each condition represented with a dotted line. Data represents average and
379 standard deviation of 3 biological replicates. Data was analyzed with an unpaired, one-tailed Student's t-test to assess
380 statistical significance versus the WT.

381

382 Our examination of the ability of different *L. pneumophila* isolates and mutants to survive in the
383 presence of copper had primarily focused on bacterial cells surviving independently in water. But
384 in the HWDS environment, *L. pneumophila* does not replicate freely in the water, instead
385 replicating within protozoan hosts (Kwaik et al., 1998; Rowbotham; 1980). Initially, both the
386 amoeba *V. vermiformis* and *T. pyriformis* were used for this experiment and PYNF media was
387 used in the cocultures. Both host cell types survived well in this media, but the PYNF media
388 attenuated copper toxicity (Supplementary Figure S2). Presumably, the copper ions bound to
389 components of the media due to their high reactivity. Previous coculture experiments with *T.*

pyriformis and *L. pneumophila* used sterile tap water, so we selected Fraquil as the media for cocultures to avoid this issue (Fields et al., 1984). However, in Fraquil *V. vermiformis* cells died before *L. pneumophila* could infect them. Only *T. pyriformis* was used for this experiment as we could not find a system compatible with both *V. vermiformis* and copper.

There was no significant difference in *L. pneumophila* replication within a ciliate host between cocultures of *T. pyriformis* and *L. pneumophila* Philadelphia-1 with and without $CuCl_2$, while survival of both Philadelphia-1 and *ΔdotA* in single species suspensions with copper decreased significantly within 24 hours, indicating that the presence of host cells protects the bacteria from copper stress. The role of host cells in protecting *L. pneumophila* from harmful environmental stressors has been previously observed. Storey et al. (2004) demonstrated that interaction between *Acanthamoebae* hosts and Legionella protects the bacteria from thermal treatment of water systems (Storey et al., 2004). However, interactions between amoeba hosts and *L. pneumophila* was shown to increase sensitivity to chlorination. Unlike amoebas, however, ciliates like *T. pyriformis* do not form protective cysts to survive environmental stresses (Salazar-Ardiles, Asserella-Rebollo, and Andrade, 2022; Nanney and McCoy, 1976; Lynn and Doerder, 2012). It is likely that the host cells are buffering the available copper. Copper ions are highly reactive, and readily bind to a wide variety of substrates (Solioz, 2018). It is likely the Cu^{2+} ions added into the coculture in the form of $CuCl_2$ bound to the negatively charged cell membrane of *T. pyriformis*, reducing the amount of available copper. This would explain why the Δ*dotA* mutant was able to persist at low levels in coculture with *T. pyriformis* and $CuCl_2$ but dies off when in a monoculture with copper. The possible ability of ciliates to attenuate available copper ions will need to be confirmed in a future study. Compared to *L. pneumophila, T. pyriformis* are

413 less sensitive to $CuCl_2$: concentrations higher than. 200 mg/L (equivalent to 1.48 mM) are
414 required to inhibited *T. pyriformis* growth (Niclau et al., 1999). This is much higher than the
415 concentration of $CuCl_2$ needed to inhibit *L. pneumophila,* which has an MIC of around 0.5 mM
416 (Supplementary Figure 3). Because of the difference in concentration of copper required to kill
417 the bacteria and *T. pyriformis,* it is also possible *T. pyriformis* shelters *L. pneumophila* from
418 copper toxicity. At $CuCl_2$ concentrations that are subinhibitory for ciliates, *L. pneumophila* cells
419 inside of the ciliate would be protected from the effects of copper toxicity.
420
421 Protection of *L. pneumophila* by hosts from copper toxicity could also explain the diversity in
422 responses to copper stress observed in the ST378 and ST2859 isolates (Fig. 1). Different
423 microenvironments within the HWDSs could favour the growth of host cells, such as dead-end
424 pipes, which have lower flow rates than the rest of the system. The higher concentration of hosts
425 within these microenvironments could protect *L. pneumophila* from copper toxicity, thus
426 removing the selective pressure applied on the bacteria by copper. As such, *L. pneumophila*
427 isolates from these environments would be less resistant to copper than isolates from
428 microenvironments with less host cells to protect the bacteria. Niclau et al. (1999) found that
429 subinhibitory concentrations of copper stimulate grazing by *T. pyriformis* (Niclau et al., 1999).
430 The presence of copper within different microenvironments within the built environment would
431 increase grazing by ciliate hosts, increasing protection of *L. pneumophila* as uptake of bacteria
432 increased. This could also explain why environments with high concentration of copper still
433 harbor *L. pneumophila*.
434
435 **4. Conclusions**

436 Copper in freshwater environments within EWSs has a major impact on *L. pneumophila*,
437 whether that be copper leaching from copper pipes or copper ions released in CSI to control
438 bacterial populations. When exposed to 0.08 mM $CuCl_2$ environmental isolates from three
439 different copper-containing HWDSs had diverse copper resistance phenotypes. No correlation
440 between location, isolate source, or sequence type and copper resistance could be determined.
441 When mutants lacking stress regulators were exposed to copper, it was found that the LetAS
442 system is essential for copper resistance. This is likely because entry into the transmissive phase
443 of *L. pneumophila*'s biphasic life cycle is required for copper resistance, as cultures of *L.*
444 *pneumophila* in transmissive phase were more resistant to copper than cultures in transmissive
445 phase. When *L. pneumophila* was cocultured with *T.pyriformis,* the presence of the host cell
446 sheltered the bacteria from the stressor, as there was no significant difference between cocultures
447 with and without copper.
448

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