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Transcriptomic Adaptation of *Legionella pneumophila* to Transient Heat Shock

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A natural inhabitant of freshwater microbial ecology, *Legionella pneumophila* is an opportunistically pathogenic bacteria that has found a niche in hot water distribution systems (HWDS) where it challenges hospitals with the spread of nosocomial infection. Superheat-and-flush is a high-temperature pasteurization which can be temporarily effective, but systems often test positive for contamination soon after pasteurization. Because of the centrality of pasteurization in HWDS sanitation schemes, an understanding of the *L. pneumophila* intrinsic heat shock response may help improve the strategies used to counter its proliferation and ability to trigger nosocomial outbreaks. We tested a group of strains to assess differences in heat shock tolerance between clinical, laboratory, and environmental strains of different origin. The transcriptome of a model Philadelphia-1 *L. pneumophila* strain in response to heat shock was determined using microarray as an exploratory analysis of the heat shock response: 401 genes were induced, including genes involved in determining protein fate and ribosome biosynthesis while 43 genes were downregulated. Mutants of 3 individual genes were tested to find their direct effects on heat shock tolerance. Deletions of *dksA* or *rpoS*, genes involved in the regulation of life cycle switching and important for surviving long-term nutrient deprivation in freshwater lowered heat shock tolerance, suggesting an overlap in the pathways required to tolerate these stressors. Surprisingly, the deletion of *htpG*, the 90-kilodalton heat shock protein, was found to increase the ability to survive under transient heat shock. Taken altogether, our results show that *L. pneumophila* exhibits most components of the conserved bacterial heat shock response. Based on this exploratory transcriptomic study, we have provided data that can act as a platform for the research of *L. pneumophila*'s survival to pasteurization in hot water systems.

Keywords: ***Legionella pneumophila*, heat shock response, transcriptome, water distribution systems, pasteurization, water-borne infection, disinfection**

INTRODUCTION

Since the Philadelphia outbreak in 1976 that gave its name, *Legionella pneumophila* has steadily risen in prominence as a public health challenge in the ensuing years (McDade et al., 1977; Herwaldt and Marra, 2018). In a retrospective (Winn, 1988) on the unmasking of the "Philly Killer," Winn details the CDC-led effort to trace the Legionnaires' Disease Bacterium, culminating in the isolation of the then-novel waterborne pathogen. *L. pneumophila* infection most often presents either as Legionnaires' Disease—a life-threatening pneumonia—or as Pontiac Fever—a mild respiratory illness (Cianciotto, 2001; Cunha et al., 2016), though cases of extrapulmonary legionellosis are also known (Padrnos et al., 2014; Chitasombat et al., 2018). A compilation of waterborne disease burden in 2014 in the United States found that Legionnaires' Disease had the second highest cost per hospital visit, costing an estimated total of 402 million 2014 dollars−12.1% of all direct healthcare costs attributed to waterborne disease. Cases were found to have a 98.1% hospitalization rate and 9.0% fatality rate, causing 15.0% of US domestic deaths due to waterborne disease (Collier et al., 2021). *L. longbeachae*, *L. micdadei*, and other members of the genus are also more rarely associated with human disease (Gomez-Valero et al., 2014; Chambers et al., 2021).

The aerosolization of contaminated water—commonly from cooling towers, showers, faucets, or some medical instruments—spreads the bacteria (Principe et al., 2017). Fortunately, *L. pneumophila* is exclusively transmitted directly from environmental reservoirs to humans [with one presumed instance of human-to-human transmission (Borges et al., 2016; Correia et al., 2016)] and therefore outbreaks are locally contained (Phin et al., 2014). Due to the dynamics of airborne transmission, a single source of *L. pneumophila* contaminated aerosols can seed infection in many exposed individuals (Addiss et al., 1989; David et al., 2017). Colonization of hot water distribution systems (HWDS) by *L. pneumophila* has been reported by hospitals worldwide, where they are associated with inpatient and staff infections (Darelid et al., 1994, 2002; Berthelot et al., 1998; Kool et al., 1999; Falkinham et al., 2015; Bédard et al., 2016a). As a result of comorbidities in hospital inpatients, nosocomial case fatality rates are higher than those for community-acquired infections (Soda et al., 2017).

Routine suppression of *L. pneumophila* populations in HWDS relies heavily on control of water temperatures within them (Springston and Yocavitch, 2017; Van Kenhove et al., 2019). Though operational guidelines vary across global health systems (reviewed in Van Kenhove et al., 2019), an example best practice can be drawn from the European Working Group for *Legionella* Infections (EWGLI). Recommendations include the setting of hot water heaters to above 60°C, that water throughout the plumbing network hold a temperature >50°C, and that hot water at terminal outlets be able to reach 70°C if necessary (Van Kenhove et al., 2019). The superheat-and-flush process, similar to pasteurization, is commonly used to clear *L. pneumophila* populations from water distribution systems because of its low upfront costs (Whiley et al., 2017). *L. pneumophila* has proven to be able to re-populate pasteurized water systems, despite adherence to these guidelines (Allegra et al., 2008, 2011). This regrowth could be associated with structural issues in water system topology such as dead ends and stagnation zones where reduced fluid velocity allows biofilm harboring *L. pneumophila* to develop (Boppe et al., 2016). Incomplete pasteurization of the system can be caused by water use patterns and plumbing design flaws that mix recently heated water from recirculation loops and cooler water in stagnant pipes (Rhoads et al., 2015, 2016; Ji et al., 2017). Direct comparisons of culturability after heat shock between 4 strains of *L. pneumophila* (two commercial type strains and two environmental strains collected from a HWDS) and one type strain of *L. longbeachae* showed disimilarities in their abilities to tolerate heat shock (Cervero-Aragó et al., 2015). *L. longbeachae* was more sensitive to heat shock than any of the *pneumophila* strains, but no clear pattern emerged concerning the heat shock tolerance of the *pneumophila* strains exposed to temperatures between from 50 to 70°C. Nutrient deprivation in HWDS may contribute to heat resistance, as water-suspended cells show an increased expression of heat shock protein genes, as well as other hallmarks of the canonical stringent response (Li et al., 2015; Trigui et al., 2015). Transition to the VBNC stage is associated with long-term nutrient deprivation, as well as with exposure to sanitation procedures like free chlorine or heat treatment (Dietersdorfer et al., 2018; Schrammel et al., 2018; Cervero-Aragó et al., 2019). These VBNC cells are under-counted in standard culture-based methods to assess contamination, but can be resuscitated by passage through the natural eukaryotic hosts of *L. pneumophila*, and are likely to contribute to survival and re-growth after superheat-and-flush (Casini et al., 2018; Dietersdorfer et al., 2018; Nisar et al., 2020).

The bacterial heat shock response is an integrated network of molecular pathways that works toward the maintenance of cellular homeostasis (Lema et al., 1988; Richter et al., 2010). Studies in *E.* coli have identified RpoH, or σ^{32}, an alternative sigma factor which regulates transcription of the heat shock regulon as the high-level transcriptional regulator of the heat shock response (Cowing et al., 1985; Jenkins et al., 1991; Chakraborty et al., 2014). Many of the heat shock proteins of the heat shock response are highly-conserved, such as the 90, 70, and 40-kilodalton heat shock proteins—known as Hsp90, Hsp70, and Hsp40 in eukaryotes and as HtpG, DnaK, and DnaJ in bacteria, respectively (Spence et al., 1990; Feder and Hofmann, 1999; Sørensen et al., 2003). Though their precise mechanisms of action vary between species and are often unclear, their general functions are to refold misfolded polypeptides and resolve toxic protein aggregates (Bepperling et al., 2012; Choi et al., 2012; Johnson, 2012; Honoré et al., 2017).

Early radiolabelling studies in *L. pneumophila* found five high temperature-induced proteins (Lema et al., 1988). HtpB, a *Legionella* homolog of the *E. coli* GroEL chaperonin, has received particular attention due to its diverse but crucial roles in bacterial protein folding, aquatic survival, and eukaryotic host infection (reviewed in Garduño et al., 2011). HtpB is under transcriptional control by both the heat shock σ^{32} and housekeeping σ^{70} promoters (Garduño et al., 2011). Compared to its homolog in *E. coli* whose expression is low under steady-temperature conditions, the HtpB protein is abundant in *L. pneumophila* at

all temperatures that support growth (Hoffman et al., 1989). This difference in regulation suggest a different and potentially more significant role of HtpB in *L. pneumophila* than in other bacteria (Hoffman et al., 1989, 1990).

Because of the importance of pasteurization in the control of *L. pneumophila*, we conducted a study into its response to transient heat shock in order to understand transcriptomic factors contributing to its survival in hospital hot water systems. Because epidemiological investigations have traced *L. pneumophila* contamination to a number of different source types, we looked to confirm prior studies that showed differences in the heat shock resistance of strains from different backgrounds by comparing a group of environmental isolates to the laboratory model strain, Philadelphia-1. Though prior research has shown a broadly-conserved bacterial heat shock response, there have been, to the best of our knowledge, no recent studies conducted using *L. pneumophila*. To address this gap, we performed an exploratory investigation into the transcriptomic response of *L. pneumophila* to a transient heat shock by microarray to provide insight into the ability of *L. pneumophila* to resist clearance from HWDS in the face of superheat-and-flush treatments. To validate the transcriptomic assessment of its heat shock response, we also directly characterized the importance of the heat shock and regulatory proteins encoded by *htpG*, *dksA*, and *rpoS* by direct genetic means.

MATERIALS AND METHODS

Culture Conditions

All strains used in this study are listed in **Table 1**. All strains were stored at −80°C. Cultures of *E. coli* were grown in LB broth or on LB agar (Li et al., 2015), supplemented where indicated with 25 μg/ml chloramphenicol, and incubated overnight at 37°C. Cultures of *L. pneumophila* were grown on N-(2-Acetamido)2-aminoethanesulfonic acid (ACES)-buffered Charcoal Yeast Extract (BCYE) Agar plates or in ACES-buffered Yeast Extract (AYE) broth (Li et al., 2015). *L. pneumophila* was routinely grown at 37°C for 72 h on BCYE plates or in liquid culture at 37°C with shaking at 250 rpm to post-exponential phase, as determined by a dark-brown pigment in the culture tube. Where indicated, media were supplemented with 25 μg/ml kanamycin, 5 μg/ml chloramphenicol, and/or 1 mM IPTG.

Heat Shock Tolerance Assay

To assess the heterogeneity of the heat shock response, multiple strains of *L. pneumophila* were tested for differences in their abilities to survive heat shock. Strains, listed in **Table 1**, were chosen to represent different cross-section of *L. pneumophila* diversity. Philadelphia-1, ATCC 33152, represents the 1976 outbreak in Philadelphia (McDade et al., 1977)—though it is important to note that the strain available from the ATCC is slightly divergent from the canonical Philadelphia-1 strain from the CDC (i.e., the first wholly sequenced strain of *L. pneumophila* and the ancestor of laboratory domesticates JR32 and KS79) (Rao et al., 2013; Mercante et al., 2016). In addition, we tested strains collected from different environmental sources, including cooling towers, hot water plumbing, and clinical isolates (Lévesque et al., 2014; Bédard et al., 2021), as well as a control *letS* mutant known to be extremely sensitive to heat shock.

Survival of *L. pneumophila* was evaluated by CFU counts from heat shocked suspensions and from unheated suspensions through serial dilutions in Fraquil and spot plating on BCYE plates. A single colony of *L. pneumophila* was used to inoculate 10 mL AYE broth and grown overnight to post-exponential phase, as described above. Cells were pelleted by centrifugation for 5 min at 5,000 × *g* and washed 3 times in Fraquil, a defined artificial freshwater (Li et al., 2015). After washing, bacteria were suspended in Fraquil at a OD_{600nm} of 1.0 and incubated at room temperature (20–25°C) for 24 h to starve the cells in a low-nutrient environment. Suspensions were dispensed in 1 ml aliquots into 13 ml polypropylene tubes (Sarstedt) and were maintained at room temperature throughout or heat shocked by immersion in a 55°C circulating water bath for 5 or 20 min and subsequently cooled passively to room temperature and used immediately afterwards to perform CFU counting. Significance analyses were conducted on log-transformed data using Student's two-tailed *t*-test with unequal variances using GraphPad Prism version 8.3.1.

Sample Preparation for Microarray

Single colonies of *L. pneumophila* were grown in AYE and suspended in 10 mL Fraquil aliquots at a OD_{600nm} of 1.0, as described above, and incubated at room temperature for 1 h. To minimize the time elapsed before reaching the target temperature, 125 ml glass Erlenmeyer flasks were pre-heated to 55°C in a circulating water bath. Ten milliliter cell suspensions were poured into the flasks and incubated at 55°C for 5 min. At the end of heat shock periods, heat-treated cultures were removed from the water baths and 8 ml from each replicate suspension was pelleted by centrifugation at 2,000 × *g* for 5 min for RNA extraction. Control samples were prepared by pelleting 8 mL of suspensions which had been kept at room temperature.

Nucleic Acid Extraction

Pellets collected from samples treated as above were used for RNA extraction using TRIzol reagent (Invitrogen), following the manufacturer's protocol, and RNA was stored in nuclease-free water (Ambion). Purity and concentration of RNA samples were evaluated using a NanoDrop 1000 spectrophotometer. Genomic DNA (gDNA) was extracted from overnight cultures of Philadelphia-1 using the Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's protocol, and stored in nuclease-free water.

Microarray Analysis

Transcriptomic analysis of the heat shock response was performed using a microarray following a two-color common reference design (Talaat et al., 2002; Faucher and Shuman, 2013). Fifteen micrograms of RNA were labeled with aminoallyl dUTP during reverse transcription into complementary DNA (cDNA) using random hexamer primers and ProtoScript II reverse transcriptase [New England Biolabs (NEB)]. Aminoallyl dUTP labeling was

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain	Description	References	Sequence type
Philadelphia-1^{ATCC}	**Philadelphia 1976** Clinical	ATCC33152	36
1427.B.F	**Sherbrooke 2014** Faucet biofilm	Bédard et al., 2021	1427-like
1427.W.HE	**Sherbrooke 2014** Heat exchanger water	Bédard et al., 2021	1427-like
1427.B.HE	**Sherbrooke 2014** Heat exchanger biofilm	Bédard et al., 2021	1427-like
LSPQ120292	**Quebec 2012** Outbreak source cooling tower	Lévesque et al., 2014	62
LSPQ120329	**Quebec 2012** Non-outbreak environmental	Lévesque et al., 2014	62
ΔrpoS	JR32 rpoS::Tn903dGent; Gen^r	Hales and Shuman, 1999	36
ΔletS	KS79 letS::aptII, Kan^r	Hovel-Miner et al., 2009	36
JR32	Philadelphia-1^{CDC} Tra$^-$ str^R *lpg1237*^{mut}	Sadosky et al., 1993; Rao et al., 2013	36
KS79	JR32 ΔcomR	De Felipe et al., 2008	36
ΔhtpG	KS79 htpG::Kan^r	This study	36
ΔdksA	KS79 dksA::Kan^r	This study	36
E. coli DH5α	supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Invitrogen	
Plasmid	**Description**	**Source**	
pMMB207c	RSF1010 derivative, IncQ, lacI q Chl^r Ptac oriT ΔmobA	Charpentier et al., 2008	
pMMB207c::DksA	*lpg2338* in pMMB207c	This study	
pXDC39	pMMB207c ΔPtac, ΔlacI, Chl^r	Xavier Charpentier	
pXDC39::HtpG	*lpg1369* in pXDC39	This study	
pSF6	pCR2.1-rrnb	Faucher et al., 2011	

L. pneumophila strains are listed with source information and sequence type—a global standard sequence-based typing classificiation.

also performed on 2.5 μg of gDNA with random primers and an exonuclease-negative Klenow fragment. Labeled cDNA was coupled with AlexaFluor-647 and labeled gDNA was coupled with AlexaFluor-546, following the manufacturer's protocol, and purified into nuclease-free water *via* MinElute column (QIAgen).

Microarray slides (GPL19458) were designed with probes complementary to 2,994 open reading frames (ORFs) and 251 small RNA (sRNA) products in the *L. pneumophila* genome, as previously described (Trigui et al., 2015). Before DNA hybridization, slides were prepared by immersion in pre-hybridization buffer (5x saline sodium citrate, 0.1% SDS, 1 mg/mL bovine serum albumin), as described in Trigui et al. (2015). Microarray slides were hybridized with gDNA, as a reference channel, and cDNA before scanning fluorescence in an InnoScan microarray scanner.

Data analysis was performed in R 3.6.1 using package limma v3.41.2 (Ritchie et al., 2015; Phipson et al., 2016). Background fluorescence was corrected independently for each channel using normexp, an adaptive background correction method. Normalization within and between arrays was performed by variance-stabilizing normalization [implemented in limma as normalizeVSN()]. A linear model was fit to expression data, and an empirical Bayes method was used to compute significance statistics (Benjamini-Hochberg false discovery rate). Differentially regulated transcripts were considered to be those with a magnitude fold-change of ±2 or greater and an adjusted *P*-value of <0.05. Data deposited in Gene Expression Omnibus, accession number GSE196713.

RT-qPCR

Microarray analysis was validated using quantitative reverse transcription PCR (RT-qPCR) targeted at eight genes identified as differentially regulated—5 upregulated and 3 downregulated—under heat shock. Primer pairs (**Table 2**) designed using NCBI Primer-BLAST had their amplification efficiencies confirmed using Philadelphia-1 gDNA dilutions between 10 and 0.001 ng/μl. All primers showed efficiencies of >90% (90.02–95.11%). RT-qPCR was conducted as described previously (Trigui et al., 2015). One microgram of RNA was reverse transcribed using Protoscript II reverse transcriptase (NEB). qPCR analysis was performed using iTaq Universal SYBR Green (Bio-Rad) and a CFX Connect Real-Time PCR Detection System (Bio-Rad). Analysis of RT-qPCR expression data was done using the ddCT method normalized to 16S RNA, as described previously (Li et al., 2015).

TABLE 2 | Primers used in this study.

Primer	Sequence 5′-3	Source
Primers for qPCR		
lpg0104-F	GACTGCCATGATCACAAAATTGC	This study
lpg0104-R	TATGAACAAGTTTCCGCCGGG	This study
lpg1247-F	AATTAATGGCGGTTTCGAGCG	This study
lpg1247-R	AAGTTCAAGCCAGCAAAACCC	This study
lpg1283-F	TCTTCCTCTGGTTGGCTATGG	This study
lpg1283-R	CATAAGCCACAACTCCACTCG	This study
lpg1369-F	TGGGGACAATTGCAAAATCCG	This study
lpg1369-R	ATCCTCAGGTTGTAACCCAGC	This study
lpg2025-F	CTTCTTCAGCATGCGATTGGG	This study
lpg2025-R	TGTTTCTGCCAAAGACAAGGC	This study
lpg2524-F	GCGCCTGGTATAAAGAACTGC	This study
lpg2524-R	TGAAGACAGGTTTTCAGCAACC	This study
lpg2841-F	GTGGTGCTTCCTGACTGTAGC	This study
lpg2841-R	GCGATTAAATAAGCGGGAGGC	This study
lpg2934-F	ATGCTCAACCAGACGTTTTGC	This study
lpg2934-R	AGAAGTAACGGAAATGCAGCG	This study
16S-F	AGAGATGCATTAGTGCCTTCGGGA	Li et al., 2015
16S-R	ACTAAGGATAAGGGTTGCGCTCGT	Li et al., 2015
Primers for mutant construction		
htpG-UF	CTTGCACATCTTGGAAAAGGAGG	This study
htpG-UR	CAGTCTAGCTATCGCCATGTACTCCCCGTTAATAGTTGTGTTCG	This study
htpG-KnF	CGAACACAACTATTAACGGGGAGTACATGGCGATAGCTAGACTG	This study
htpG-KnR	GCTCATCCCGGATAATGACTTGTACCCAACTGATCTTCAGCATC	This study
htpG-DF	GATGCTGAAGATCAGTTGGGTACAAGTCATTATCCGGGATGAGC	This study
htpG-DR	CAATTATTGCCTTACTGCCTGGG	This study
dnaK-UF	ATTTCACCGCTGGTAACTAATGG	This study
dnaK-UR	CAGTCTAGCTATCGCCATGTACATGACGATTTCAAGCTATCACCC	This study
dnaK-KnF	GGGTGATAGCTTGAAATCGTCATGTACATGGCGATAGCTAGACTG	This study
dnaK-KnR	CCTTCTTCCGCCCTCTTAAATCAACCCAACTGATCTTCAGCATC	This study
dnaK-DF	GATGCTGAAGATCAGTTGGGTTGATTTAAGAGGGCGGAAGAAGG	This study
dnaK-DR	AACTACCTTGCATAGCAAATCGC	This study
dksA-UF	GGATGCTTTGGTTTCTCCTGAAC	This study
dksA-UR	CAGTCTAGCTATCGCCATGTATGCCAGACCCTATATTTCATTCTG	This study
dksA-KnF	CAGAATGAAATATAGGGTCTGGCATACATGGCGATAGCTAGACTG	This study
dksA-KnR	CTTGTAATAATAAACTCCCGCCTGACCCAACTGATCTTCAGCATC	This study
dksA-DF	GATGCTGAAGATCAGTTGGGTCAGGCGGGAGTTTATTATTACAAG	This study
dksA-DR	CAGAGAGCTTAAAGAGGCTCAGG	This study
Primers for complementation		
KpnI-HtpG-F	TCATCAGGTACCTGAATCGAGAGGGTTTGAGTTGG	This study
XbaI-HtpG-R	TCATCATCTAGAGGATAATGACTTGTTGTAATCCGGG	This study
EcoRI-DksA-F	TCATCAGAATTCTACAGAATGAAATATAGGGTCTGGC	This study
XbaI-DksA-R	TCATCATCTAGAGGCAAGAAAGAATCAAGCACC	This study
pXDC39-F	GCTTCCACAGCAATGGCATCC	Li et al., 2015

Mutant Construction

Deletion mutants of *htpG* (*lpg1369*) and *dksA* (*lpg2338*) were constructed through allelic exchange (Mendis et al., 2018). Efforts to construct a deletion mutant of *dnak* (*lpg2025*) were not successful. Flanking regions of each gene ∼1 kbp in length were amplified from purified Philadelphia-1 gDNA using OneTaq polymerase (NEB) with their respective upstream region UF-UR primer pair and downstream region DF-DR primer pair (listed in **Table 2**). A kanamycin resistance cassette was amplified from pSF6 using oligonucleotide primers (*gene*-Kn-F and *gene*-Kn-R) that partially overlap with each gene's flanking sequence. Flanking region and kanamycin cassette PCR products were purified using

the QIAquick gel extraction kit (QIAgen). DNA fragments were ligated with Phusion polymerase (NEB) using terminal UF-DR primer pairs. Purified ligation products were introduced into *L. pneumophila* KS79 *via* natural transformation and resulting transformants were selected on BCYE supplemented with kanamycin. Successful transformation was confirmed by fragment size analysis of PCR amplicons. KS79 was used as the wild-type control strain for phenotypic assays.

To generate hemi-diploid mutants with transcomplementation of the deleted alleles, we re-introduced plasmid-borne copies of each gene. The gene *htpG* was amplified with its promoter from the Philadelphia-1 genome using primers KpnI-HtpG-F and XbaI-HtpG-R. The amplicon and the plasmid pXDC39 were digested using KpnI and XbaI (NEB) restriction enzymes. Purified digests were ligated using T4 DNA ligase (NEB) and transformed into *E. coli* strain DH5α made chemically competent with CCMB, as per (Mendis et al., 2018). Plasmid-carrying bacteria were selected for on LB supplemented with chloramphenicol. The first attempt to clone *dksA* with its own promoter was unsuccessful. Therefore, we cloned it under the control of the *Ptac* promoter in pMMB207c (Charpentier et al., 2008). Primers EcoRI-DksA-F and XbaI-DksA-R were used to amplify *dksA* without its promoter. The amplicons and the plasmid were digested with EcoRI and XbaI (NEB) restriction enzymes, ligated and transformed into *E. coli* as described above. Each plasmid was checked for proper orientation of insert using the pXDC39-F primer and a gene-specific reverse primer. Plasmids were introduced into KS79 and into the deletion mutants through electroporation, as previously described (Mendis et al., 2018), and selected for on BCYE supplemented with chloramphenicol.

RESULTS

Strain-Wise Differences in Heat Shock Tolerance

To support the use of Philadelphia-1 as a model strain of heat shock survival, we tested multiple strains of *L. pneumophila* to compare their heat resistances. Between-strain differences in ability to tolerate high temperatures were apparent after 20 min of exposure to 55°C (**Figure 1**). At this time point, the model strain Philadelphia-1 suffered a 1.69 $\log_{10}$-fold drop in culturability. Our group has previously constructed a Δ*letS* mutant (Mendis et al., 2018), with an inactive LetA/S two-component signal transduction system causing metabolic dysregulations that render it more sensitive to heat and starvation stresses. Included as a control, this strain did show a significantly greater drop in culturability than Philadelphia-1 (4.99 $\log_{10}$-fold after 20 min). Three of the tested strains, 1427.B.F, 1427.W.HE, and 1427.B.HE, are closely related serotype 1427-like strains isolated from the hot water system of a hospital in Sherbrooke, Canada during the investigation of an outbreak in 2014 (Bédard et al., 2016b, 2021). 1427.W.HE, isolated from the water in a heat exchanger, experienced a loss of culturability comparable to Philadelphia-1 at 5 and 20 min of heat shock. Two biofilm-derived isolates, 1427.B.F and 1427.B.HE, isolated from a faucet and a heat exchanger, respectively, suffered greater losses of culturability—2.05 and 2.77 $\log_{10}$-fold drops, respectively. LSPQ120292 and LSPQ120329 were collected during an outbreak in 2012 in Québec City, Canada (Lévesque et al., 2014). They were isolated from the epidemic source cooling tower and from an unrelated cooling tower, respectively. Compared to Philadelphia-1, the surviving population of LSPQ120292 was not significantly different at either time point. In contrast, LSPQ120329 experienced 1.14 and 3.84 $\log_{10}$-fold drops in culturability after 5 and 20 min, both significantly greater than that of Philadelphia-1. These results show that Philadelphia-1, despite its origin as a cooling tower-derived strain and subsequent history as a laboratory strain, maintains a high level of heat tolerance compared to contemporary environmental isolates and is a usable model strain for our transcriptomic study.

The Early Heat Shock Regulon

RNA was extracted from starved Philadelphia-1 suspensions before and after experiencing 55°C temperatures for 5 min. Microarray analysis showed that most genes were not differentially regulated (absolute $\log_2$ fold-change $\geq$ 1 and Benjamini-Hochberg-adjusted $p \leq 0.05$) in these conditions (**Figure 2A**, **Supplementary Table S1**). Of the 444 regulated genes, 43 were significantly downregulated (selected genes listed in **Table 3**) while 401 were upregulated (selected genes listed in **Table 4**). Global transcriptomic analysis by microarray was validated through RT-qPCR (**Figure 2B**) on a set of eight differentially regulated genes, selected to represent both upregulated and downregulated genes in multiple cellular pathways. Except for one gene (*lpg2934*), the direction of change was concordant overall between the two methods (Spearman's rho = 0.5476, $p = 0.171$).

To discriminate patterns in gene expression, differentially regulated genes were classified by functional categories based on their annotated GenBank functions (**Figure 3A**). Like with the individual genes (**Figure 2A**), there was a global increase in expression of most functional categories (**Figure 3A**) based on their mean fold-changes. Only transcripts associated with biosynthesis of secondary metabolites, chemotaxis/motility/cell division, and rRNA were not significantly upregulated, with only rRNA transcripts showing an overall (non-significant) down-regulation. Genes involved in detoxification/adaptation and cell envelope synthesis had the second and fourth highest median fold changes in expression, respectively, as could be expected during the early repair of heat shock-induced damage to the cell (Guyot et al., 2010; Meyer and Baker, 2011; Li and Gänzle, 2016). Though patterns in the strict count of genes under regulation (**Figure 3B**) in each functional category is complicated by variations in the number of transcripts under each category and by the annotation of the *L. pneumophila* genome leaving many ORFs and proteins of unknown function, the category with the highest number of downregulated genes is transport and binding. Normalizing by percentage to account for variation in category size (**Figure 3C**), we find that tRNA transcripts are the most

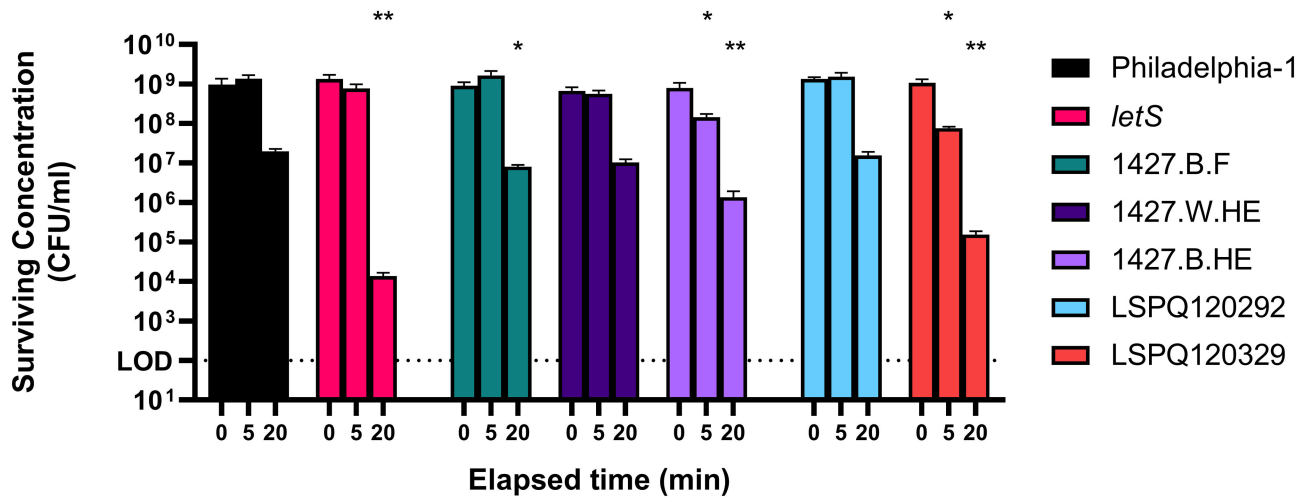

FIGURE 1 | Survival of *L. pneumophila* strains at 55°C. Isolates were washed in Fraquil and acclimated for 1 h and heat shocked at 55°C for 5 or 20 min. Error bars indicate SD of three biological replicates. Significance is shown relative to population concentration of Philadelphia-1 after the same elapsed time and is calculated using a two tailed Student's *t* statistic with log-transformed data (ns = not significant, * < 0.05, ** < 0.01 with Bonferroni's correction for 7 comparisons).

up-regulated (30.0%) and that protein fate/hydrolases/secretion transcripts are the most down-regulated (3.12%).

Genes With the Same Expression Scheme Have Different Impacts on Heat Shock Survival

To further dissect the *L. pneumophila* response to heat shock, three upregulated genes were chosen for further investigation (**Table 4**). *HtpG* encodes a bacterial heat shock protein of the Hsp90 family. While homologs of HtpG are strongly conserved across the bacterial domain, its functions are diverse and difficult to characterize (Spence et al., 1990; Genest et al., 2011; Choi et al., 2012; Honoré et al., 2017; Grudniak et al., 2018). *Lpg2025* encodes a heat shock protein homologous to the Hsp70-family DnaK (Genest et al., 2011). Finally, *lpg2338* encodes DksA, a protein implicated in the regulation of the stringent response and life phase switching in *L. pneumophila* (Dalebroux et al., 2010). Using allelic exchange, single deletion mutants of KS79 were produced by replacing each gene in turn with a kanamycin resistance cassette. No deletion mutants of *lpg2025* were successfully produced, even when the temperature of incubation during mutant construction was lowered from 37 to 25°C to allow potentially thermo-sensitive mutants to grow.

The two successfully constructed mutant strains were tested for their heat shock tolerances at the same temperatures used in the microarray protocol. Though both strains were found to significantly differ from the wild type in their culturability after heat shock, the effects differed directionally. The Δ*htpG* mutant, unexpectedly, maintained culturability to a significantly greater degree than its ancestor—a 1.29 $\log_{10}$-fold higher survivability at 20 min of heat shock (**Figure 4A**). In contrast, the Δ*dksA* mutant showed a lower heat shock tolerance, with the surviving population reaching the lower limit of detection after 20 min (**Figure 4B**). Both phenotypes were successfully complemented by expressing the deleted gene *in trans* (**Figures 4A,B**).

Because RpoS and DksA are known to collaborate in regulation of life cycle switching in *L. pneumophila* (Dalebroux et al., 2010), we extended prior research (Hales and Shuman, 1999; Trigui et al., 2015) on the stress resistance role of RpoS to our present study conditions. To measure the role of RpoS on heat shock survival, we used a insertion mutant strain constructed from a JR32 ancestor strain (Hales and Shuman, 1999). This mutant showed a diminished survivability in response to heat stress, consistent with the role of RpoS in *E. coli* and *Pseudomonas aeruginosa* (Hengge-Aronis et al., 1991; Suh et al., 1999). Compared to the JR32 strain, which had a 4.17 $\log_{10}$-fold drop in culturability after 20 min of exposure to 55°C, the RpoS deletion mutant had a 6.20 $\log_{10}$-fold drop (**Figure 4C**). This phenotype was partially complemented by IPTG induction of plasmid-borne RpoS expression (**Figure 4C**).

DISCUSSION

The Influence of Environmental Background on Heat Shock Tolerance

Legionella control regimes in hot water distribution systems are most often centered around baseline temperature set-points and remedial pasteurization processes (Whiley et al., 2017). We hypothesized that strains collected from hot water systems, which had presumably adapted to higher temperatures from exposure during hot water use or from failed pasteurizations, would show higher heat tolerances than clinical isolates or isolates collected from non-hot water systems, which are more likely to have adapted to other circumstances. We tested a panel of six isolates representing three outbreaks: the original 1976 Philadelphia outbreak from which *L. pneumophila* was identified (McDade et al., 1977; Mercante et al., 2016), a 2012 cooling

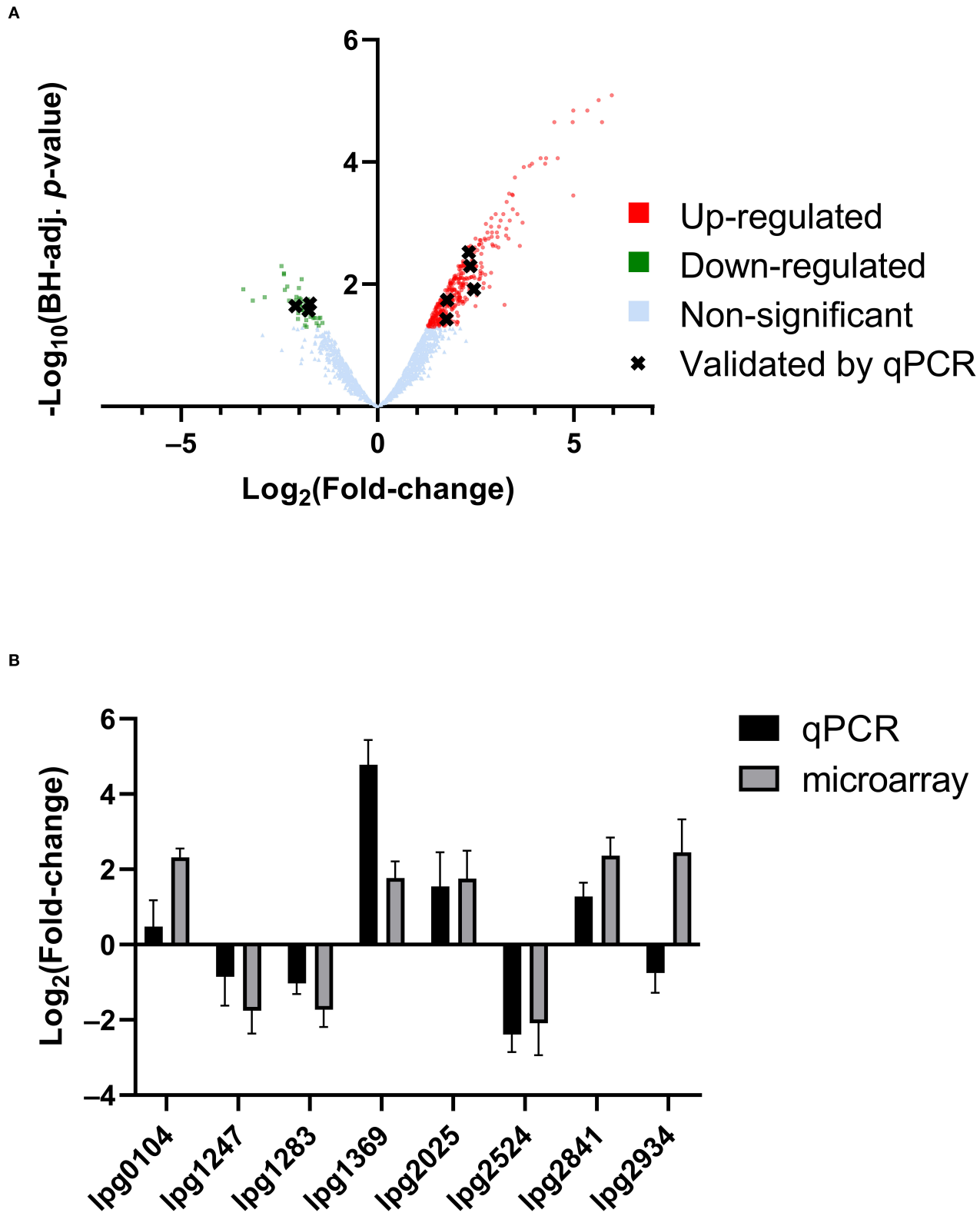

FIGURE 2 | Transcriptomic response of *L. pneumophila* to heat shock. **(A)** Volcano plot of microarray data, showing significantly differentially regulated genes. **(B)** qPCR validation of microarray expression data, showing overall agreement of up-regulation and down-regulation profiles.

TABLE 3 | Selected downregulated genes identified by microarray.

lpg number	Gene name	Gene	Log_2 FC	Adjusted *p*-value
lpg2099	MsrA2—peptide methionine sulfoxide reductase		−2.29	0.0109
lpg2524	Transcriptional regulator, LuxR family		−2.09	0.0228
lpg1022	Thymidine phosphorylase (TdRPase) (thymidine/pyrimidine-nucleoside phosphorylase)	deoA	−2.08	0.0186
lpg0267	Magnesium and cobalt transport protein CorA	corA	−2.06	0.0161
lpg2686	DotA	dotA	−2.02	0.0371
lpg0230	Sensor histidine kinase (two component response regulator) PleD	pleD	−2.02	0.0297
lpg0156	Signal transduction protein (EAL/GGDEF domain protein)−2 component response regulator (c-di-GMP phosphodiesterase A)		−2.00	0.0115
lpg1993	Polysaccharide deacetylase		−2.00	0.0218
lpg2471	Hydrogenase expression/formation protein HypE	hypE	−1.94	0.0082
lpg0499	Carboxy-terminal protease		−1.84	0.0470
lpg2405	Mutator mutT protein	mutT	−1.84	0.0317
lpg1283	Lipoprotein NlpD		−1.73	0.0207
lpg1705	Carboxypeptidase G2 (peptidase, M20/M25/M40 family)		−1.71	0.0218
lpg2267	Prolidase (aryldialkylphosphatase) (Xaa-pro dipeptidase)		−1.70	0.0343
lpg1914	Fimbrial protein, type IV pilin, PilE	pilE	−1.65	0.0324
lpg0121	ABC transporter, permease protein (nitrate permease)		−1.63	0.0358
lpg2698	N-acetylmuramoyl-L-alanine amidase	amiB	−1.46	0.0351
lpg1792	Flagellar protein (flagellar motor switch protein FliM)	fliM	−1.40	0.0429

Genes listed are significantly downregulated with a Benjamini-Hochberg adjusted $p \leq 0.05$ and a <2-fold decrease in expression after exposure to 5 min of heat shock at 55°C. This table lists only genes whose annotations are informative and well-supported. Full data in ***Supplementary Table S1****.*

tower-associated outbreak in Quebec City (Lévesque et al., 2014), and a 2014 HWDS-associated outbreak in Sherbrooke (Bédard et al., 2016b, 2021). After acclimatization to starvation in water, heat shock at 55°C assay revealed differences in these strains' abilities to tolerate heat shock. Of the related 1427-like isolates collected from spatially separated locations in a hospital HWDS, the isolate collected from heat exchanger water (1427.W.HE) was more resistant to heat shock than related isolates collected from the biofilm in the heat exchanger or from an internal faucet surface. Biofilm isolates, collected from fouled metal surfaces, were found to have a higher resistance to copper attributed to local adaptation to the heavy-metal rich environment (Bédard et al., 2021); we speculate that the higher heat resistance in 1427.W.HE was due to similar *in situ* adaptation to transient heat shocks while in water, which is more closely mimicked in our experimental conditions. Surprisingly, the two clinically-isolated strains, Philadelphia-1 and LSPQ120292, were the most robust, while the remaining four environmental isolates suffered greater decreases in culturability at 55°C. Though this contradicts our hypothesis that the heat exchanger strains with prior exposure to heat shock conditions would be the most heat resistant, it is consistent with the findings of Epalle et al., who found a higher retention of culturability and viability in a clinical strain of *L. pneumophila* compared both to either a laboratory or an environmental strain when exposed as saline suspended cells to temperatures between 50 and 70°C (Epalle et al., 2015).

L. pneumophila Engages a Core Bacterial Heat Shock Response

Our microarray study of the *L. pneumophila* Philadelphia-1 transcriptional response to heat shock investigates an important aspect of its resistance to microbial control strategies. We determined that the *L. pneumophila* early heat shock response involves a co-ordinated up-regulation of genes necessary to cope with the immediate challenges of sudden stress (**Figure 2A**, **Table 4**), highlighted by an up-regulation in the transcription of *rpoH*, an alternative sigma factor canonically recruited by bacteria to direct transcription toward a heat shock response profile (Cowing et al., 1985; Gruber and Gross, 2003; Chakraborty et al., 2014). These include canonical heat shock proteins (such as HtpG and GrpE) and proteases (such as HtrA and ClpB) which help resolve protein damage either by promoting the re-folding of damaged polypeptides or by recruiting proteolytic machinery (Guisbert et al., 2008;

TABLE 4 | Selected upregulated genes identified by microarray.

lpg number	Gene name	Gene	Log_2 FC	Adjusted *p*-value
lpg1211	Transporter, LysE family (threonine efflux)		5.62	0.0000
lpg0583	Phosphate transporter (phosphate permease)		3.23	0.0218
lpg2026	Heat shock protein GrpE	grpE	3.20	0.0007
lpg1177	Riboflavin biosynthesis protein RibD	ribD	3.13	0.0009
lpg0491	Amino acid (glutamine) ABC transporter, periplasmic amino acid binding protein		3.01	0.0007
lpg2667	RNA polymerase sigma-32 factor (sigma factor RpoH)	rpoH	2.89	0.0065
lpg0811	Rod shape determining protein MreB (regulator of FtsI)	mreB	2.64	0.0032
lpg0492	Amino acid (glutamine) ABC transporter, permease	yqiY	2.64	0.0022
lpg2988	ATP synthase F0, A subunit (ATP synthase A chain)	atpB	2.64	0.0045
lpg2094	Carbon storage regulator RsmA (controls glycogen synthesis, could also regulate swarming and quorum sensing)	csrA	2.54	0.0028
lpg2651	50S ribosomal protein L21	rplU	2.47	0.0079
lpg2934	Transcription termination factor rho		2.46	0.0121
lpg2295	Ribosomal large subunit (23S rRNA) pseudouridine synthase C	rluC	2.38	0.0077
lpg2841	Major facilitator superfamily (multi-drug resistance translocator, bicyclomycin resistance)		2.36	0.0050
lpg2658	Ferrous iron transporter A	feoA	2.33	0.0031
lpg0104	Peptide methionine sulfoxide reductase		2.32	0.0030
lpg2595	Peptide deformylase (polypeptide deformylase)	def	2.31	0.0051
lpg1903	Hydrolase (esterase/lipase/thioesterase)		2.27	0.0034
lpg2903	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE		2.27	0.0049
lpg0821	Lipopolysaccharide biosynthesis glycosyltransferase (beta 1,4-glucosyltransferase) (UDP glucose-lipooligosaccharide beta 1,4-glucosyltransferase)		2.25	0.0049
lpg2345	ATP-dependent RNA helicase (cold shock DEAD box protein A)	deaD	2.17	0.0042
lpg2493	Small heat shock protein HspC2 (molecular chaperone)		2.16	0.0079
lpg2031	Arginyl tRNA synthetase	argS	2.07	0.0144
lpg0610	Major facilitator family transporter (multidrug efflux transporter)		2.00	0.0077
lpg2797	Ribosomal RNA large subunit methyltransferase J (cell division protein FtsJ)		1.99	0.0078
lpg1509	D-alanyl-D-alanine carboxypeptidase		1.98	0.0218
lpg0780	Metallo-beta-lactamase family protein, COG0491: Zn dependent hydrolases, including glyoxylases		1.96	0.0122
lpg2980	DNA topoisomerase IV subunit A	parC	1.96	0.0152

(Continued)

TABLE 4 | Continued

lpg number	Gene name	Gene	Log_2 FC	Adjusted *p*-value
lpg1862	Trigger factor TF (FKBP-type peptidyl prolyl cis-trans isomerase)	tig	1.94	0.0097
lpg2789	NADH dehydrogenase I, A subunit (NADH-ubiquinone/plastoquinone oxidoreductase chain 3)	nuoA	1.94	0.0085
lpg1417	DNA gyrase, A subunit	gyrA	1.88	0.0125
lpg1208	Transcriptional regulator MarR family		1.87	0.0158
lpg2530	3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (2-dehydro-3-deoxy-phosphoheptonate aldolase)	aroF	1.86	0.0102
lpg0640	Heat shock protein, HslVU, proteasome-related peptidase subunit	hslV	1.85	0.0086
lpg1186	Competence lipoprotein ComL		1.85	0.0093
lpg1967	Transcriptional regulator, TetR family (tetracyclin resistance)		1.84	0.0314
lpg1393	3-oxoacyl-(acyl carrier protein) synthase II FabH	fabH	1.84	0.0234
lpg0192	Heat shock hsp20 (alpha crystallin) proteins family		1.82	0.0177
lpg1369	Chaperone Hsp90 HtpG (heat shock protein)	htpG	1.77	0.0181
lpg2025	Chaperone protein DnaK, heat shock protein Hsp70	dnaK	1.76	0.0372
lpg2338	DnaK suppressor protein	dksA	1.75	0.0330
lpg0945	Gala protein type 1 or 3 (caspase recruitment domain, leucine rich repeat?)	legL1	1.74	0.0181
lpg0741	Inosine-5′-monophosphate dehydrogenase (IMP dehydrogenase)		1.73	0.0207
lpg2646	Response regulator GacA (global activator)/transcription activator	letA	1.73	0.0297
lpg1946	Transcriptional regulator LuxR	lpnR2	1.72	0.0125
lpg3004	Ribonuclease P protein component	rnpA	1.71	0.0200
lpg2466	Hydrogenase expression/formation protein (F420-non-reducing hydrogenase II)	vhtD	1.68	0.0275
lpg1750	ClpB protein (heat shock protein) Clp ATPase (ATP-dependent protease binding subunit)	clpB	1.67	0.0214
lpg0615	Polypeptide deformylase (formylmethionine deformylase)		1.65	0.0207
lpg1331	Protease DO (periplasmic serine endoprotease)	htrA	1.65	0.0189
lpg1392	Fatty acid/phospholipid synthesis protein PlsX	plsX	1.57	0.0411
lpg2763	Mg2+ and Co2+ transporter CorB, hemolysin		1.53	0.0214
lpg1801	RecA bacterial DNA recombination protein (recombinase A)	recA	1.52	0.0297
lpg1546	Fimbrial biogenesis and twitching motility protein PilF		1.50	0.0245
lpg2597	DNA processing enzyme DprA (SMF family) (Rossman fold nucleotide binding protein)		1.49	0.0282
lpg0928	Type IV pilus biogenesis protein PilN	pilN	1.46	0.0476

(Continued)

TABLE 4 | Continued

lpg number	Gene name	Gene	Log_2 FC	Adjusted *p*-value
lpg2839	SsrA (tmRNA) binding protein (small protein B), protein quality control system	smpB	1.46	0.0259
lpg1362	Type II protein secretion LspG (general secretion pathway protein G)	gspG	1.45	0.0328
lpg1463	Preprotein translocase; secretion protein SecA	secA	1.45	0.0295
lpg2859	MoxR protein (ATPase) methanol dehydrogenase regulatory protein		1.40	0.0352
lpg1277	ABC transporter ATP binding protein (abcT3) (multidrug resistance ABC transporter) (hemolysin secreting ATP binding protein)	abcT3	1.39	0.0317
lpg2009	Guanosine-3,5-bis(diphosphate)-3-pyrophosphohydrolase	spoT	1.37	0.0443
lpg2661	3-methyl-2-oxobutanoate hydroxymethyltransferase (ketopantoate hydroxymethyltransferase)	panB	1.35	0.0386
lpg0400	Signal recognition particle protein Ffh (GTP-binding export factor)	ffh	1.35	0.0486

Genes listed are significantly downregulated with a Benjamini-Hochberg adjusted $p \leq 0.05$ and a >2-fold increase in expression after exposure to 5 min of heat shock at 55°C. This table lists only genes whose annotations are informative and well-supported. Full data in ***Supplementary Table S1****.*

Genest et al., 2011). Other upregulated proteins include FeoA (*lpg2658*), involved in ferrous iron transport, and two polypeptide deformylase genes *lpg2595* and *lpg0615*. These proteins have been shown to play roles in re-establishing polypeptide structure and function (Cianciotto, 2001; Imlay, 2013). Genes related to DNA integrity were also identified in the heat shock up-regulon, including GyrA, RecA, and ParC, highlighting the stresses to the cell caused by heat shock (Piao et al., 2006; Almahmoud et al., 2009; Murata et al., 2011, 2018).

Can these transcriptomic results explain the differences in thermosensitivity observed between clinical and environmental strains of *L. pneumophila* or the known thermosensitivity of other *Legionella* species, such as *L. longbeachae* (Cervero-Aragó et al., 2015)? Though the Legionella genus pan-genome is large and highly variable (Gomez-Valero et al., 2019), the bacterial heat shock response and the heat shock proteins involved are highly conserved and it seems unlikely that the presence or absence of these genes could explain the differences seen in heat shock tolerance. Instead, we find it more plausible that more subtle regulatory changes are responsible for this variation. This is supported by the reduced heat shock resistance of mutants with disruptions in *dksA*, *rpoS*, or *letS*—genes important in the central regulation of virulence and life phase switching (Dalebroux et al., 2010; Trigui et al., 2015; Mendis et al., 2018). We propose therefore that mutations in regulators or promoter of heat shock proteins could most likely explain the variability in heat shock resistance seen between strains.

Transcriptional Involvement of Ribosome Biogenesis Genes

The molecular functions of several significantly upregulated genes (**Table 4**) implicate a possible role for ribosome biogenesis in the resolution of early heat shock including *lpg2295* (*rluC*), *lpg2345* (*deaD/csdA*), and *lpg2797* (*ftsJ/rrmJ/rlmE*). In addition, the BipA ribosome-associated GTPase encoded at locus *lpg2822* is also upregulated, though its corrected significance of 0.0529 falls just outside of a $p < 0.05$ statistical cut-off. Heat shock is known to independently cause the accumulation of immature ribosomal particle precursors, likely through titration of the DnaK/DnaJ/GrpE chaperone complex away from a putative thermosensitive ribosomal assembly factor (Alix and Guérin, 1993; René and Alix, 2011). One of the early irreversible denaturations during the thermal inactivation of *E. coli* is the disassembly of the 30S ribosomal subunit, with the remainder of the superstructure sequentially disassembling as temperature increases (Mackey et al., 1991).

BipA is a well-studied protein in *E. coli* whose deletion is associated with a number of phenotypic changes including virulence defects, hypermotility, and, interestingly, impaired growth at suboptimal temperatures (Farris et al., 1998; Pfennig and Flower, 2001; Krishnan and Flower, 2008; Choudhury and Flower, 2015; Choi and Hwang, 2018; Gibbs et al., 2020; Goh et al., 2021). The major function of BipA at low temperatures appears to be the assembly of the 50S subunit with deletion mutants accumulating 44S precursors that fail to integrate the L6 ribosomal protein (Choudhury and Flower, 2015; Choi and Hwang, 2018). The cold-sensitivity of

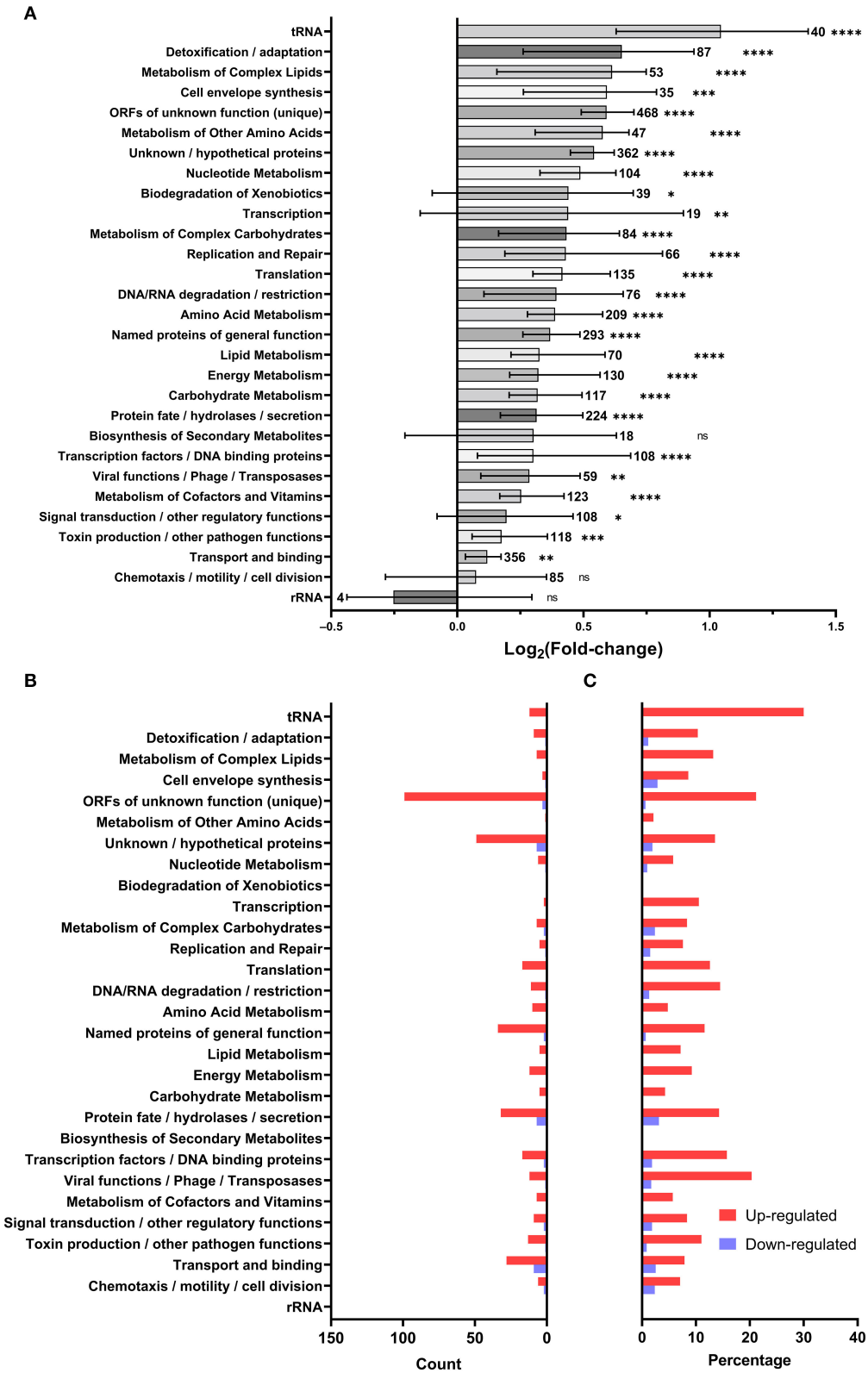

FIGURE 3 | Microarray data summarized by GenBank annotated function. Each gene was assigned to the GenBank categories included in its annotated function: **(A)** the distribution of $\log_2$ fold-change of genes included in each category (bars and error bars show mean and 95% confidence interval; sample size adjacent to each bar; a one-sample two-tailed Wilcoxon signed rank test in Prism 9.3.1 was used to evaluate the significance of discrepancy between the observed $\log_2$ fold-changes and a hypothetical $\log_2$ fold-change of 0; ns = not significant, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001), **(B)** count of differentially regulated genes in each Genbank category, **(C)** percentage of genes differentially regulated in each Genbank category.

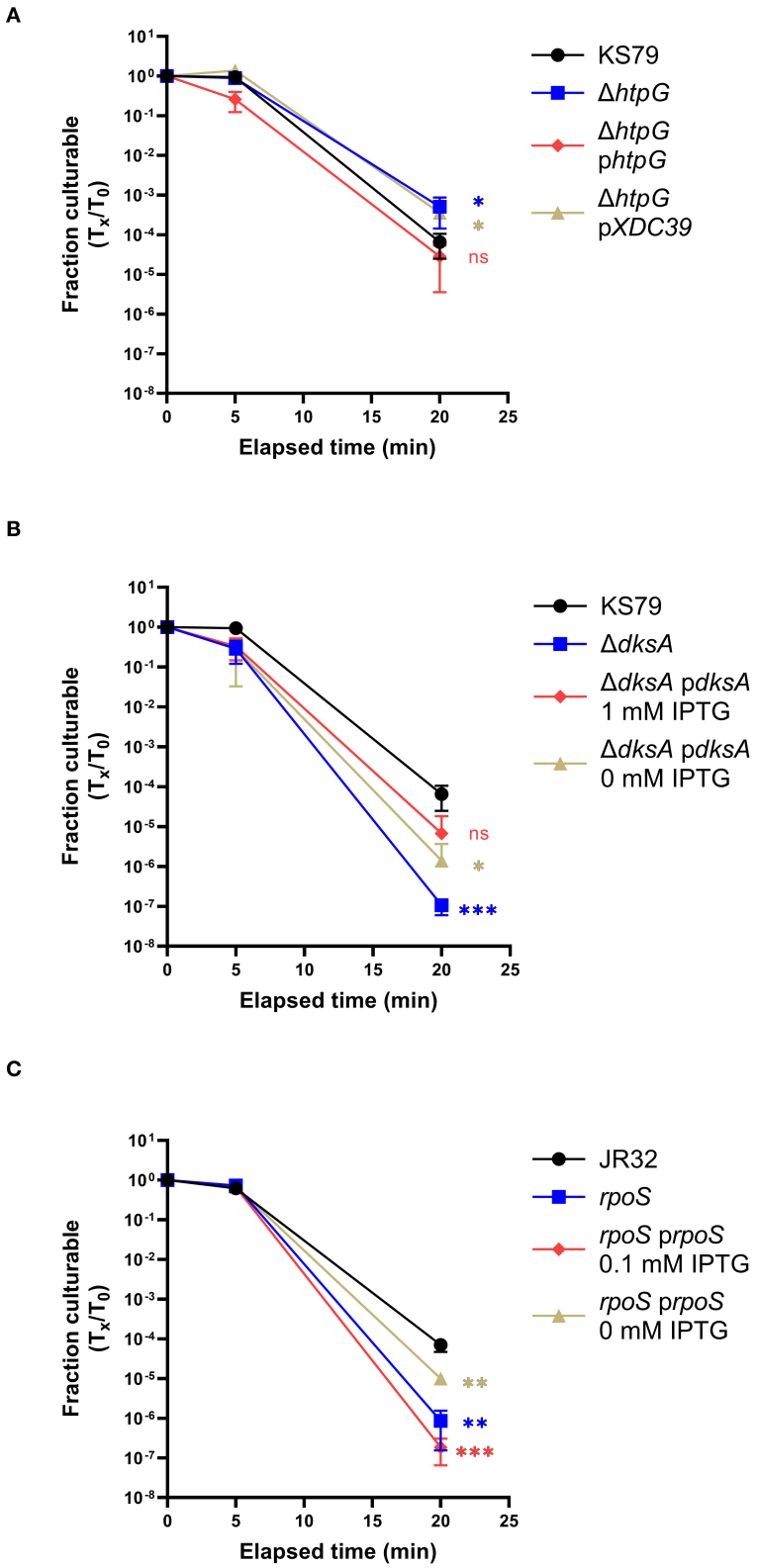

FIGURE 4 | Effects of *htpG*, *dksA*, and *rpoS* on heat shock tolerance. Strains washed in and acclimated to Fraquil were heat shocked at 55°C for 5 or 20 min. **(A)** Δ*htpG* was compared with its ancestor and complemented with a plasmid-borne copy of *htpG* [*phtpG* or with an empty vector control (pXDC39)].

(Continued)

FIGURE 4 | (B) Δ*dksA* was compared with its ancestor and complemented with a plasmid-borne copy induced with 1 mM IPTG. **(C)** An RpoS-null mutant was compared to its ancestor strain JR32 and complemented with a plasmid-borne copy induced with 0.1 mM IPTG. Error bars indicate SD, $n = 3$ biological replicates. Significance was measured compared to the ancestral strain at the same time point using a two-tailed Student's *t* statistic on log-transformed values (ns = not significant, * < 0.05, ** < 0.01, *** < 0.001 with Bonferroni's correction for 3 comparisons).

Δ*bipA* mutants is complemented by the additional deletion of RluC, a pseudouridine synthase that modifies the peptidyl transferase center in the 50S subunit (Krishnan and Flower, 2008; Choudhury and Flower, 2015). CsdA, a DEAD-box RNA helicase, is another protein involved in the ribosomal biogenesis in *E. coli* through its effects in the maturation of the 50S subunit (Choudhury and Flower, 2015). Like with BipA, Δ*CsdA* mutants accumulate a 40S immature precursor of the 50S subunit at low temperatures (Charollais et al., 2004; Peil et al., 2008). Double mutants lacking both CsdA and BipA experience greater growth defects at low temperature and a more severe accumulation of immature ribosome subunit fractions, suggesting independent molecular mechanisms with overlapping effects (Choudhury and Flower, 2015). RrmJ is a methyltransferase whose role in ribosome biogenesis is also in the assembly of the 50S subunit (Caldas et al., 2000; Arai et al., 2015; Wang et al., 2020). Knockout of its methylation function causes delayed growth at lower temperatures.

Regulatory Protein Mutants Have Altered Heat Shock Tolerances

DksA, RpoS, and LetA/S are all involved in regulatory systems that control *L. pneumophila*'s response to starvation and cellular stress, while influencing virulence and shifts into the transmissive or stationary phases (Dalebroux et al., 2010; Trigui et al., 2015; Mendis et al., 2018). Prior work has shown the importance of the LetA/S-RsmY/Z-CsrA regulatory cascade in suppressing growth-related gene transcription in water-suspended bacteria and, reciprocally, that a deletion of LetS causes pre-mature loss of culturability in water and increases susceptibility to heat shock (Mendis et al., 2018). It was also shown that the production of RsmY and RsmZ, downstream small regulatory RNA effectors of LetA/S, involves RpoS control (Hovel-Miner et al., 2009; Mendis et al., 2018). RpoS, an alternative sigma factor, is a major organizer of the bacterial general stress response and, in *L. pneumophila*, alters gene transcription under nutritional deprivation and allows the bacteria to differentiate from a replicative state to a more resistant transmissive form (Hughes et al., 2019). Proper function of RpoS is required for *L. pneumophila* to replicate in protozoan hosts (Hales and Shuman, 1999; Hovel-Miner et al., 2009) and to maintain long-term culturability in freshwater (Trigui et al., 2015). RpoS-null mutants of *L. pneumophila* retain a stationary-phase advantage in resistance to osmotic, oxidative, and acid stressors (Hales and Shuman, 1999) unlike in *E. coli* where RpoS is necessary to adopt this resistant form. While this appears to stand at odds with our observations of increased heat shock susceptibility in the tested RpoS-null mutants, we note that Hales and Shuman studied *L. pneumophila* cells immediately after harvest from post-exponential growth in broth while our results were found using cells that had been starved for 24 h in freshwater. We suggest that the pre-mature death seen when RpoS-null mutants are suspended in freshwater (Trigui et al., 2015) reflects a cell that is more sensitive to other stressors, including heat shock. Alternatively, it may be that heat resistance depends on pathways more deeply tied to RpoS function than osmotic, oxidative, or acid stresses, and so the loss of RpoS causes a greater phenotypic change.

DksA is a bacterial transcriptional regulator that binds to RNA polymerase and influences its affinity for different gene promoter sequences. DksA works with the alarmone ppGpp during starvation to allow the bacteria to transition from its replicative life stage to a motile transmissive form (Dalebroux et al., 2010), in co-ordination with RpoS. Both ppGpp and DksA are also involved in a negative feedback loop centered on CsrA, a post-transcriptional regulator that plays simultaneous roles in the control of iron homeostasis, thiamine biosynthesis, quorum sensing, flagellar biosynthesis, central carbon flux, virulence, and the general stress response (Sahr et al., 2017). *L. pneumophila* lacking DksA show a premature loss of viability in stationary phase compared to wild-type and also develop a filamentous cell shape (Dalebroux et al., 2010). Filamentation is also seen in sessile *L. pneumophila* cultured at higher temperatures, while planktonic cells and cells grown at 25°C retain a rod-shaped morphology (Piao et al., 2006). The deletion mutant strain lacking DksA showed a greater sensitivity to heat shock conditions than its wild-type ancestor.

HtpG Deletion Causes Unanticipated Increase in Heat Shock Tolerance

HtpG is a homolog of the ubiquitous 90 kilodalton bacterial heat shock protein. In bacterial model systems, it responds to heat shock, and other forms of cell stress, through a coordinated chaperone response that disaggregates protein tangles and resolves misfolded polypeptides (Genest et al., 2011, 2015). In other bacterial species, HtpG is involved in numerous cell functions including virulence, hot and cold tolerance, and motility (Choi et al., 2012; Grudniak et al., 2018). Surprisingly, given its historically designated role as a heat shock protein, we observed that the deletion of the HtpG-coding locus in *L. pneumophila* improved heat-shock tolerance. A similar phenomenon was shown by Fauvet et al. in a proteomic study of *E. coli*, where the deletion of *htpG* improved growth at elevated temperatures in a temperature-sensitive Δ*dnaKdnaJ* double mutant (Fauvet et al., 2021). DnaK and DnaJ are, respectively, *E. coli* homologs of HSP70 and HSP40 which act cooperatively with HtpG to direct re-folding of misfolded or of nascent unfolded polypeptides (Amemura-Maekawa and Watanabe, 1997; Richter et al., 2010). When DnaK/J foldase activities are overwhelmed by massive disruptions of proteostasis during severe cellular

stress or outright missing, as in a double mutant, the authors suggest that HtpG directs its substrates toward proteolysis which depletes the cellular inventory of proteins necessary for survival and growth under these conditions. A similar phenomenon may be occurring in our bacterial system, with an overzealous HtpG protein causing off-target proteolysis during transient heat shock.

CONCLUSION

As a natural member of the freshwater microbiome, *L. pneumophila* has been able to expand to similar niches in the human built environment (Kirschner, 2016; Lesnik et al., 2016). These ecosystems have repeatedly demonstrated the ability to seed *L. pneumophila* outbreaks of tremendous public health consequence (McDade et al., 1977; Lévesque et al., 2014). Despite the best efforts of premise plumbing managers, populations can often survive pasteurization (Allegra et al., 2011; Whiley et al., 2017) making it important to understand the heat shock response in *L. pneumophila*. The present study is, to our knowledge, the first transcriptomic study of *L. pneumophila*'s response to heat shock under starvation conditions simulating a HWDS. As an exploratory platform for study, the microarray has narrowed the scope of possible genes involved in heat shock resistance for future experimental or comparative genomic studies to explain the differences in heat resistance seen in different strains and species of *Legionella*. We show the recruitment of canonical bacterial heat shock factors, as well as a possible role for ribosome biogenesis in resolving the disruption of protein homeostasis caused by a sudden change in temperature. The ability of the bacterium to accommodate thermal stress could be related to the overall organization of its life cycle, with genes such as *letS*, *dksA*, and *rpoS* that are involved in the life cycle control (Sahr et al., 2017) also affecting thermotolerance when genetically disrupted. Though this study gives a preliminary exploration of factors underlying the heat shock tolerance of *L. pneumophila*, there are further research paths that it leaves unaddressed. Though strain-wise differences are observed in heat shock tolerances, transcriptomic and genetic studies of a larger corpus of strains could give a deeper understanding of the pathways and genes involved in heat resistance that this study could not fully resolve. In conclusion, we find a complex regulatory network underlying the heat shock resistance of *L. pneumophila* that allows it to persist in engineered water systems following pasteurization.

DATA AVAILABILITY STATEMENT

The microarray datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: GSE196713].

AUTHOR CONTRIBUTIONS

JL and SF conceived the project, designed experiments, prepared manuscript, and edits. JL performed experiments and data analysis. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frwa.2022.891477/full#supplementary-material

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