Protein Homeostasis in Survival and Persistence of *Escherichia coli*, *Salmonella* Typhimurium and *Cronobacter sakazakii* at Alkaline pH and after Desiccation

by

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Abstract

Bacteria have evolved a protein homeostasis network to maintain the integrity of their proteome. Chaperones and proteases of the core genome can be complemented by accessory genes encoding additional elements of the protein homeostasis network. The transmissible locus of stress tolerance (tLST) confers exceptional tolerance to high temperature, chlorine, oxidative chemicals and high hydrostatic pressure by reducing protein aggregation. Highly expressed tLST proteins include intracellular small heat shock proteins sHsp20 and $sHsp_{GI}$, disaggregase $ClpK_{GI}$, and periplasmic stress chaperones PscA and PscB. The tLST also encodes $KefB_{GI}$, a Na^+/H^+ antiporter. This study aimed to determine the effects of improved protein homeostasis that is mediated by the tLST on bacterial tolerance to alkaline pH and desiccation.

The expression of the Cpx, a two-component regulatory system in *Escherichia coli* which responds to envelope stress, was not altered by the presence of the tLST. The tolerance or resistance of *E. coli* to alkaline pH in the pH range of 6.9 to 9.2 and at pH 11, respectively, were also not changed by the presence of the tLST. The presence of the tLST improved, however, survival at pH 11 in presence of chlorine stress; this effect was attributed to $KefB_{GI}$ rather than protein homeostasis.

The impact of protein homeostasis on desiccation tolerance was characterized in *Salmonella enterica* serovar Typhimurium, *Cronobacter sakazakii* and *E. coli.* The cloning and expression of the *shsp20*, $shsp_{GI}$ and $clpK_{GI}$ decreased desiccation tolerance in *S.* Typhimurium and *C. sakazakii* but not in *E. coli*. Protein aggregates were visualized *in vivo* using an IbpA-Yfp fusion protein, demonstrating that cloning of *shsp20*, $shsp_{GI}$ and $clpK_{GI}$ reduced intracellular protein aggregates in *S.* Typhimurium and *C. sakazakii* but not in *E. coli*. The presence of the elements intensively consuming cellular resources including high copy plasmids as well as cloning of highly expressed proteins had a detrimental effect on desiccation tolerance irrespective of the function of the

expressed proteins. Abolishing the ATP-hydrolysis function of $ClpK_{GI}$ by substitution of two amino acids (E383A/E723A) increased cell counts of *S.* Typhimurium after desiccation more than 100-fold, demonstrating a direct contribution of protein aggregation to desiccation tolerance. Dry storage of *S.* Typhimurium and *C. sakazakii* for 112 days revealed that cloning of the tLST or of its protein homeostasis module decreased survival during desiccated storage in infant formula. In conclusion, additional protein quality control provided by tLST does not aid in alkaline resistance or tolerance in *E. coli*. The desiccation tolerance of *S.* Typhimurium and *C. sakazakii* positively correlates with the formation of protein aggregates but are negatively impacted by the presence of the elements intensively consuming cellular resources. Taken together, this study improves the understanding of how protein homeostasis affects bacterial stress tolerance by increasing tolerance to some stressors while reducing tolerance to others.

Preface

This thesis is the original work by Tongbo Zhu.

Chapter 2 has been published as: Zhu, T., Wang, Z., McMullen, L. M., Raivio, T., Simpson, D. J., & Gänzle, M. G. (2021). Contribution of the Locus of Heat Resistance to Growth and Survival of *Escherichia coli* at Alkaline pH and at Alkaline pH in the Presence of Chlorine. Microorganisms, 9(4), 701. doi: 10.3390/microorganisms9040701. The results in Chapter 2 have been presented as a poster at the ASM World Microbe Forum 2021 online worldwide. The term of locus of heat resistance (LHR) has been used in this Chapter to be identical with the publication.

Chapter 3 has been submitted for publication and is currently under major revision. The title of the manuscript is "Protein aggregation as a bacterial survival strategy in *Salmonella* Typhimurium and *Cronobacter sakazakii* under desiccation" with authors Tongbo Zhu, Zhiying Wang, Lynn M. McMullen, Tracy L. Raivio, Michael G. Gänzle, and David J. Simpson.

Dedication

To Luca,

Thanks for being a “chaperone”. All is well.

Acknowledgements

I believe the most fortunate in my 2019 was I started my journey in Lab 2-50. The key word of my life here is “Story”. As a story-lover, in the past three years, I have learned how to understand research as a story, and how to tell my scientific work to others as a story.

I sincerely appreciate my supervisor, Dr. Michael Gänzle, for accepting me as a master student and all his guidance, patience and encouragement that unwind my mind when I struggled with my experiments. I would like to thank my co-supervisor, Dr. Tracy Raivio, for her knowledge and support since my undergraduate. I am grateful to have Dr. Lynn McMullen as my committee member; thank you for the helpful advice on my statistics, scientific writing and career development. I also want to acknowledge Dr. Lisa Stein, for agreeing to be my arm’s length examiner, I really appreciate your input.

I sincerely appreciate all the help from my past and present lab members. Especially I would like to thank Dr. David Simpson for providing the research idea and letting me realize that I am not suited for the PhD program. I want to thank Dr. Zhiying Wang for being my close collaborator in the lab, enjoying or suffering all the happiness, sadness, pain and joy with me from our experiments.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Amp	Ampicillin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
a_W	Water activity
BLAST	Basic local alignment search tool
Cam	Chloramphenicol
CFU	Colony forming units
CPEC	Circular polymerase extension cloning
eGFP	Enhanced green fluorescent protein
GSH	Glutathione
GSSG	Glutathione disulfide
HOCl	Hypochlorous acid
IPTG	Isopropyl β-d-1-thiogalactopyranoside
Kan	Kanamycin
LB	Luria-bertani
LHR	Locus of heat resistance
OD	Optical density
ORF	Open reading frame
PCR	Polymerase Chain Reaction
(p)ppGpp	Guanosine pentaphosphate/tetraphosphate
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-qPCR	Quantitative reverse transcription PCR
sHSP	Small heat shock protein
STEC	Shiga toxin-producing *Escherichia coli*
TA	Toxin-antitoxin
Tet	Tetracycline
TF	Trigger factor
tLST	Transmissible locus of stress tolerance
TSA	Tryptic soy agar

TSB	Tryptic soy broth
VBNC	Viable but non-culturable

Chapter 1. Introduction

1.1 Bacterial protein homeostasis and stress response

Accurate protein folding is essential for bacterial physiology. Bacteria have evolved a sophisticated molecular system, collectively named as protein homeostasis or proteostasis network, to maintain the proteome integrity according to the rapidly changed living environment. Various cellular and environmental stresses including the accumulation of toxic chemicals, oxidative stress, high pressure, heat shock and extreme pH can result in disturbance of protein homeostasis which leads to protein misfolding, unfolding and aggregation, and therefore, reduced cell viability (Ezraty et al., 2017; Govers & Aertsen, 2015; Kishore et al., 2012; Lund et al., 2014; Richter et al., 2010). In response to the proteotoxic stresses, bacteria deploy a protein quality control machinery with its central constituents of chaperones and proteases functioning to maintain the dynamic equilibrium from protein synthesis, folding, refolding, transport to timely degradation of irreparably damaged proteins (Castanié-Cornet et al., 2014; Mogk et al., 2011; Schramm et al., 2014). In prokaryotes, the major elements in this machinery are highly conserved.

The highly conserved bacterial chaperone systems include the ribosome-bound trigger factor (TF), DnaKEJ and GroESL complex (Fig. 1.1) (Castanié-Cornet et al., 2014; Wong & Houry, 2004). Chaperones prevent protein misfolding and aggregation by shielding their exposed hydrophobic surfaces from the aqueous cytosol, thereby protecting these residues from forming aberrant interactions (David et al., 2016). The ATP-independent TF interacts with newly translated nascent polypeptides to promote stable formation of secondary and tertiary structures of protein (Agashe et al., 2004; O'Brien et al., 2012). In contrast, ATP-dependent chaperones, DnaK and GroEL, aid not only during *de novo* protein synthesis and protein transport but also inspect the conformational

status of existing cytoplasmic proteins (Calloni et al., 2012; Hartl & Hayer-Hartl, 2009). The

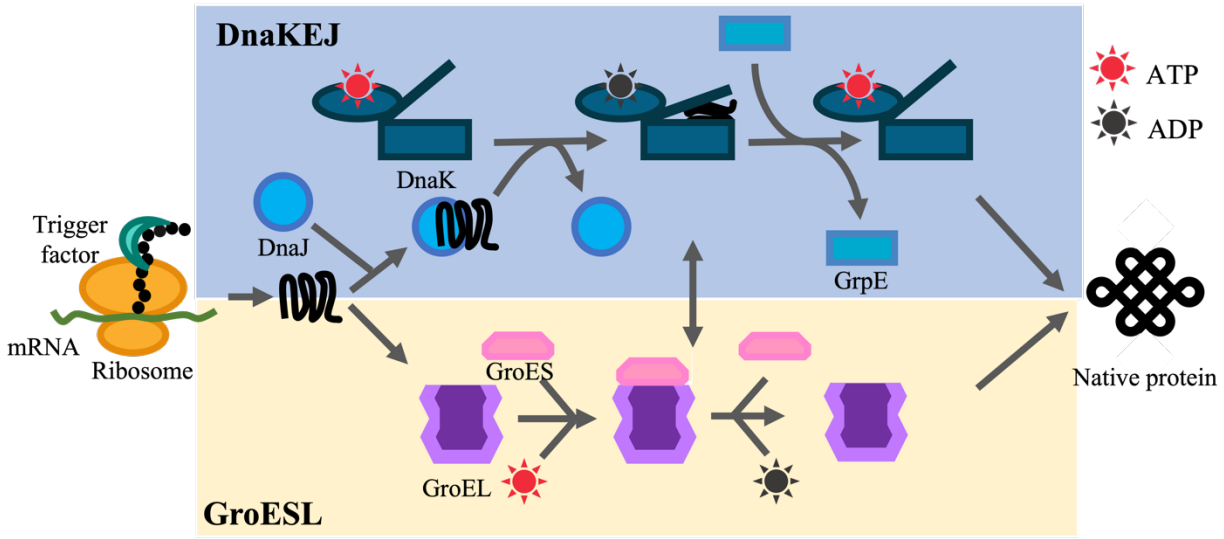

Figure 1.1. The bacterial chaperone network of ribosome-bound trigger factor (TF), DnaKEJ and GroESL for *de novo* protein synthesis. TF interacts with newly translated polypeptides and hands them over to the DnaKEJ or GroESL systems. In the DnaKEJ machinery, DnaJ holds and presents the non-native protein substrate to DnaK which folds the substrate into its native conformation in an ATP-dependent manner. GrpE functions to reset DnaK for the following cycle. In the GroESL machinery, GroES presents non-native protein substrate to one side of GroEL, which folds the substrate into its native conformation in an ATP-dependent manner. The subtracts of the DnaKEJ or GroESL systems can be interchanged.

Escherichia coli DnaK is constitutively expressed and is stress inducible under the control of σ32, and interacts with more than 700 cytosolic proteins, functioning as a central hub in the chaperone network. In the reaction cycle, DnaJ holds and presents the non-native protein substrate to ATP-bounded DnaK; After ATP-hydrolysis, GrpE catalyzes ADP release and subsequent new ATP binding triggers substrate dissociation, which resets DnaK for the next cycle (Calloni et al., 2012). Although GroESL expressed ten times lower than DnaK, it is essential for growth of *E. coli* at all temperatures (Fayet et al., 1989). GroESL-depleted strains exhibit defective transcription,

translation and a filamentous morphology (Fujiwara & Taguchi, 2007). GroES binds unfolded protein substrate to one side of the GroEL complex which then undergoes a dramatic conformational change, shifting from closed to open state (Tyagi et al., 2009). In the central cavity of GroEL, the unfolded protein is refolded into its native conformation and the subsequent dissociation of GroES releases the trapped substrate (Tyagi et al., 2009).

The protein quality control network also has disaggregation chaperone machineries to resolve existing protein aggregates (Barnett et al., 2000). ClpB, a highly conserved disaggregase that is widespread in Gram-negative bacteria, improves survival under various stress conditions, e.g. osmotic shock, oxidative stress, heat, acidity, ethanol and nutrient depletion (Alam et al., 2021; Meibom et al., 2008; Thomas & Baneyx, 1998; Tripathi et al., 2020). Together with DnaK, ClpB mediates the ATP-dependent re-solubilization of aggregated proteins (Clark et al., 2013). The ATP-independent small heat shock proteins (sHSP) associate with partially unfolded proteins (Webster et al., 2019a). sHSPs are holdases that interact with exposed hydrophobic residues to prevent aggregation until the proteins are refolded by other chaperones (Bakthisaran et al., 2015; Haslbeck & Vierling, 2015).

Damaged or unneeded proteins that are misfolded beyond repair are degraded by proteases (Fig. 1.2). The proteolysis reactions are mainly conducted by the finely tuned energy-dependent AAA+ (ATPases Associated with a variety of cellular Activities) proteases (Mahmoud & Chien, 2018). Proteases are exceptionally sensitive and selective in recognizing substrates. The widespread and highly conserved proteases in bacteria includes the Lon, FtsH and ClpP (Mahmoud & Chien, 2018; Sauer & Baker, 2011). These ATP-fueled enzymes contain AAA+ domains that are assembled into a hexameric ring that translocate unfolded polypeptides into its degradation chamber (Gur & Sauer, 2008; Sauer & Baker, 2011). In *E. coli*, at least 50% of the misfolded proteins are degraded

by Lon (Kowit & Goldberg, 1977); the membrane anchored FtsH is essential for the quality control of membrane proteins and plays a role in regulation of the heat shock response (Langklotz et al., 2012). The ClpP protease system is required for survival and infection of hosts of Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Mycobacterium* tuberculosis and *Listeria monocytogenes* (Illigmann et al., 2021; Msadek et al., 1998). For Gram-negative bacteria, ClpP in particular is important for the growth of *Salmonella enterica* serovar Typhimurium under low pH, elevated temperature and high salt conditions (Thomsen et al., 2002).

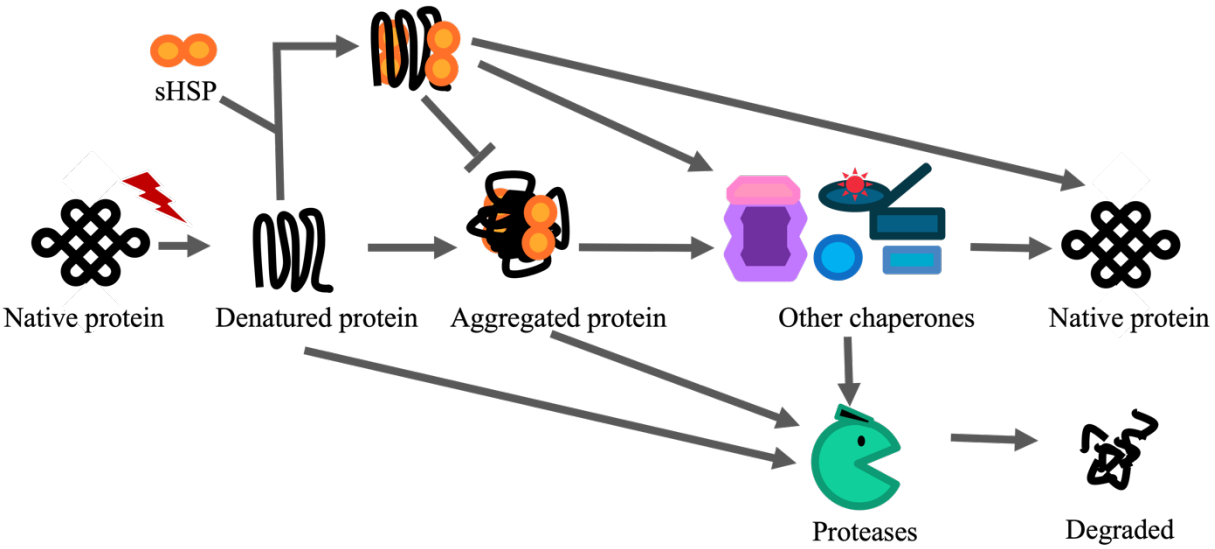

Figure 1.2. Function of chaperones and proteases in response to disruption of protein homeostasis. sHSP interacts with the denatured proteins in their early stage to stabilize the structure and prevent further aggregation until the proteins are refolded by themselves or by other ATP-dependent chaperones. Chaperones like ClpB require co-existing DnaK to form a bi-chaperone system whereas its homolog ClpK can function independently. The denatured or aggregated proteins which are beyond repair are degraded by proteases.

In *E. coli*, heat shock ranging from 42 to 47°C, the maximum growth temperature, increases the requirement for protein homeostasis and most of the chaperones and proteases are strongly upregulated (Richter et al., 2010). Both DnaKJE and GroESL are upregulated following

temperature elevation (Chapman et al., 2006; Tomoyasu et al., 2001). Severe oxidative stress causes inactivation of DnaK but the activation of Hsp33, a redox-regulated chaperone, improves bacterial tolerance to H_2O_2 and hypochlorous acid (Wholey & Jakob, 2012). Additionally, the ATP-independent chaperones HdeA/B, which are located in the periplasm, can be induced under acidic pH; Their overexpression promotes survival from pH 2 to 4 (Dahl et al., 2015). Therefore, maintaining protein homeostasis becomes particularly important when bacteria are faced with stresses that destroy the structural integrity of proteins. Rapid repair or clearance of the defective proteins by the protein quality control machinery ensures bacterial survival, furthermore rendering ecological benefits in competition with other microorganisms for limited resources.

Although bacteria have existing protein quality control machineries to cope with fluctuating proteostasis, protein aggregation not only occurs in the presence of acute stress, which can overwhelm the machinery, but also occurs naturally, which has been proposed as inevitable and accruing intracellular waste bins. The protein aggregates are disposed asymmetrically at the older cell pole during cell division and progeny that inherit the old pole experience declines in growth and reproduction rates, which have been defined as cellular aging (Lindner et al., 2008). This mechanism has been proposed as a more cost-effective way for rejuvenation of bacterial populations; however, subsequent studies linked the aged subpopulation with the formation of persister cells and an enhanced stress tolerance (Govers et al., 2018). The concept of persister cells and how protein aggregates contribute to their formation will be explored further in subsequent sections.

1.2 Bacterial persister cells

Stress is an unavoidable part of the life of free-living bacteria. In addition to evolving resistance mechanisms and actively adaptation through complex transcriptional and post-transcriptional

regulatory networks, bacteria can stochastically produce a phenotypically variant subpopulation of individuals without undergoing genetic changes; this subpopulation are termed as persisters (Wood et al., 2013). The concept of persister cells, or persisters, was first described in 1940s when it was reported that 1% of a staphylococci population survived penicillin treatment (Bigger, 1944). Hence, bacterial persistence is typically observed when the majority of an isogenic bacterial population is sensitive to antibiotic treatment, while a subpopulation of individuals survives for a much longer period of time (Brauner et al., 2016). Therefore, the formation of persisters has been postulated as a bet-hedging strategy where a genetically identical bacterial population diversifies its phenotypes to distribute the risk from fluctuating environments (Veening et al., 2008).

The function of persisters is survival of a small part of the population. They are dormant cells, i.e. non-multiplying and lacking transcription, translation, and proton-motive force (Kwan et al., 2013). Persisters can transiently escape the effects of antibiotics by exhibiting transient tolerance, therefore they are distinguishable from permanently antibiotic-resistant bacteria that acquire resistance though horizontal gene transfer or genetic mutations and can proliferate in the presence of the bactericidal or bacteriostatic agents (Brauner et al., 2016). Unlike resistance, bacterial persistence is a non-inherited trait. After removal of antibiotics, the persisters can be re-cultured to a population that retains the same sensitivity to the antibiotics as the original population containing a similar proportion of cells as persisters (Keren et al., 2004).

Critically, the persister state arises not only from antibiotic treatment but also under different growth stages, nutrient deprivation and oxidative stress (Leszczynska et al., 2013; Paranjape & Shashidhar, 2019; Wu et al., 2012). The persister phenotype has been well-documented in a wide variety of bacterial species including *E. coli*, *S.* Typhimurium and *Pseudomonas aeruginosa* with highly redundant proposed mechanisms via toxin-antitoxin systems, the ppGpp pathway and

protein aggregate formation (Fig. 1.3).

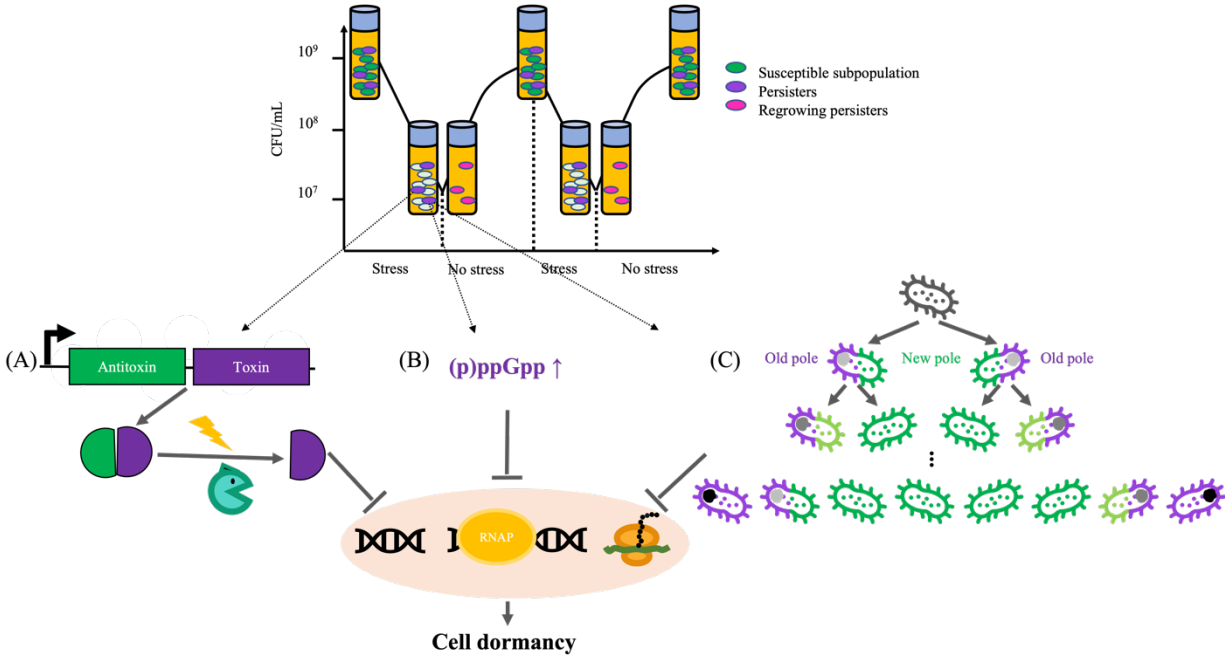

Figure 1.3. Three proposed mechanisms for the formation of persister cells. (A) Persisters formed via the chromosomally encoded toxin-antitoxin (TA) systems. The labile antitoxins are degraded by stress-induced proteases to release free toxins. (B) Persisters formed via elevated level of (p)ppGpp. (C) Persisters formed via protein aggregation. Protein aggregates form naturally in the cell but are accumulated and inherited asymmetrically by the daughter cells with the older cell poles. The presence of free toxins, high levels of (p)ppGpp and intensive protein aggregates inhibits DNA replication, transcription and translation, which arrests cell growth and lead to cell dormancy. Persister cells arise due to the state of dormancy.

1.3 Toxin-antitoxin systems

Toxin-antitoxin (TA) systems play important roles in the formation of persister cells. These systems were first described in the 1980s as a mechanism that forces maintenance of low-copy number plasmids (Gerdes & Rasmussen, 1986; Ogura & Hiraga, 1983). The systems usually consist of two adjacent genes encoding a stable toxin that arrests host metabolism and a labile antitoxin. Since reproduction of the host cells depends on the presence of the antitoxin, cells that

do not replicate the plasmids no longer reproduce (Ogura & Hiraga, 1983). Currently, most of the identified toxins are proteins whereas the antitoxins are either RNA or proteins that alter the translational level, stability or activity of their cognate toxins (Jurėnas et al., 2022). The identified TA systems have been classified into 8 types based on the mode of action of the antitoxins and summarized in Table 1.1.

Table 1.1. The working mechanism of TA systems.

TA systems	Toxin type	Anti-toxin working mechanism	Reference
Type I	Proteins	Small RNAs preventing the transcription of the toxin	(Kawano et al., 2002)
Type II		Proteins directly binding and neutralizing the toxin	(Tam & Kline, 1989)
Type III		Small RNAs forming the protein–RNA duplex with toxin	(Fineran et al., 2009)
Type IV		Proteins stabilizing the cellular target of toxin	(Jimmy et al., 2020)
Type V		RNase specifically degrading the mRNA of toxin	(X. Wang et al., 2012)
Type VI		Proteins promoting the degradation of toxin by proteases	(Aakre et al., 2013)
Type VII		Proteins post-translational modifing the toxin	(X. Wang et al., 2021)
Type VIII	RNAs that blocks gene expression	Antisense RNAs repressing the expression of the toxin RNA	(Choi et al., 2018)

Theses TA systems are also frequently distributed in the chromosome of bacteria and commonly occur with multiple copies present in each replicon. In *E. coli* K-12 there are 8 well-characterized and 28 putative TA systems (Yamaguchi & Inouye, 2011). The first TA system linked to bacterial persistence was the type II TA system, encoded by the *hipBA* module, found in *E. coli*, with HipA

toxin inactivating the translation factor EF-Tu through phosphorylation (Moyed & Bertrand, 1983). The TA loci contribute cumulatively to persistence as the deletion of all type II TA loci dramatically reduced persistence of *E. coli*. In contrast, overproduction of type II toxins induced a dormancy state that was resolved by the induction of cognate antitoxin genes (Maisonneuve & Gerdes, 2014; Pedersen et al., 2002). Under stress conditions, the labile antitoxins are preferentially degraded by stress-induced proteases, which leaves the toxin to arrest cellular activity therefore switching into a persister state (Page & Peti, 2016). In addition, single-cell analysis showed that the transcriptional of the TA operon in *E. coli* can be stochastically turned on in a small proportion of cells (Maisonneuve & Gerdes, 2014). The gene expression in individual cells is influenced by both intrinsic and extrinsic molecular noise that arises from stochastic nature of biochemical reactions and results in pronounced phenotypic diversity (Veening et al., 2008).

1.4 ppGpp

In *E. coli*, the *hipA7* gain-of-function mutation in the *hipA* toxin gene enhances persistence by 100 to 1,000-fold (Moyed & Bertrand, 1983). However, this phenotype is diminished in *relA spoT* double null mutants, which lack the ability to synthesize the enzymes producing the alarmone guanosine pentaphosphate/tetraphosphate, (p)ppGpp (Korch et al., 2003). Therefore, (p)ppGpp has been suggested as a critical metabolic regulator for toxin-antitoxin modules. (p)ppGpp is the effector in the stringent response that is triggered under nutrient starvation, such as amino acid or fatty acid starvation (Steinchen et al., 2020). In Gram-negative bacteria, (p)ppGpp inhibits DNA replication, transcription and synthesis of rRNA, which in turn dramatically alters the global gene expression profile. The downregulation of genes for growth and the upregulation of genes for stress tolerance result in a slow growth rate and cell dormancy (Durfee et al., 2008; Gaca et al., 2015). In addition, the persistence phenotype also depends on the regulation of efflux pumps and reactive

oxygen species (ROS) by (p)ppGpp where (p)ppGpp acts as a central regulator (Chuang et al., 2015; Nguyen et al., 2011). However, not all type II TA systems require the activation of stringent response to function in persister formation (Ramisetty et al., 2016). The (p)ppGpp deficient strains still generate persister cells (Chowdhury et al., 2016).

1.5 Protein aggregation

While the accumulation of aggregated protein is commonly considered as detrimental to host cells, the presence of protein aggregates also aids in bacterial stress tolerance by promoting cell dormancy, or in other words, the possible generation of persisters. In *E. coli*, persister cells that survive through antibiotic treatment more often contain protein aggregates than the non-dormant antibiotic-susceptible subpopulation (Cesar et al., 2022; J. Yu et al., 2019). Removal of the aggregates by the DnaK-ClpB bichaperone system resuscitates the persisters to actively growing cells (Pu et al., 2019). The protein aggregates are developed gradually, which is correlated to the degree of dormancy, termed as "dormancy depth", at the single cell level (Dewachter et al., 2021; Pu et al., 2019). Certain levels of protein aggregation are required for the cells to enter the dormant state but too much aggregation also prevents resuscitation after stress is removed (Dewachter et al., 2021). A low intensity of aggregates is associated with cells trapped at a shallow dormancy depth and can rapidly recover from antibiotic treatment, while deep dormant cells contain more intense aggregates that would require a longer recovery time (Pu et al., 2019). Despite the increasing number of studies demonstrating the protective effects of protein aggregates on host cells against antibiotic stress, induced protein aggregation also aids in bacterial survival under heat and desiccation treatments, which suggests accumulating protein aggregates can be a bacterial general stress response mechanism (Govers et al., 2018; X. Wang et al., 2020).

Protein aggregates can start to form naturally at any bacterial growth stage but are disposed at the

older cell pole during cell division (Lindner et al., 2008). Growth arrest has been commonly observed in daughter cells that inherited the older cell pole and subsequently enter the dormant state (Lindner et al., 2008). The correlation between protein aggregation and bacterial dormancy suggests that accumulation of protein aggregates may directly cause the formation of persisters. Proteomic analysis revealed that protein aggregates sequester a wide range of proteins functioning in a variety of cellular metabolic activities from energy production to cell proliferation (Leszczynska et al., 2013; Pu et al., 2019).

1.6 tLST as a tool to manipulate protein aggregation

The transmissible locus of stress tolerance (tLST), previously known as the locus of heat resistance (LHR), is a 14-19 kb mobile genomic island horizontally transferred among diverse species of beta- and gamma-Proteobacteria (Boll et al., 2017; Kamal et al., 2021; Mercer et al., 2015). Several variants of tLST have been identified in *Enterobacteriaceae* including tLSTa, tLST1 and tLST2; tLST1 is the shortest tLST variant providing its bacterial host with tolerance phenotypes towards elevated temperature, chlorine, oxidative chemicals and high hydrostatic pressure (Fig. 1.4) (C. Lee et al., 2018; Li et al., 2020; Mercer et al., 2017; Z. Wang et al., 2020a). Genes encoded by the three fragments of the tLST function in reducing protein aggregation, protein oxidation, and the oxidation of membrane lipids respectively. (Li et al., 2020; Z. Wang et al., 2020a). Highly expressed tLST proteins include sHsp20, $ClpK_{GI}$, $sHsp_{GI}$, PscA and PscB (Li et al., 2020). The small heat shock proteins (sHsps) and disaggregase ClpK prevent the aggregation of misfolded proteins and disaggregate the aggregated proteins (Haslbeck, 2016; C. Lee et al., 2015a). The sHsp20, $sHsp_{GI}$, and $ClpK_{GI}$, belonging to the tLST protein homeostasis module, aid in *E. coli* survival under heat and high pressure by reducing the amount of protein aggregation (Li et al., 2020). Therefore, the tLST or its protein homeostasis module can be used as a tool to manipulate

the levels of protein aggregates within the cells in order to determine the impact of protein homeostasis on bacterial stress tolerance. In addition, PscA and PscB (Periplasmic Stress Chaperones), previously named YfdX1 and YfdX2, demonstrate chaperone-like activity, preventing protein aggregation and pH-dependent stoichiometric conversion (H. S. Lee et al., 2018; Saha et al., 2016). These observations suggest their possible function in pH adaptation. The predicted translocation signal peptide in the N-terminus of YfdX indicates its localization in the periplasmic space (Saha et al., 2016). Maintaining protein homeostasis is vital for bacterial survival under alkaline condition as exposing the bacteria to extreme high pH can break the structural integrity of proteins (Mendonca et al., 1994). The tLST1 also encodes a Na^+/H^+ antiporter, $KefB_{GI}$, that may function to maintain a polarized membrane at alkaline conditions and thus contribute to alkaline adaptation (Mercer et al., 2017; Z. Wang et al., 2021). Therefore, this study aimed to examine the function of protein homeostasis in survival and persistence of bacteria under alkaline pH and desiccation conditions.

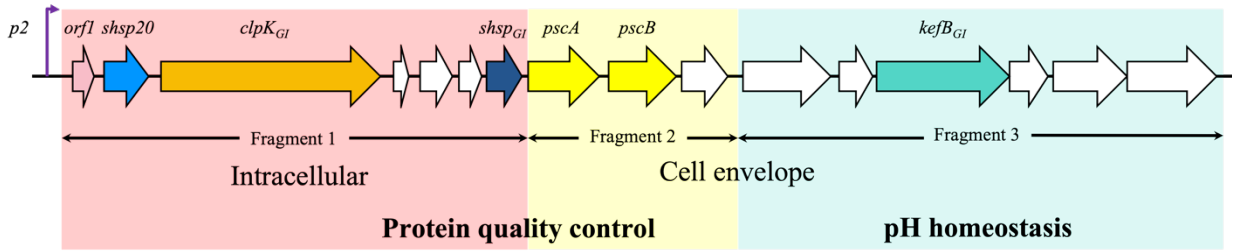

Figure 1.4. Schematic representation of the transmissible locus of stress tolerance 1 (tLST1). sHSP20, $ClpK_{GI}$ and $sHSP_{GI}$ are intracellular chaperones. PscA and PscB are chaperones localized to the periplasmic space. $KefB_{GI}$ is an inner membrane associated Na^+/H^+ antiporter which may contribute to alkaline adaptation.

1.7 Hypothesis

Maintaining the protein homeostasis and formation of persister cells are important strategies for bacterial stress tolerance. Persisters have been exclusively studied in antibiotic research as they

are transiently tolerant to killing by antibiotics. The accumulation of protein aggregates in persisters provides proof of concept that the protein aggregation caused by disruption of protein homeostasis may increase stress tolerance through generation of persisters. This concept remains to be evaluated for chemical and physical stressors other than antibiotics. Experimentation described in this thesis therefore aimed to test the hypothesis that bacterial protein homeostasis improves tolerance to alkaline pH but reduces desiccation tolerance. Specifically, the tLST was used as a tool to manipulate protein homeostasis in *E. coli*, *S.* Typhimurium and *C. sakazakii*. It was determined whether the tLST has opposite effects on bacterial survival at alkaline conditions or in the dry state.

Chapter 2. Contribution of the locus of heat resistance to growth and survival of *Escherichia coli* at alkaline pH and at alkaline pH in the presence of chlorine

2.1 Introduction

Escherichia coli are commensals in the intestine of humans and animals, but the species also includes pathogenic strains that cause infections of the gastrointestinal and urinary tracts (Croxen et al., 2013; Johnson & Russo, 2002). Food contamination by pathogenic *E. coli* may occur at any step of the farm-to-fork continuum and is considered a contributor to foodborne disease (Baker et al., 2016; S. C. Yang et al., 2017). In food processing plants, alkaline chlorinated cleaners and sanitizers are used to sanitize food contact surfaces (Sharma & Beuchat, 2004). The chlorine and alkaline treatments function through synergistic mechanisms. Hypochlorous acid (HOCl), the active component of chlorine, oxidizes cellular components and permeabilizes the cytoplasmic membrane (Dukan & Touati, 1996; Hurst et al., 1991; Venkobachar et al., 1977). Extreme alkaline pH also denatures proteins and results in membrane permeabilization (Mendonca et al., 1994). Mechanisms that allow strains of *E. coli* to resist chlorine at alkaline pH include the locus of heat resistance (LHR), a mobile genetic element that improves survival of *E. coli* after chlorine treatment under alkaline conditions (Z. Wang et al., 2020a; Zhi et al., 2016).

LHR is a 15-kb genomic island flanked by mobile genetic elements, which allow transmission among diverse species of *Enterobacteriaceae* (Boll et al., 2017; Mercer et al., 2015). Collectively, genes encoded by the LHR confer resistance to heat, chlorine and oxidative stress by reducing protein aggregation, protein oxidation, and by reducing the oxidation of membrane lipids (C. Lee et al., 2018; Li et al., 2020; Mercer, Nguyen, et al., 2017; Z. Wang et al., 2020a). Proteomic analysis demonstrated that 11 of the 16 putative open reading frames of the LHR are expressed; highly expressed proteins include sHsp20, $ClpK_{GI}$, $sHsp_{GI}$, $YfdX1_{GI}$ and YfdX2 (Li et al., 2020).

The small heat shock protein sHsps and disaggregase ClpK prevent the aggregation of misfolded proteins or disaggregate and refold the denatured proteins (Haslbeck, 2016; C. Lee et al., 2015a). YfdX with predicted signal peptide in the N-terminus is likely localized in the periplasmic space demonstrating chaperone-like activity (Saha et al., 2016). The LHR also encodes for $KefB_{GI,}$ a Na^+/H^+ antiporter that may function to maintain a polarized membrane at alkaline conditions (Mercer, Nguyen, et al., 2017) and thus contribute to alkaline resistance, i.e. growth at alkaline pH, or alkaline tolerance, i.e. survival after lethal challenge with alkaline (Padan et al., 2005; Zwietering et al., 1990a), however, a role of $KefB_{GI}$ in alkaline tolerance or alkaline resistance has not been verified experimentally.

Although close homologs of several highly expressed LHR-encoded proteins have been shown or implied to function on bacterial cell envelope, the contribution of LHR to this location remains to be investigated (Ferguson et al., 1995; Li et al., 2020; Saha et al., 2016). The cell envelope of Gram-negative bacteria consists of the cytoplasmic membrane, the periplasm, a thin layer of peptidoglycan, and an outer membrane. Homeostasis of the cell envelope in response to perturbations is provided by the envelope stress responses. Cpx, a two-component regulatory system consisting of CpxA, a membrane-associated sensor kinase and the response regulator CpxR is a widely conserved regulator of the envelope stress response in Gram-negative bacteria (Jianming et al., 1993; Weber & Silverman, 1988). The most commonly accepted model suggests that Cpx system detects periplasmic misfolded proteins and maintains the integrity of the cytoplasmic membrane (Raivio et al., 2013; Raivio & Silhavy, 1999). Therefore, the Cpx is a suitable reporter system to monitor putative protective effects of LHR-encoded proteins on the cell envelope. In addition, a functional overlap between LHR and Cpx response also suggests the possibility of crosstalk between these two mechanisms of stress resistance. This study therefore

aimed to determine the function of LHR on maintaining the bacterial cell envelope homeostasis, the regulation of the genes comprising LHR and whether the LHR responds to solely alkaline pH challenge in *E. coli*.

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids and growth conditions

All strains and plasmids used in this study are listed in Table 2.1. Unless otherwise stated, strains of *Escherichia coli* strains were grown on LB (Luria-Bertani, BD Biosciences, USA) plates or in LB broth with at 37°C with antibiotics added when necessary.

Table 2.1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Description	Reference
E. coli MG1655	*E. coli* K-12 derivatives	
E. coli MG1655 *lacZ*::LHR	Full-length LHR with its promoter inserted into MG1655 *lacZ*	This study
E. coli MG1655 Δ*cpxR*::Kan	*E. coli* MG1655 with chromosomal *cpxR* replaced by kanamycin resistance cassette	This study
E. coli MG1655 *lacZ*::LHR Δ*cpxR*::Kan	*E. coli* MG1655 *lacZ*::LHR with chromosomal *cpxR* replaced by kanamycin resistance cassette	This study
E. coli MG1655 *lacZ*::LHR Δ$kefB_{GI}$::*FRT*	*E. coli* MG1655 *lacZ*::LHR with LHR $kefB_{GI}$ replaced by FRT scar site	This study
E. coli MG1655 *lacZ*::LHR Δ$kefB_{GI}$::*FRT* Δ*cpxR*::Kan	*E. coli* MG1655 *lacZ*::LHR with LHR $kefB_{GI}$ replaced by FRT scar site and chromosomal *cpxR* replaced by kanamycin resistance cassette	This study
E. coli MG1655 *lacZ*::LHR Δ*evgA*::Kan	*E. coli* MG1655 with chromosomal *evgA* replaced by kanamycin resistance cassette	This study

(Continuous of Table 2.1)

pJW15	Promoterless luminescence reporter plasmid containing *luxCDABE* operon, ori_{p15A}; Kan^r	(MacRitchie et al., 2008)
pJW25	pJW15 plasmid containing *cpxP* promoter; Kan^r	(MacRitchie et al., 2008)
pLHR	Low copy plasmid containing the LHR	(Mercer et al., 2015)
pKDsg-lacZ	Plasmid containing crispr targeting sequencs for lacZ	This Study

2.2.2 Construction of derivatives of *E. coli* MG1655

The strain *E. coli* MG1655 used in this study is an *E. coli* K-12 derivative. The chromosomal integrated LHR positive strain MG1655 *lacZ*::LHR was constructed by the Scarless Cas9 Assisted Recombineering system (Reisch & Prather, 2015). pLHR was digested by the DraI enzyme (Thermofisher, USA) to linearize the pLHR plasmid which contains the LHR fragment flanked by parts of the *lacZ* gene (Mercer et al., 2015). The λ-Red was pre-induced in a MG1655 strain containing both pKDsg-lacZ and pCas9cr4 followed by an electroporation of the LHR fragments. After recovery, the culture was grown overnight with chloramphenicol (34μg/ml), spectinomycin (50μg/ml) and anhydrotetracycline (100ng/ml). The overnight culture was treated at 60°C for 5min and plated on LB agar containing IPTG (Isopropyl β-d-1-thiogalactopyranoside, 0.2 mM) and X-gal (40mg/mL). White colonies were screened with LHR-16-F/lacZ-upstream, LHR-2-R/lacZ-downstream and *yfdX1*-check-F/R primers in Table 2.2 to get the target mutant followed by plasmids lost.

MG1655 *lacZ*::LHR Δ$kefB_{GI}$ was constructed using the λ-Red system(Datsenko & Wanner, 2000).

The donor DNA was PCR-generated possessing chloramphenicol cassette flanked by sequences of up- and downstream of *kefB*$_{GI}$. pKD46 was transformed into MG1655 *lacZ*::LHR and λ-Red was induced to facilitate the genomic integration of donor DNA. The mutant was picked from plates containing chloramphenicol (25μg/ml) and checked with primers listed in Table 2.2. pCP20 was used to flipped out the chloramphenicol cassette (Cherepanov & Wackernagel, 1995).

Mutant constructions of Δ*cpxR* and Δ*evgA* were done by P1 transduction based on the previous description (Thomason et al., 2007). Briefly, the P1 *vir* phage was first replicate on the donor KEIO collection single-gene knockout strain and the result P1 lysate was used to infect the recipient strains (Baba et al., 2006). The potential mutants were selected on LB plates containing low concentration kanamycin (25μg/ml) followed by the purification with high concentration condition (50μg/ml). Mutations were confirmed by PCR using the primers listed in Table 2.2

Table 2.2. Primers used in this study.

Primer	Sequence (5'-3')	References
sgRNA-lacZ-F	GGCCAGTGAATCCGTAATCAGTTTTAGAGCTAGAAATAGCAAG	This study
sgRNA-lacZ-R	TGATTACGGATTCACTGGCCGTGCTCAGTATCTCTATCACTGA	
Targeting sequence	GGCCAGTGAATCCGTAATCA	
LHR-16-F	CGGTATCGCCGTCGACGACG	
lacZ-upstream	GCTGTTGCCCGTCTCACTGG	
LHR-2-R	GCCGGAATTTCCCCGTGTGC	
lacZ-downstream	GGACGACGACAGTATCGGCC	

(Continuous of Table 2.2)		
yfdX1-check-F	TCGGTAAAGAAAGCGGTCAAG	
yfdX1-check-R	CATCGGAAGGTTGTCGGTTT	
kefB-P2	CATCGTGCGCTGGACGTCGACGCAAGTGGG ACGCTGACCGATGGGAATTAGCCATGGTCC	
kefB-P1	TGGTCACGTAAGACCTGAAATGGGTTAAGG CGTGTTGATTGTGTAGGCTGGAGCTGCTTC	
kefB-check-F	TTGCTGGGGTATCTCTCTGT	
kefB-check-R	CAGCCACATCAATAGCAGGA	
cpxR F	CTATGCGCATCATTTGCTCC	
cpxR R	CATGCTGCTCAATCATCAGC	
k1	CAGTCATAGCCGAATAGCCT	(Datsenko & Wanner, 2000)
evgA F	GACGCCTTATGTCTGTATTAC	This study
evgA R	GTTGCTGCGAATCGGTATG	
Orf1-F	GGTGATTTTCACGCTCGATG	
Orf1-R	TCGGATGACTTCTGCTGTTC	
ORF8-F	TCGGTAAAGAAAGCGGTCAAG	(Ma & Chui, 2017)
ORF8-R	CATCGGAAGGTTGTCGGTTT	(Ma & Chui, 2017)

Orf13-F	TTGCTGGGGTATCTCTCTGT	This study
Orf13-R	CAGCCACATCAATAGCAGGA	
gapA-F	GTTGACCTGACCGTTCGTCT	(Fang et al., 2017)
gapA-R	ACGTCATCTTCGGTGTAGCC	

2.2.3 Phylogenetic tree of the CpxR response regulator

To construct a phylogenetic tree, the amino acid sequence of CpxR from *E. coli* MG1655 was used as the query to blast against the NCBI non-redundant reference proteins database. The coverage of 70% and identity of 60% were used as the cut-off values. One sequence of each species was picked from the family *Enterobacteriaceae* with the sequence from *Vibrio cholerae* as outgroup to generate the tree. The sequences were aligned by MUSCLE (www.ebi.ac.uk/Tools/msa/m), the tree was constructed using maximum likelihood by MEGAX (Stecher et al., 2020) (v10.1.8) and viewed by iTol (itol.embl.de).

2.2.4 Determination of the Cpx activity by bioluminescence assay

Cpx activity was determined by a luminescent reporter assay as described (Price & Raivio, 2009). Single colonies of MG1655 and MG1655 *lacZ*::LHR carrying either low copy number plasmids pJW15 or pJW25 were inoculated overnight in LB broth and then subcultured 1:100 in 5mL of LB with 25μg/ml kanamycin at 30°C with aeration at 200rpm. The Cpx activities were induced at different conditions. Exponentially growing cultures were harvested at an OD_{600nm} of 0.4; stationary phase cultures were harvested at OD_{600nm} of 1.0. To determine the influence of aeration of Cpx activity, half the volume of each subculture was cultivated at aerobic conditions, the other aliquot was incubated in a 2mL microcentrifuge tube which was sealed with parafilm to reduce the diffusion of oxygen. The subcultures were incubated together at 30°C 200rpm for 18h before

measurements. To determine the impact of alkaline pH, cells from 2mL culture were harvested and resuspended in 2mL LB with pH adjusted to 7.0, 8.0, 8.5 and 9.0 by adding NaOH (Sigma-Aldrich, Canada). The bacteria were induced for 1h before the readings were taken. For other experiments, exponentially growing bacteria were induced for 2h with the following inducing reagents (final concentration): 1mM $ZnSO_4$, 1mM $CuSO_4$ and 6mM 2-phenylethanol (Sigma-Aldrich, Canada); cultures without inducing agent served as reference. Luminescence and cell optical density at 600nm (OD_{600}) were measured by plate readers (luminescence: Victor X4, Perkin Elmer, USA; OD_{600}: Varioskan Flash, Thermo Scientific, USA) in the volume of 200µL with 96-well clear-bottom microtiter plates (Corning, USA). The normalized luminescence value was calculated by standardizing the luminescence intensity to the OD_{600}. The values obtained from the strains with pJW15 were used as blank, which were subtracted from the readings acquiring from the same strains containing pJW25. The fold change was calculated from dividing induced conditions by the uninduced condition of the same strain. Bioluminescence assays were performed in triplicate independent experiments.

2.2.5 Measurement of LHR gene expression by RT-qPCR

The bacteria overnight cultures were subcultured 1:100 in LB buffered to a pH of 7.1 and 8.2 by addition of Tris and phosphate (50mM each) (Sigma-Aldrich, Canada) and were grown to an OD_{600nm} of 0.5. The cells were harvested, and the RNA isolation was performed using RNAprotect bacteria reagent and the RNAeasy mini kit (Qiagen, Canada). The gDNA was removed by RQ1 RNase-Free DNase (Promega, USA) and the reverse transcription of the RNA to cDNA was done by QuantiTect® reverse transcription kit (Qiagen, Canada). QuantiTect SYBR® Green PCR Kits (Qiagen, Canada) and 7500 fast real-time PCR system (Applied Biosystems®, USA) were used to measure the LHR expression with the primers targeting *orf1*, $yfdX1_{GI}$ and $kefB_{GI}$ (Table 2.2). The

glyceraldehyde-3-phosphate dehydrogenase A gene (*gapA*) was used as the house-keeping gene and the DNase digested RNA served as the negative control. The $\log_2$-normalized relative gene expression level was calculated by comparing the values obtained from either MG1655 *lacZ*::LHR Δ*cpxR* or MG1655 *lacZ*::LHR Δ*evgA* to MG1655 *lacZ*::LHR grown under the same pH. Data shown are means of three independent experiments.

2.2.6 Determination of the growth rates

Tris-phosphate (50mM each) buffered LB was prepared at two different pH levels each at two different osmolarity levels and filter sterilized. The low salt media was made by adding 10g tryptone, 5g yeast extract, 4.1g NaCl, 6.1g trizma base and 7.1g Na_2HPO_4 (Sigma-Aldrich, Canada) to 1L distilled water with a final pH of 6.5 or 10.0. The normal salt media was made as described above except including 10g NaCl. To obtain the working media, the two pH LB of each salt concentration were mixed at different ratios in clear-bottom 96-well plates (Corning) to achieve a pH gradient. The resulting pH in media containing the high pH and low pH media in different ratios was measured with a glass electrode (Thermofisher, USA). The bacteria were grown for 24h in 5mL LB broth and subcultured 1:1000 in a final volume of 155µL. OD_{600nm} was measured every 30min for 16h by plate reader while incubating at 37°C with a rotation diameter of 6mm/s. The growth rates were calculated by fitting experimental data to the logistic growth curve (Zwietering et al., 1990b) in SigmaPlot (v12.5, Systat Software Inc., USA) with the data representing three biological replicates.

2.2.7 Determination of the tolerances to extreme alkaline pH with or without chlorine treatment

To determine the bacterial tolerance to extreme alkaline pH, filter-sterilized carbonate-bicarbonate (50mM) buffered LB was prepared by adding 0.525g sodium bicarbonate and 4.637g sodium

carbonate (anhydrous) into 1L LB broth with the final pH of 11.2. Cells were pelleted from 500μL of the overnight cultures and resuspended in 500μL of carbonate-bicarbonate buffered LB for 5min. Sterile water (4.5mL) was added to the mixture and the after-treatment pH was measured. Alkaline pH tolerance with addition of chlorine was determined by resuspending the bacterial pellet the same volume of filter-sterilized pH 11 LB including 10mM NaClO (5% *w/w*, Sigma-Aldrich). After 5min, cells were harvested by centrifugation and resuspended in fresh LB. The viable cell count was determined by serial dilution and surface plating using LB broth and agar plate for both treated and untreated samples. The values were then log-transformed to obtain the reduction of cell count [$\log(N_0/N)$]. Data represent three independent experiments performed with technical repeats.

2.2.8 Statistical analysis

Statistical analysis of data was performed using RStudio (v1.2.1335, R Core Team, Austria). Differences among Cpx activity and gene expression were determined with a one-sample two-tailed student's *t*-test ($P<0.05$); Differences among cell count reductions were determined by one-way ANOVA followed by Tukey's HSD ($P<0.05$).

2.3 Results

2.3.1 Co-existence of CpxR response regulator and LHR

In *Enterobacterales*, to date, LHR has only been found in the family *Enterobacteriaceae* and the genus *Yersinia* (Mercer et al., 2015). To provide an initial assessment of cross-talk between CpxR and LHR, we determined whether genomes of those species that encode for the LHR also encode for CpxR. The CpxR response regulator is present in all species possessing the LHR except *Citrobacter braakii*, indicating that crosstalk between the Cpx pathway and LHR is feasible (Fig.

2.1).

2.3.2 The presence of LHR does not alter Cpx pathway activity

To determine whether LHR plays a role in maintaining protein homeostasis in the bacterial periplasms or inner membrane integrity, we assessed the Cpx activity in *E. coli* MG1655 and MG1655 *lacZ*::LHR under various stress conditions. The Cpx response is activated by the stationary phase, aerobic conditions, elevated pH and the presence of zinc, copper and ethanol (Clarke & Voigt, 2011; Danese & Silhavy, 1998; De Wulf et al., 1999; L. J. Lee et al., 2005; Nakayama & Watanabe, 1995; Raivio et al., 2013; Yamamoto & Ishihama, 2006). As a member of the Cpx regulon, *cpxP* is one of the most upregulated genes when Cpx is activated (Danese & Silhavy, 1998). Therefore, pJW15, the promoterless vector control carrying *lux* operon, and its derivative pJW25 with *cpxP'-lux* reporter were used to monitor Cpx activity. The Cpx pathway was highly upregulated after induction relative to the reference conditions in both *E. coli* MG1655 and *E. coli* MG1655 *lacZ*::LHR strains (Fig. 2.2); however, this induction was not altered (P>0.05) by the presence of the LHR (Fig. 2.2).

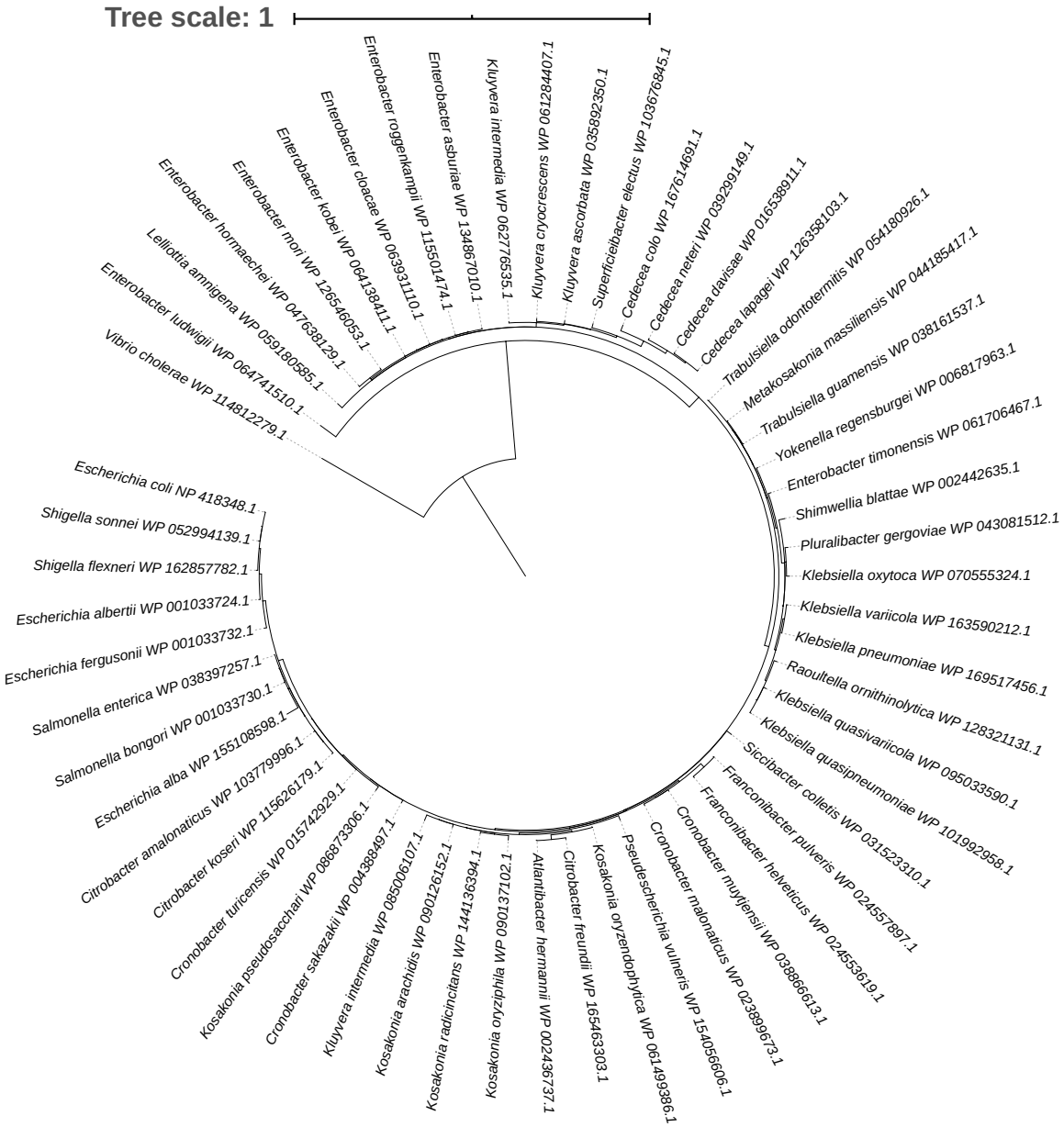

Figure 2.1. A phylogenetic tree showing the distribution of CpxR response regulator in family *Enterobacteriaceae* with *Vibrio cholerae* as the outgroup.

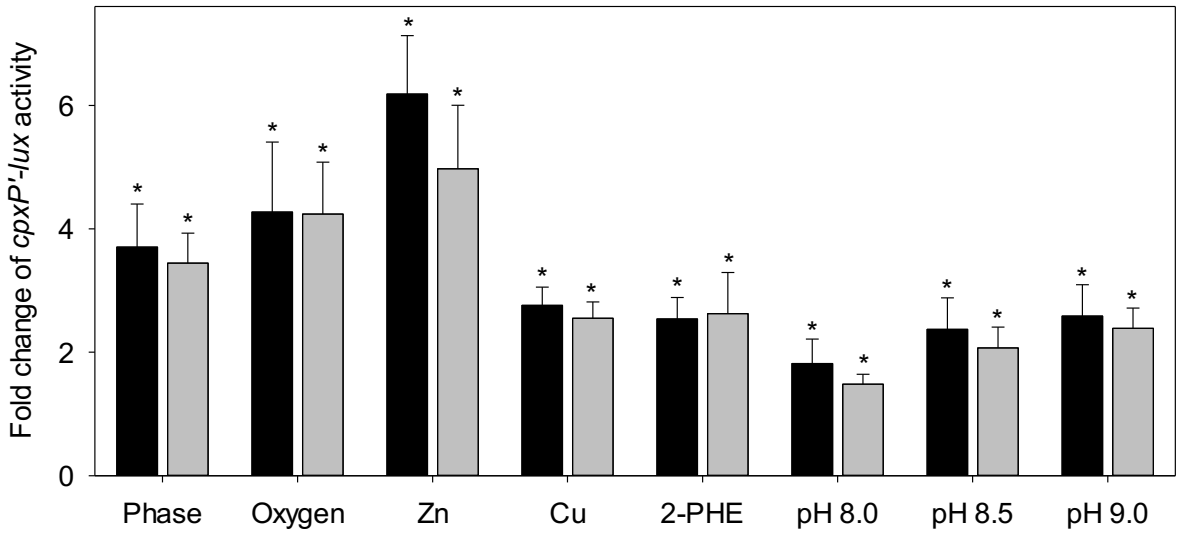

Figure 2.2. The Cpx responses in *E. coli* MG1655 (black bars) and MG1655 *lacZ*::LHR (grey bars) under different treatments. The fold change of *cpxP'*-lux activity was calculated relative to the reference conditions as follows: stationary phase vs. log phase (reference); aerobic vs. anaerobic incubation (reference); 1mM $ZnSO_4$ vs. no addition (reference); 1mM $CuSO_4$ vs. no addition (reference); 6mM phenylethanol vs. no addition (reference), pH 8.0, 8.5 and 9.0 vs. 7.0 (reference for all three pH values). An asterisk indicates a significant difference of experimental conditions to the respective reference conditions (P<0.05). Data are mean ± standard deviation of three independent experiments.

2.3.3 LHR transcriptional level is affected by CpxR but not EvgA response regulator under alkaline pH

To determine whether the expression of LHR is directly or indirectly dependent on EvgA or CpxR response regulators, the mRNA of three fragments of the LHR, *orf1*, $yfdX1_{GI}$ and $kefB_{GI}$(Mercer, Nguyen, et al., 2017), were quantified by RT-qPCR in *E. coli* MG1655 *lacZ*::LHR, MG1655 *lacZ*::LHR Δ*cpxR* and MG1655 *lacZ*::LHR Δ*evgA*. At pH 7.1, no difference can be detected on LHR expression without either of the response regulators. At a pH of 8.0, lack of functional EvgA did not affect LHR transcription; however, deletion of CpxR upregulated *orf1*, $yfdX1_{GI}$ and $kefB_{GI}$

(Fig. 2.3). Results indicate that alkaline condition activates LHR expression in the absence of CpxR response regulator.

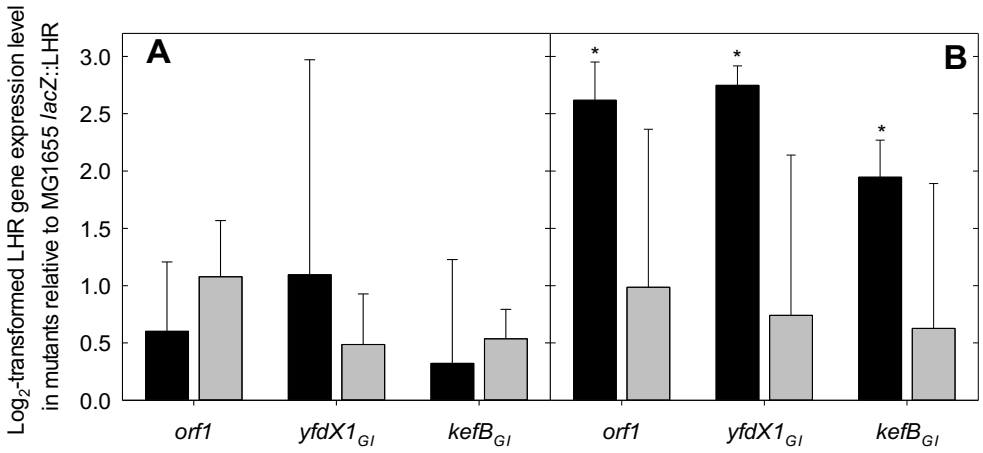

Figure 2.3. The expression level of LHR in *E. coli* MG1655 *lacZ*::LHR with either *cpxR* (black bars) or *evgA* knocked out (grey bars) relative to the expression in MG1655 *lacZ*::LHR. (**Panel A**) pH=7.1. (**Panel B**) pH=8.0. The LHR expression was measured at the level of three putative fragments represented by *orf1*, $yfdX1_{GI}$ and $kefB_{GI}$ with RT-qPCR by using MG1655 *lacZ*::LHR as reference. Asterisks indicate means that were significantly different from the respective reference conditions ($P<0.05$). Data are mean ± standard deviation of three independent experiments.

2.3.4 Cpx but not the LHR is necessary for growth under alkaline pH

Cpx response has been implicated in elevated pH adaptation and Δ*cpxR* mutation in *E. coli* resulted in impaired growth under alkaline condition (Danese & Silhavy, 1998). KefB is a glutathione-regulated potassium/proton antiporter protecting bacteria from electrophile toxicity (Ferguson et al., 1993). Therefore, to determine the role of LHT in bacterial alkaline pH resistance and the contribution of $kefB_{GI}$, we assessed the growth rates of *E. coli* MG1655, MG1655 *lacZ*::LHR, MG1655 Δ*cpxR*, MG1655 *lacZ*::LHR Δ*cpxR* and MG1655 *lacZ*::LHR Δ$kefB_{GI}$ in buffered LB at pH 6.9 to 9.2. The growth rates of *E. coli* MG1655, MG1655 *lacZ*::LHR and MG1655 *lacZ*::LHR Δ$kefB_{GI}$ at neutral or alkaline were not different (Fig. 2.4). The growth rates of Δ*cpxR* LHR-

negative and -positive strains were comparable to the strains with a functional CpxR at pH values below 8.0, however, deletion of *cpxR* reduced growth at alkaline pH and reduced the maximum pH-value of growth from 9.2 to 8.2 irrespective of the presence of the LHR (Fig. 2.4). Comparable results were obtained in both low and normal salt media (Fig. 2.4). Therefore, the functional Cpx pathway but not the presence of LHR is required for bacterial growth at mild alkaline pH.

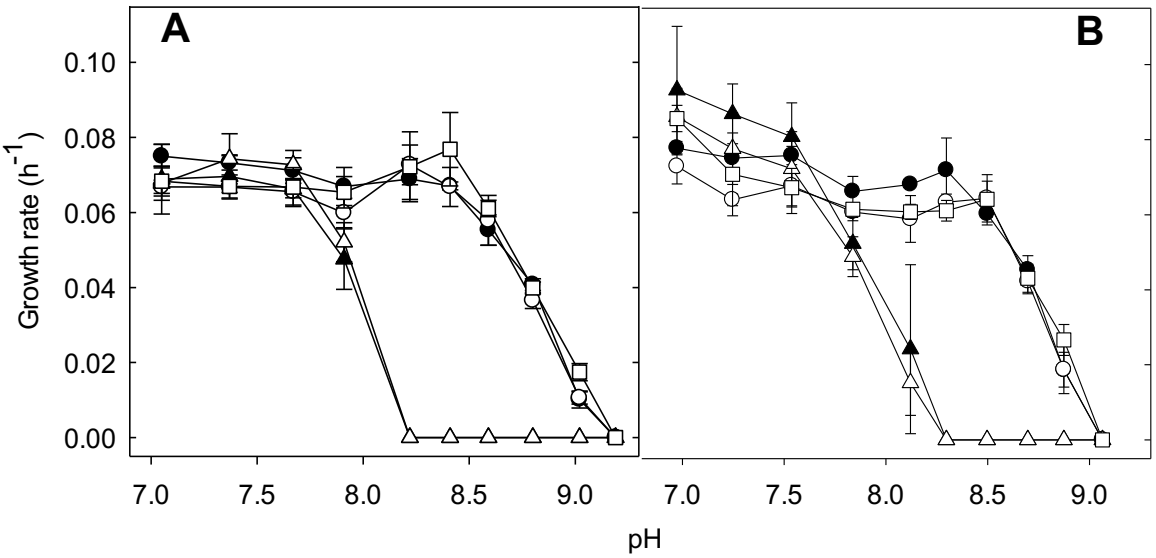

Figure 2.4. The growth rates of *E. coli* MG1655 and MG1655 *lacZ*::LHR and gene knockout derivative strains at pH-values ranging from 6.9 to 9.2. (**Panel A**) Low salt: the strains were incubated in Tris-phosphate (50mM+50mM) buffered low salt LB, with NaCl reduced in the corresponding amount. (**Panel B**) Normal salt: the strains were incubated in Tris-phosphate (50mM+50mM) buffered normal salt LB. All the strains were subcultured at 1:1000 from overnight cultures and incubated at 37 °C for 16 hours. (●) MG1655, (○) MG1655 *lacZ*::LHR, (▲) MG1655 Δ*cpxR*, (Δ) MG1655 *lacZ*::LHR Δ*cpxR*, (□) MG1655 *lacZ*::LHR Δ$kefB_{GI}$. Data are mean ± standard deviation of three independent experiments.

2.3.5 LHR improves bacterial survival to extreme alkaline pH in the presence of chlorine

To further explore the role of CpxR and the LHR at alkaline conditions, survival of *E. coli* MG1655, MG1655 *lacZ*::LHR, MG1655 Δ*cpxR*, MG1655 *lacZ*::LHR Δ*cpxR*, MG1655 *lacZ*::LHR Δ$kefB_{GI}$

and MG1655 *lacZ*::LHR Δ*cpxR* Δ$kefB_{GI}$ under extreme alkaline condition in the absence or presence of chlorine was determined. All strains survived challenge at pH 11.0 for 5 min with a reduction of viable cell counts of less than 1 log (cfu/mL); challenge at pH 11.3 reduced cell counts of all strains to levels below the detection limit (Fig. 2.5). When treated with carbonate-bicarbonate buffered LB at pH 11.2, the viable cell counts of all strains was reduced by about 1-2 log (cfu/mL) (Fig. 2.6A). *E. coli* MG1655 and its *cpxR* null derivative demonstrated a similarly high level of sensitivity to pH 11.0 with addition of chlorine (Fig. 2.6). The wildtype strain harbouring LHR was more tolerant (P<0.05) to alkaline conditions in the presence of chlorine; however, deletion of *cpxR* or $kefB_{GI}$ or both diminished tolerance (Fig. 2.6). In summary, LHR improves bacterial survival under extreme alkaline pH only in the presence of chlorine, which is also *cpxR* and $kefB_{GI}$ dependent.

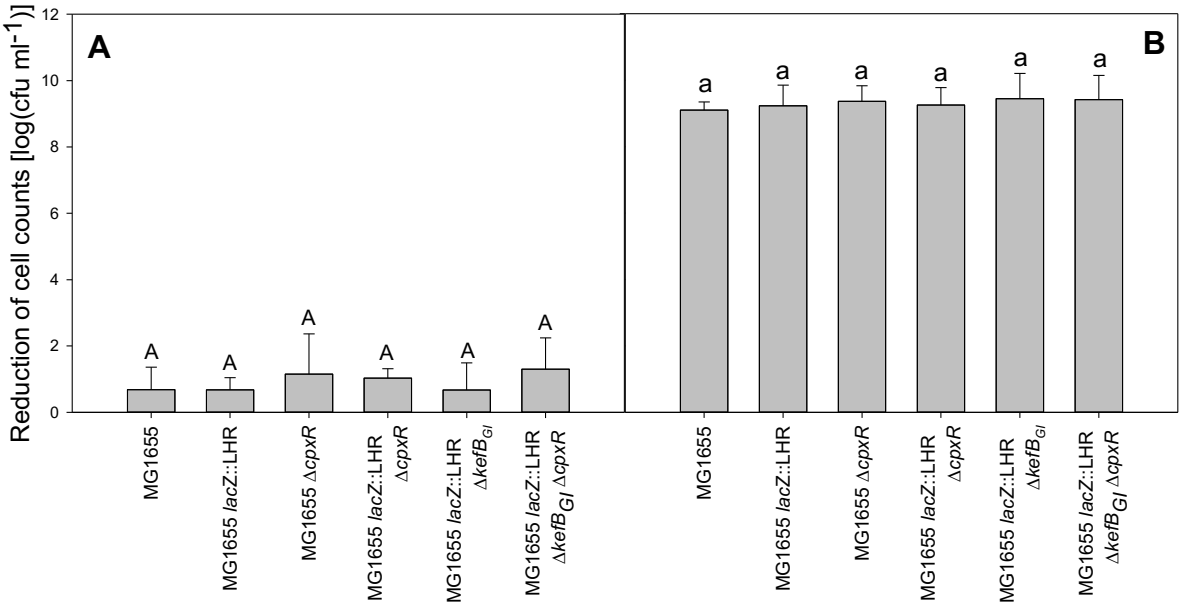

Figure 2.5. Reduction of cell counts of *E. coli* MG1655 and MG1655 *lacZ*::LHR and gene knockout derivative strains under different pH. **(A)** The overnight cultures of all the tested strains were treated with 50mM Carbonate-bicarbonate buffered LB under pH 11.0 condition for 5min. **(B)** The overnight cultures of all the tested strains were treated under pH 11.3 condition for 5min. Bars with different superscripts are statistically different in each panel. Data are mean ± standard deviation of three independent experiments.

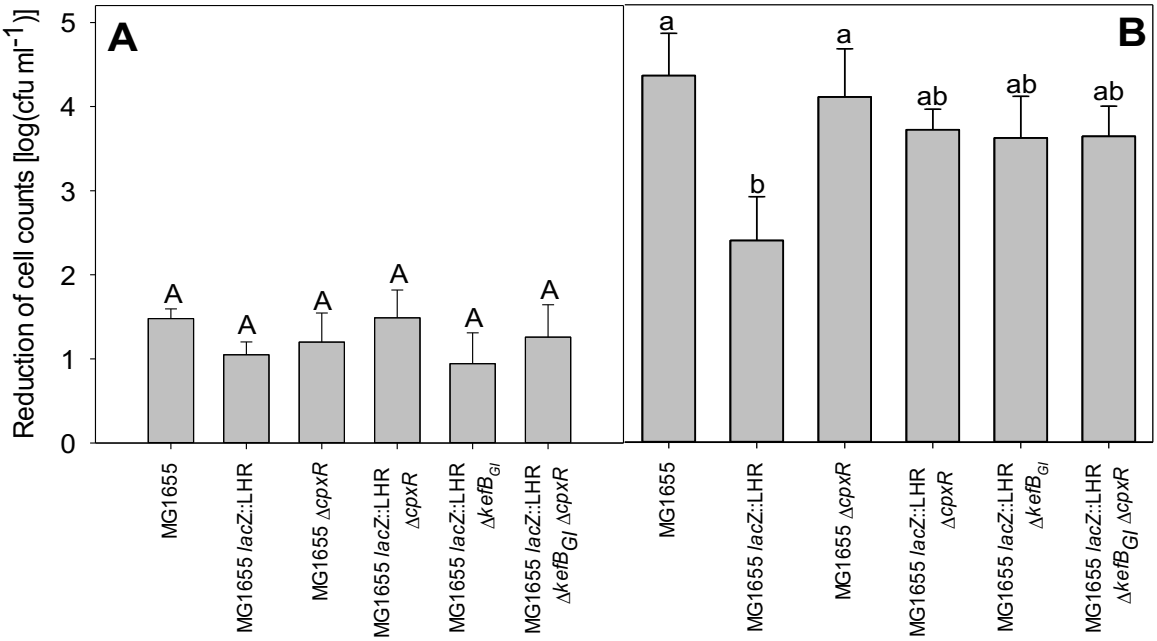

Figure 2.6. Reduction of cell counts of *E. coli* MG1655 and MG1655 *lacZ*::LHR and gene knockout derivative strains under different killing conditions. **(A)** Extreme alkaline pH killing: the overnight cultures of all the tested strains were treated with 50mM Carbonate-bicarbonate buffered LB under pH 11.2 condition for 5min. **(B)** Extreme alkaline pH plus chlorine killing: the overnight cultures of all the tested strains were treated under pH 11 with 10mM NaClO condition for 5min. Bars with different superscripts are statistically different in each panel. Data are mean ± standard deviation of three independent experiments.

2.4 Discussion

2.4.1 The functional overlap between LHR and Cpx response

We hypothesized in this study that proteins encoded by the LHR contribute to cell envelope homeostasis, therefore, promoting bacterial survival under various stress conditions. EvgAS is a two-component regulatory system has been shown related to osmotic adaptation, acid and antibiotic resistance by regulating the genes encode chaperone-like proteins and drug transporters (Masuda & Church, 2002; Nishino & Yamaguchi, 2001). The expression of the *yfdX* gene on *E. coli* chromosome is significantly induced by the overexpression of EvgA response regulator (Nishino & Yamaguchi, 2001). YfdX demonstrates chaperone-like activity preventing protein aggregation and exhibits a pH-dependent stoichiometric conversion between dimeric and tetrameric states at pH 10.0 and 7.5 respectively, which suggests its possible function involved in pH adaptation (H. S. Lee et al., 2018; Saha et al., 2016). YfdX possesses a predicted signal peptide in the N-terminus indicating its localization in the periplasm (Saha et al., 2016). A deletion of *yfdX* could be complemented by overexpressing of the periplasmic chaperone HdeA, supporting the above prediction (Id et al., 2019). $YfdX1_{GI}$ in LHR also includes a predicted Sec-dependent protein secretion signal peptide at its N-terminus (SignalP, v5.0, Denmark (Almagro Armenteros et al., 2019)). LHR confers significant chlorine resistance (Z. Wang et al., 2020a). Chlorine can non-selectively oxidize cellular components including proteins and nucleotides, and also destruct the cytoplasmic membrane permeability (Dukan & Touati, 1996; Hurst et al., 1991; Venkobachar et al., 1977). Protein denaturation and aggregation in both cytoplasm and periplasm or the membrane leakage induced by chlorine treatments can eventually lead to cell death. However, our results suggest LHR does not function to mitigate the impact of those stressors that activate the Cpx response, however, expression of LHR in *E. coli* is under the control of CpxR. The LHR reduces

the oxidation of membrane lipids (Z. Wang et al., 2020a); the present study thus suggests that oxidation of membrane lipids is not an inducing signal for the Cpx pathway.

Bioinformatic analyses demonstrated CpxR response regulator is present in *Enterobacterales,* including all of those species that include strains which possess the LHR except *Citrobacter braakii*. Deletion of the CpxR response regulator did not alter LHR expression at neutral pH but increased its transcription level at pH 8.0. The Cpx pathway responds to elevated extracellular pH. Although growth of the *cpxR* null mutant strain was comparable to the wildtype strain at neutral pH, it's growth was impaired at alkaline conditions and failed to grow at pH 9.0 to 9.2 (Danese & Silhavy, 1998; Price & Raivio, 2009). These results suggested that LHR may only confer the cells with tolerance to lethal alkaline conditions and that Cpx response may act as a "switch" which can shift the bacterial adaptation from the mild to lethal stresses: Under moderate stress conditions, the activation of the Cpx pathway mitigates periplasmatic stress and maintain the LHR expression at a low level to reduce fitness cost. At extreme stress conditions, LHR expression is de-repressed or activated directly or indirectly to improve the bacterial survival.

2.4.2 The function of LHR on bacterial alkaline pH adaptation

The adaptation to mild alkaline pH in mesophilic bacteria includes increasing acid production and ATP generation in the central carbon metabolism, enhanced expression of transporters, and modification of membrane properties (Dilworth & Glenn, 1999; Enomoto & Koyama, 1999; Hicks et al., 2010; Krulwich et al., 1998; Maurer et al., 2005; Mayer et al., 2010; Padan et al., 2005; Y. Yang et al., 2014). Among these, proton pumps and cation/proton antiporters are main contributors to the ability of bacteria to maintain their internal pH. This supports growth at an external pH of 5.5–9.0 while maintaining the cytoplasmic pH in the narrow range of 7.5–7.7 (Hunte et al., 2005; Krulwich et al., 2009, 2011; Nakamura et al., 1984; Padan et al., 2005; Padan & Schuldiner, 1994;

Saito & Kobayashi, 2003; Slonczewski et al., 2009). To date, very few reports demonstrate how mesophilic bacteria survive under extreme alkaline pH. The LHR did not enhanced the resistance or tolerance of *E. coli* to alkaline pH; however, the presence of both the Cpx pathway and the LHR enhanced the tolerance of *E. coli* to lethal challenge with alkaline. The Cpx system in conjunction with σ^E and σ^{32} responses can sense and regulate gene expression in response to oxidative stress and high pH (Danese & Silhavy, 1998; Dartigalongue & Raina, 1998; Raivio & Silhavy, 1999). In *Salmonella enterica* Typhimurium, CpxR is required to cope with bacterial oxidative damage (López et al., 2018). That suggests us the function of LHR to alkaline tolerance may require oxidative stress as the inducing signal. The *E. coli* genomic KefB is a potassium/proton antiporter repressed by glutathione (GSH) and the glutathione-deficient mutants exhibit reduced cytoplasmic pH at 7.35 comparing to its parent strain at 7.85 (Elmore et al., 1990; Ferguson & Booth, 1998). The Kef channels are regulated by their C-terminal NAD-binding domains containing a single binding site for GSH (Roosild et al., 2010). The GSH can stabilize an interdomain association between two NAD-binding folds therefore inhibit Kef activity (Roosild et al., 2010). GSH, a predominant low-molecular-weight thiol in most gram-negative bacteria, has been proposed to protect cells from oxidative damage. In *vitro*, 1 mol GSH can react with 3.5 to 4.0 mol HOCl (Harwood et al., 2006). In *E. coli*, the glutathione-deficient strain is twice as sensitive to killing by chlorine comparing to its isogenic glutathione-sufficient strain (Chesney et al., 1996). The lethal level of oxidative stress can dramatically increase the amount of intracellular glutathione disulfide (GSSG) and decrease the ratio of GSH/GSSG (Smirnova et al., 2000). Therefore, the addition of chlorine can consume the GSH in the bacterial cell then activate $kefB_{GI}$ leading to cytoplasmic acidification which protects bacteria from lethal effect during electrophilic attack (Ferguson et al., 1995). Hence, the functional $kefB_{GI}$ plays an essential and specific role in protecting the bacteria

from the extreme alkaline condition with the presence of chlorine. Taken together, our results are consistent with the proposed model demonstrating the role of the Cpx pathway and LHR on sensing and protecting the bacteria from the lethal alkaline pH with the presence of oxidative stress. Notably, we also observed that bacterial survival and death under extreme alkaline conditions occurred within a very narrow pH, which is opposite to its survival under extreme acidic pH. Bacterial survival at low pH is well understood as microbes encounter acid environments in many situations including low pH foods and during gastrointestinal transit. *E. coli* withstands exposure to the extreme acidic condition—less than 1 log cell reduction at pH 2.5 for at least 2 h—as it possesses several amino acid-dependent extreme acid resistance mechanisms (Bhat & Alex, 1998; De Biase & Lund, 2015; Lund et al., 2014; Richard & Foster, 2004). The acid stress response maintains the bacterial intracellular pH through the amino acid-dependent decarboxylase/ antiporter activities and protects the proteins from acid damage by inducing the periplasmic chaperone expression (Castanie-Cornet et al., 1999; Iyer et al., 2003; Kern et al., 2007; Lund et al., 2014; Meng & Bennett, 1992). However, how bacteria tolerant the extreme alkaline pH is poorly documented in the literature. Therefore, our discovery on defining the bacteria alkaline pH killing range: *E. coli* MG1655 can withstand the exposure to alkaline pH until 11.2 with significant survival whereas exhibiting dramatic killing at pH 11.3 (Fig. 2.5). The mechanism behind this phenomenon remains to be investigated.

2.5 Conclusion

In conclusion, the known species possessing LHR also have functional Cpx response suggesting the possible crosstalk between the Cpx pathway and LHR. LHR does not functionally relate with solely alkaline pH tolerance but needs the presence of chlorine, which also depends on Cpx response. In contrast with the Cpx two-component regulatory system which contributes to bacterial

stress adaptation under non-lethal conditions, LHR functions on protecting the bacteria from lethal challenges. CpxR response regulator negatively regulates LHR transcription at normal growth conditions to reduce the fitness cost; in addition, Cpx response may act as a ‘switch’ sensing the lethal stress and turning on the LHR expression to improve the bacterial survival.

Chapter 3. Protein aggregation as a bacterial survival strategy in *Salmonella* Typhimurium and *Cronobacter sakazakii* under desiccation

3.1 Introduction

Salmonella and *Cronobacter sakazakii* are well-known for their desiccation tolerant lifestyles. As facultative intracellular pathogens, *Salmonella* have adapted to the gastrointestinal tract of mammalian, avian, and reptilian hosts but fecal-oral transmission requires their ability to persist in dry environments when transitioning between host individuals or host species (Malik-Kale et al., 2011). *C. sakazakii* naturally associates with plants which also requires its rapid adaptation to dry environment during transmission (Schmid et al., 2009). In contrast, strains of *Escherichia coli* are more phenotypically diverse and may live in different environments including vertebrate hosts, plants, and aquatic environments (Gordienko et al., 2013).

Water activity (a_W) is a major factor in preventing or limiting microbiol growth, while *Salmonella* and *C. sakazakii* may remain viable for lengthy periods of time due to their high level of desiccation tolerance in low-a_W foods (a_W<0.85) (Beuchat et al., 2013). Over the past several decades, processed ready-to-eat low-a_W foods including powdered infant formula (PIF), breakfast cereal, chocolate, tree nuts and tree nut-containing products, dry seasonings, paprika-powdered potato chips, dried mixed fruit and peanut butter have been associated with outbreaks of salmonellosis worldwide (Cahill et al., 2008; Centers for Disease Control and Prevention, 1998, 2004, 2009; Jernberg C et al., 2015; Johansen et al., 2021; Lehmacher et al., 1995; Schmitt et al., 2018; Werber et al., 2005). *C. sakazakii* is notorious for its ability to cause life-threatening health complications in neonates and infants after consumption of contaminated PIF (Henry & Fouladkhah, 2019). After infection, death can occur within hours of the onset of symptoms with mortality rates ranging from 31% to 80% (Drudy et al., 2006). Therefore, understanding the

mechanisms of desiccation tolerance in bacteria will provide information for the development of control strategies to reduce contamination of foods with dry-tolerant pathogens.

Bacteria have evolved a variety of mechanisms to mitigate the detrimental effects of drying including the accumulating compatible solutes, the use of alternative sigma factors to upregulate genes involved in combatting oxidative stress, regulation of lipid and DNA metabolic processes, energy conservation, lipopolysaccharide modifications, and capsule and biofilm formation (Burgess et al., 2016; Garmiri et al., 2008; Gibson et al., 2006; Kempf & Bremer, 1998; Kragh & Truelstrup Hansen, 2020; Mandal & Kwon, 2017; Ophir & Gutnick, 1994; Pratt et al., 2016; Schnider-Keel et al., 2001; Truelstrup Hansen & Vogel, 2011). Surprisingly, protein aggregation also contributes to desiccation tolerance in *Acinetobacter baumannii* (X. Wang et al., 2020). This discovery is unanticipated since the formation of cellular protein aggregates are considered detrimental as it often indicates that the proteome is under stress. The capacity of the cell to synthesize, fold, transport and degrade proteins depends on its protein homeostasis network (David et al., 2016). In all domains of life, the primary driving force of protein folding is non-covalent interactions involving both the polypeptide back-bone and the amino acid side chains that allow the proteins to rapidly reach their native three-dimensional conformation (Dill et al., 2008). However, transient folding-intermediates are generated during the folding process that are vulnerable to perturbations of the proteostasis state triggered by environmental and cellular stresses and thus tend to unfold or misfold to form protein aggregates (Schramm et al., 2014). The role of protein aggregates in desiccation tolerance of *C. sakazakii* and *Salmonella* and the corresponding mechanisms is unknown.

The transmissible locus of stress tolerance (tLST), formerly known as the locus of heat resistance (LHR), is a plasmid-encoded or chromosomal genomic island widely transmitted across diverse

species of beta- and gamma-Proteobacteria (Kamal et al., 2021; Mercer et al., 2015; Mercer, Nguyen, et al., 2017). The tLST highly expresses the protein homeostasis module which contains the small heat shock proteins sHsp20 and $sHsp_{GI}$, and the disaggregase $ClpK_{GI}$, all of which prevent protein aggregation or disaggregate and refold denatured proteins (Li et al., 2020). The tLST can be expensive for strains to maintain (Li et al., 2020; Z. Wang et al., 2021). Its contribution to protein homeostasis increases bacterial tolerance to heat, high pressure and oxidative stress (Li et al., 2020; Mercer et al., 2015; Z. Wang et al., 2020b). However, the effect of the tLST on bacterial desiccation tolerance by reducing protein aggregates or other unknown pathways remains elusive. In this study, we take advantage of the tLST, specifically the components comprising its protein homeostasis module, to create different levels of protein aggregates within the cell to determine the impact of bacterial protein aggregation on desiccation tolerance of *S. enterica* serovar Typhimurium and *C. sakazakii* in comparison to *E. coli*. As the small heat-shock protein IbpA co-aggregates with the aggregated protein preventing its irreversible denaturation, the *ibpA-yfp* fusion construction was used in this study to visualize the protein aggregates in the bacterial population (Lindner et al., 2008).

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

All strains and plasmids used in this study are shown in Tables 3.1, respectively. Bacterial strains were grown on Tryptic Soy Agar (TSA, BD Biosciences, USA) or in Tryptic Soy Broth (TSB, BD Biosciences) at 37°C with antibiotics added when necessary. Unless otherwise stated, antibiotics (Sigma-Aldrich, Canada) were used at the following concentrations: ampicillin, 100µg/ml;

chloramphenicol, 34μg/ml; kanamycin, 50μg/ml; tetracycline, 25μg/ml.

Table 3.1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Description	Reference
E. coli TOP10	Cloning strain	Invitrogen
E. coli MG1655	*Escherichia coli* K-12 derivative	
S. Typhimurium ATCC 13311	*Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 13311	ATCC
C. sakazakii FUA 10122	*Cronobacter sakazakii* FUA 10122 isolated from Daqu	(Z. Wang et al., 2018)
E. coli MG1655 *ibpA-yfp*	*E. coli* MG1655 derivative expressing a IbpA-YFP fusion protein	(Govers & Aertsen, 2015)
S. Typhimurium ATCC 13311 *ibpA-yfp*	*S.* Typhimurium ATCC 13311 derivative expressing a IbpA-YFP fusion protein	This study
C. sakazakii FUA 10122 *ibpA-yfp*	*C. sakazakii* FUA 10122 derivative expressing a IbpA-YFP fusion protein	This study
pCA24N	High copy number plasmid; Cm^R	(Kitagawa et al., 2005)
pUC19	High copy number plasmid; Amp^R	Sigma
pRK767	Low copy number plasmid; Tet^R	(Gill & Warren, 1988)
peGFP	Source of egfp gene	Clontech
pRK*p2*	tLST *p2* promoter cloned into pRK767; Tet^R	This study
pLHR	tLST *p2* promoter and the full length of tLST cloned into pRK767; Tet^R	(Mercer et al., 2015)
pRF1	tLST *p2* promoter and protein homeostasis module cloned into pRK767; Tet^R	(Mercer et al., 2015)

(Continuous of Table 3.1)		
pRK*p2*::*orf1*	LHR promoter and *orf1* cloned into pRK767; Tet^R	This study
pRK*p2*::*shsp*$_{20}$	LHR promoter and *shsp*$_{20}$ cloned into pRK767; Tet^R	This study
pRK*p2*::*clpK*$_{GI}$	LHR promoter and *clpK*$_{GI}$ cloned into pRK767; Tet^R	This study
pRK*p2*::*shsp*$_{20}$-*clpK*$_{GI}$	tLST *p2* promoter, *shsp*$_{20}$ and *clpK*$_{GI}$ cloned into pRK767; Tet^R	This study
pRK*p2*::*clpK*$_{GI}$-E383A/E723A	tLST *p2* promoter and the double-point mutant of *clpK*$_{GI}$ cloned into pRK767; Tet^R	This study
pRK*p2*::*shsp20*-*clpK*$_{GI}$-E383A/E723A	tLST *p2* promoter, *shsp20* and the double-point mutant of *clpK*$_{GI}$ cloned into pRK767; Tet^R	This study
pRK*p2*::*shsp*$_{GI}$	tLST *p2* promoter and *shsp*$_{GI}$ cloned into pRK767; Tet^R	This study
pRK*p2*::*egfp*	tLST *p2* promoter and *egfp* cloned into pRK767; Tet^R	This study
pUC*kan*	pUC19 carrying kanamycin resistance cassette; Amp^R, Kan^R	Invitrogen
pUC*p2*::*egfp*	tLST *p2* promoter, *orf1* and *egfp* cloned in pUC19; Amp^R	(Mercer et al., 2017)
pUC*p2*ΔD::*egfp*	*pUCp2::egfp* with a 5-bp deletion at positions -30 to -35 upstream from *orf1* start codon; Amp^R	(Mercer et al., 2017)

3.2.2 Determination of the frequency of tLST in *Cronobacter sakazakii*

Genome metadata was downloaded from NCBI using NCBImeta (Eaton, 2020) to identify the isolation sources of publicly available *C. sakazakii* genomes. Genomes from strains originating

from humans, powdered infant milk formula, powdered infant milk formula facilities, dried food and environmental sources were downloaded and made into blast databases. A blastn v.2.9.0 (Camacho et al., 2009) search was performed on each database using the nucleotide sequence of *shsp20* and *clpK* from the tLST found in *E. coli* AW1.7 (LDYJ01000141). The tLST positive and negative genomes were counted and a chi-squared test was performed, comparing each group to the environmental group and comparing the PIF group to the PIF facilities ($P<0.05$). The PIF facilities database included the sequences from PIF so the latter was subtracted from the former for the final table.

3.2.3 Construction of *ibpA-yfp* fusion strains

The FRT-flanked chloramphenicol resistance (*cat*) gene which followed the *ibpA*-yfp locus in *Escherichia coli* MGAY (Lindner et al., 2008) was excised by transiently equipping this strain with a Flp site-specific recombinase expressing plasmid, pFLP2 (Hoang et al., 1998). The 700-1000bp regions up- and downstream of *ibpA* of *S.* Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 were amplified and assembled with the PCR products containing the *ibpA*-*yfp*-FRT-*cat*-FRT sequences from MGAY strain through circular polymerase extension cloning (CPEC) method (Quan & Tian, 2009). The reactions were carried out using Phusion High-Fidelity PCR Master Mix (Thermo Scientific). All primers used are listed in Table 3.2. The resulting donor DNA was electroporated into *S.* Typhimurium ATCC 13311 (pSIM19) and *C. sakazakii* FUA 10122 (pSIM19), which transiently expressed the λ-Red system (Datta et al., 2006). The strains were cured of the plasmid by growing at 42°C for overnight. The potential mutants were selected at 10μg/ml followed by the purification at 34μg/ml chloramphenicol on Luria-Bertani (LB; BD Biosciences) agar plates. The FRT-flanked *cat* gene was removed as described above. All resulting mutants were tested for loss of antibiotic resistance and screened for the presence of fluorescent

foci with a fluorescence microscope.

3.2.4 Plasmid constructions

The genes on protein homeostasis module including *orf1*, *shsp20*, $clpK_{GI}$, $shsp_{GI}$, $shsp20\text{-}clpK_{GI}$ and *egfp* were amplified using pLHR (Mercer et al., 2015) and peGFP (Clotech) as templates and placed behind the predicted tLST promoter *p2* on the pRK767 plasmid. The generated amplicons containing the *p2* promoter and the desired genes were fused in-frame and inserted into KpnI/HindIII (Fastdigest, Thermofisher, USA) digested pRK767 using GeneArt™ Gibson Assembly HiFi Master Mix (Invitrogen, USA) and transformed into *E. coli* TOP10. To introduce the double-point mutations, the amplicons of $clpK_{GI}$ and $shsp20\text{-}clpK_{GI}$ were cloned into NotI/HindIII digested pCA24N with the CPEC method, adding in mutations with site-directed mutagenesis primers. The double-point mutants were inserted into pRK767 together with the *p2* promoter as described above. All of the constructed plasmids were fully sequenced to confirm the identity of the inserts. pRK767 and pU19 together with all the pRK767- and pUC19-based recombinant vectors were individually electroporated into corresponding strains. All transformants were confirmed using PCR. The primers used for PCR are listed in Table 3.2.

Table 3.2. Primers used in this study.

Used for	Primer	Sequence (5'-3')	Reference
Plasmid transformation check	*orf1*-check-F	GGTGATTTTCACGCTCGATG	(Z. Wang et al., 2021; Zhu et al., 2021)
	orf1-check-R	TCGGATGACTTCTGCTGTTC	
	sHsp20-check-F	TACAAGATTGCCCTGGAAGT	
	sHsp20-check-R	CTTGATCGAATCCTGGTTGG	
	$clpK_{GI}$-check-F	CCATTCTTATGTCGGTCCAGAG	
	$clpK_{GI}$-check-R	CCACCTTGCTGACCTGTT	
	$sHsp_{GI}$-check-F	TCCGGGAACTGGATGAATTG	
	$sHsp_{GI}$-check-R	AGATCCAGCTTGAGGAGGAA	
	yfdX1-check-F	TCGGTAAAGAAAGCGGTCAAG	
	yfdX1-check-R	CATCGGAAGGTTGTCGGTTT	
	orf11-check-F	GAAGCGATTGTCCGAGCTAAG	
	orf11-check-R	TGCTTGCCACTTCGTTATCC	
Plasmid constructions	Promoter-F	AGTCACGACGTTGTAAAACGACGGCCAGTGA ATTCGAGCTCGGCTGTCCATTGCCTGAGC	This study
	Promoter-R	AATTTCACACAGGAAACAGCTATGACCATGAT TACGCCACTCACCCTCTCCAATCGCGTC	
	orf1-R	ACAATTTCACACAGGAAACAGCTATGACCATG ATTACGCCATCAAGCCCTTTGCCTGCTG	
	Promoter-*sHSP20*-R	GAACCAGTTCCAGGGAGCCAACTTCTTGAAAT CGATGTCCATCTCACCCTCTCCAATCGC	

(Continuous of Table 3.2)

sHSP20-F	CGGAACCGCAAAGCGACGCGATTGGAGAGGG
	TGAGATGGACATCGATTTCAAGAAGTTGG
sHSP20-R	ACAATTTCACACAGGAAACAGCTATGACCATG
	ATTACGCCATCAGCCGTTGATCGGGATC
Promoter-$clpK_{GI}$-R	GTGGCGGGTTGGCCGCAGACCTGGCATTGTTT
	TCTGGCCATCTCACCCTCTCCAATCGCG
$clpK_{GI}$-F	TTGGCCGGAACCGCAAAGCGACGCGATTGGA
	GAGGGTGAGATGGCCAGAAAACAATGCCA
$clpK_{GI}$-R	TAACAATTTCACACAGGAAACAGCTATGACCA
	TGATTACGCCATCAAGATGCGTCGCTCG
Promoter-$sHSP_{GI}$-R	TCCAGTTCCCGGAAGGGGTCCCACGGAGTCAA
	TGCAGACATCTCACCCTCTCCAATCGCG
$sHSP_{GI}$-F	GGCCGGAACCGCAAAGCGACGCGATTGGAGA
	GGGTGAGATGTCTGCATTGACTCCGTGGG
$sHSP_{GI}$-R	CAGGAAACAGCTATGACCATGATTACGCCATT
	AGTTGACTGAGATTTCAATCTGTTTCGG
Promoter-*yfp*-R	ACCACCCCGGTGAACAGCTCCTCGCCCTTGCT
	CACCATCTCACCCTCTCCAATCGCGTCG
yfp-F	GGCCGGAACCGCAAAGCGACGCGATTGGAGA
	GGGTGAGATGGTGAGCAAGGGCGAGGAGC

(Continuous of Table 3.2)

	yfp-R	TTCACACAGGAAACAGCTATGACCATGATTAC GCCATTACTTGTACAGCTCGTCCATGCC	
	clpK$_{GI}$-pCA-F	ATACGGATCCGGCCCTGAGGCCTATGCGGCCG CAGTAAATGGCCAGAAAACAATGCCAGG	
	pCA-*clpK*$_{GI}$-R	GTGGCGGGTTGGCCGCAGACCTGGCATTGTTT TCTGGCCATTTACTGCGGCCGCATAGGC	
	pCA-*clpK*$_{GI}$-F	CGCAAGAAGAAGTCGGCGAGCGACGCATCTT GAAAGCTTAATTAGCTGAGCTTGGACTCC	
	clpK$_{GI}$-pCA-R	TGGATCTATCAACAGGAGTCCAAGCTCAGCTA ATTAAGCTTTCAAGATGCGTCGCTCGCC	
	clpK-E/A-P1-F	GCTGATTTTGTTCATCGACGCGGTGCACACCA TCGTCGG	
	clpK-E/A-P1-R	CCGACGATGGTGTGCACCGCGTCGATGAACAA AATCAGC	
	clpK-E/A-P2-F	GTGCTGCTGCTCGACGCGATCGAGAAGGCACA CCCTG	
	clpK-E/A-P2-R	CAGGGTGTGCCTTCTCGATCTCGCCGAGCAGC AGCAC	
Plasmid construction sequence check	pRK-seq-F	GATGTGCTGCAAGGCGATTAAG	
	pRK-seq-R	GCTTCCGGCTCGTATGTTGTG	This study

	pCA-seq-F	GAGAGGATCTCACCATCACC	
	pCA-seq-R	GAGGTCATTACTGGATCTATCAAC	
	clpK-seq-2	GATTGGTCTGGCCGAGGAAG	
	clpK-seq-3	GGAGTCCGAACTGCACCAG	
	clpK-seq-4	CCAACACCATCATCATCGCC	
	clpK-seq-6	CCGCATCAGCGAACAGTC	
	clpK-seq-7	CAGAAGGTGCTGAAGGAG	
	clpK-seq-8	GACATGTCGGAATATGGCG	
	clpK-seq-9	GATCAGACGCTGATCGAC	
	ST-*ibp*-fusion-F	TTAGCTATTTAACGCGGGACGTTC	
	ST-*ibp*-fusion-R	ATGCGTAACTTTGATTTATCCCCGC	This study
ibpA-yfp fusion	CS-1000-up-F	GCAGCATGATGGCAATGCCG	This study
strain constructions	CS-*ibp*-fusion-tail-R	CGTATCGCTATCAGCGAACGTCCCGCGTTAAA	
		TAGCTAACGGCATCGTATGAAAAGGCCC	
	ibp-fusion-CS-tail-F	AGGCCCCGCTGCGGGGGCCTTTTCATACGATG	
		CCGTTAGCTATTTAACGCGGGACGTTCG	
	ibp-fusion-CS-tail-R	GGCGATGTTAACTCTCGCTGATTTCAGGAGTA	
		TGTATGCGTAACTTTGATTTATCCCCGC	
	CS-*ibp*-fusion-tail-F	AGAACGGTAAAGCGGGGATAAATCAAAGTTA	
		CGCATACATACTCCTGAAATCAGCGAGAG	
	CS-1000-down-R	CCTGACGTTCACGCACGATC	

3.2.5 Determination of the bacterial dry tolerance in 0.1% peptone water

Bacterial dry tolerance was determined in sterile 0.1% peptone (BD Biosciences, USA) water as previously described (Seeras, 2017). Briefly, single colonies of *E. coli* MG1655, *S.* Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 carrying the desired plasmids were inoculated overnight in TSB at 37°C with aeration at 200rpm. A 100µL aliquot of each inoculum was spread onto TSA plates and incubated aerobically for 24h at 37°C. Sterile 0.1% peptone water was prepared by adding 1g peptone into 1L distilled water and sterilized. The bacterial lawns were collected from the surface of the TSA plates and washed three times with 1mL 0.1% peptone water. The harvested cells were resuspended to a final volume of 1mL. The initial viable cell counts were determined by serial dilution in 0.1% peptone water and surface plating using 0.1% peptone water on LB agar plates. A 25µL of each bacterial suspension was spread evenly into the 24-well microtiter plates (Corning) and air-dried in a biosafety cabinet for 5h. After drying, plates were transferred to air-tight containers containing saturated NaCl solution to achieve a a_W of 0.75 and stored at 21°C for up to 21 days. A 200µL aliquot of 0.1% peptone water was used to rehydrate the cells for enumeration as described above. Results are presented in log-transformed ratio of cell counts before and after drying [$\log(N/N_0)$]. Data shown are means of three independent experiments.

3.2.6 Visualization and counting the percentage of protein aggregates in the bacterial population

Cell suspensions for *E. coli* MG1655 *ibpA-yfp*, *S.* Typhimurium ATCC 13311 *ibpA-yfp* and *C. sakazakii* FUA 10122 *ibpA-yfp* carrying the desired plasmids were harvested from TSA plates and washed in 0.1% peptone as described above. The suspensions were transferred to a microscope slide, quickly mixed with pre-warmed liquid 0.8% agarose in 1:1 ratio and covered with a glass cover slip. The same position of the slide was observed with a fluorescence phase contrast

microscope to obtain both fluorescence and bright-field images (Carl Zeiss). Images were overlayered with Photoshop CC (v. 19.0, Adobe Inc.). The proportion of protein aggregates in a cell population was calculated by dividing the number of cells possessing visible fluorescent foci by the total number of cells observed under bright-field. Data shown are means of three independent experiments with analysis of at least 300 cells per strain.

3.2.7 Determination of the dry tolerance of bacteria in infant formula

Cell suspensions for *S.* Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 carrying the desired plasmids were acquired in the same way as described above. A suspension (1mL) was homogeneously mixed with 7g powdered infant formula (Enfamil A+®, Canada) in a sterile stomacher bag (Whirl-pak®, Canada). After air-drying in a biosafety cabinet for 5h, the lumpy mixture was crushed for 4min into fine powder with a stomacher. Each sample (0.5g) was aliquoted into 24-well plates and was transferred to air-tight containers containing saturated $MgCl_2$ solution to achieve a a_W of 0.33 and stored at 21°C for up to 112 days. Viable cell counts were determined before and after drying by rehydrating the powder with 2mL 0.1% peptone water followed by serial dilution and surface plating on Violet Red Bile Agar (VRBA) (BD Biosciences, USA). The cell counts were log-transformed and the reduction of cell counts [$\log(N/N_0)$] was calculated. Data shown are the mean of three independent experiments.

3.2.8 Statistical analysis

Statistical analysis of data was performed using SPSS (v. 26.0, SPSS Inc.) software. Differences in reduction of cell counts was tested with a two-way ANOVA with Tukey's HSD *post hoc* analysis (P<0.05). The effect of tLST or protein homeostasis module on protein aggregation was tested with one-way analysis of variance (ANOVA) with Tukey's HSD *post hoc* analysis (P<0.05). Results are shown as means ± standard deviations for three biological replicates. The chi-squared

test on the frequency of the tLST in *C. sakazakii* from different environments was preformed using Microsoft Excel 2016.

3.3 Results

3.3.1 Dry environments select against the tLST in *Cronobacter sakazakii*

The frequency of tLST in isolates of *C. sakazakii* from different environments was compared to investigate the possible selective pressure for tLST-positive *C. sakazakii*. In a total of 322 isolates, the tLST was enriched in isolates from humans and PIF facilities but was reduced in isolates from dried sources relative to environmental sources (Table 3.3). The PIF isolates have a lower frequency of the tLST than the isolates from facilities producing PIF ($P<0.05$), indicating that the drying process of PIF might be detrimental to survival of the tLST-positive strains (Table 3.3).

Table 3.3. The frequency of tLST in *C. sakazakii* from different environments.

	Isolation sources				
	Environmental	Dried	PIF facilities	PIF formula	Human
tLST	7	5	42	9	82
no tLST	30	66	30	21	30
tLST frequency (%)	19	7	58	30	73
χ^2 vs. environmental		3.69×10^{-11}	2.63×10^{-65}	7.05×10^{-2}	9.03×10^{-177}
χ^2 vs. PIF facilities				4.92×10^{-13}	

3.3.2 Impact of the tLST and its protein homeostasis module on desiccation tolerance

The bioinformatic results suggested a relationship between the tLST and desiccation environment and the detrimental effects of the tLST on desiccation tolerance was experimentally confirmed in *Salmonella* Typhimurium (Seeras, 2017). The current study assessed whether this phenomenon is relevant in different species of the *Enterobacteriaceae* and whether the effect is due to the tLST protein homeostasis module. The desiccation tolerance of *Escherichia coli* MG1655, *S.*

Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 was determined using derivatives of the low copy number plasmid pRK767 (Gill & Warren, 1988): pLHR carrying the tLST, pRF1 carrying the protein homeostasis module of the tLST or pRK767 itself, the vector control (Mercer et al., 2015). Peptone water was used as the drying matrix to avoid osmotic stress that would result from drying in saline (Amezaga & Booth, 1999). All strains of *E. coli* MG1655 rapidly died at the same rate regardless of the presence of plasmids (Fig. 3.1A) while expression of the tLST substantially reduced desiccation tolerance in *S.* Typhimurium ATCC 13311 (Fig. 3.1B) and moderately in *C. sakazakii* FUA 10122 (Fig. 3.1C). Remarkably, in both *S.* Typhimurium and *C. sakazakii,* the presence of the protein homeostasis module alone decreased the level of desiccation tolerance comparable to that of strains carrying the full-length tLST (Fig. 3.1B, C). Overall, the tLST, specifically the protein homeostasis module, reduces desiccation tolerance in *S.* Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 but not *E. coli* MG1655.

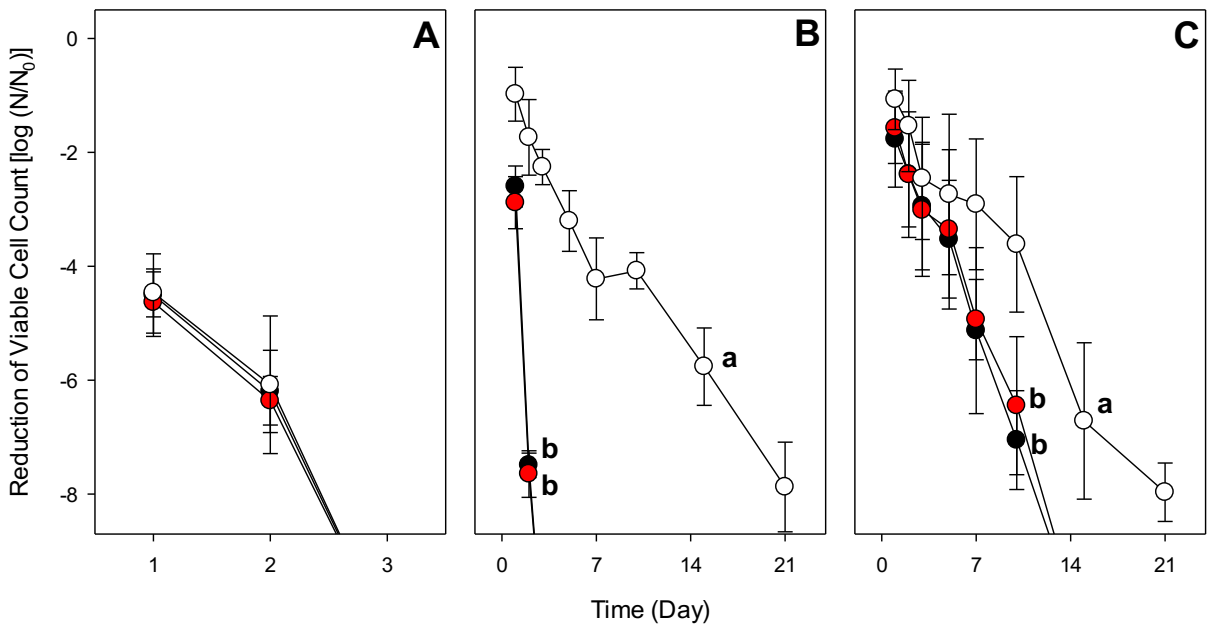

Figure 3.1. Influence of tLST or protein homeostasis module on desiccation survival of *E. coli* MG1655, *S.* Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 after exposure to a_W 0.75 at 21°C. (A) *E. coli* MG1655 pRK767 (○), pLHR (●) and pRF1 (●). (B) *S.* Typhimurium ATCC 13311 pRK767 (○), pLHR (●) and pRF1 (●). (C) *C. sakazakii* FUA 10122 pRK767 (○), pLHR (●) and pRF1 (●). Data are mean ± standard deviation of three independent experiments. In each panel, means that do not have common superscripts are significantly different. Lines dropping below the x-axis refer to cell counts below the detection limit.

3.3.3 Impact of the tLST and its protein homeostasis module on protein aggregation *in vivo*

To obtain insight into the effect of tLST on protein aggregation, the protein aggregates in *E. coli* MG1655 *ibpA-yfp*, *S.* Typhimurium ATCC 13311 *ibpA-yfp* and *C. sakazakii* FUA 10122 *ibpA-yfp* harbouring pLHR, pRF1 or pRK767 were observed by fluorescence phase contrast microscopy. All the bacteria were collected with the same methods described for the desiccation tolerance tests. Strains of *S.* Typhimurium ATCC 13311 *ibpA-yfp* and *C. sakazakii* FUA 10122 *ibpA-yfp* harbouring tLST or its protein homeostasis module had lower proportions of individual cells with protein aggregates when compared to that of strains harbouring pRK767; however, no difference

was detected among the strains of *E. coli* MG1655 *ibpA-yfp* (Fig. 3.2). Results indicated that in *S.* Typhimurium and *C. sakazakii* the tLST, and specifically its protein homeostasis module, function to reduce protein aggregate formation, which correlates with bacterial desiccation tolerance, but this effect is not observed in all *Enterobacteriaceae*.

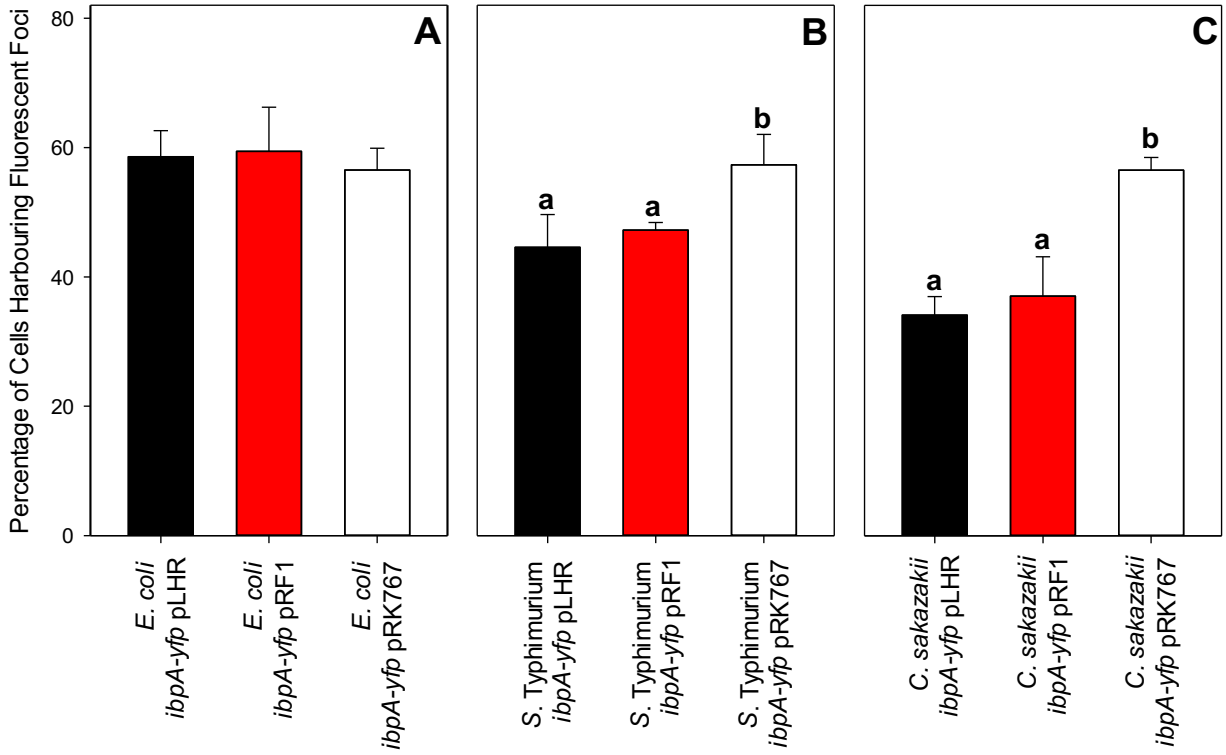

Figure 3.2. Percentage of cells with fluorescent foci representing protein aggregates. (A) *E. coli* MG1655 *ibpA-yfp* harbouring pLHR, pRF1 and pRK767. (B) *S.* Typhimurium ATCC 13311 *ibpA-yfp* harbouring pLHR, pRF1 and pRK767. (C) *C. sakazakii* FUA 10122 *ibpA*-yfp harbouring pLHR, pRF1 and pRK767. Data are mean ± standard deviation of three independent experiments. In each panel, bars that do not have common superscripts are significantly different.

3.3.4 Effect of protein aggregation on desiccation tolerance

The tLST-encoded $ClpK_{GI}$, also known as $ClpG_{GI}$ in *Pseudomonas aeruginosa*, is an ATP-

dependent autonomous disaggregase (C. Lee et al., 2018; C. Lee et al., 2015). $ClpK_{GI}$ is composed of two AAA domains, ATP hydrolysis can be inhibited with two mutations, E383A and E723A, one in each of the ATPase domains, abrogating its protein disaggregation activity *in vitro* (Katikaridis et al., 2021). In addition, ORF1 expressed from the tLST has only been reported as a transcriptional regulator of sHsp20-$ClpK_{GI}$ but lacks evidence of being directly involved in protein quality control machinery (C. Lee et al., 2015b). Therefore, to further determine the effect of the individual genes comprising the protein homeostasis module in bacterial desiccation tolerance, genes encoding ORF1, sHsp20, $ClpK_{GI}$-E383A/E723A, sHsp20-$ClpK_{GI}$, sHsp20-$ClpK_{GI}$-E383A/E723A and $sHsp_{GI}$ were cloned into pRK767 individually under the control of the native tLST *p2* promoter and transformed into *S.* Typhimurium ATCC 13311 to assess their impact on cell survival (Fig. 3.3). Interestingly, the addition of any of these genes reduced desiccation tolerance in *S.* Typhimurium ATCC 13311 when compared to the empty vector pRK767. The survival of strains expressing both of the two sHsps was lower than the strains solely expressing $ClpK_{GI}$. Eliminating the ATP hydrolysis activity in the disaggregase $ClpK_{GI}$ had no significant effect on desiccation tolerance when $ClpK_{GI}$ alone was present on the plasmid, however when present with sHsp20, the mutant $ClpK_{GI}$ had approximately 2 log(cfu/mL) greater survival after 3 days of incubation relative to the wildtype sHsp20-$ClpK_{GI}$ construct. The constructed plasmids were also transformed into *S.* Typhimurium ATCC 13311 *ibpA-yfp* to visualize the protein aggregates *in vivo* by fluorescence phase contrast microscopy. All tLST-gene expressing strains except the one with ORF1 had a lower proportion of cells with protein aggregates when compared to the vector control. No difference was detected between $ClpK_{GI}$ and $ClpK_{GI}$-E383A/E723A expression strains but the presence of wildtype sHsp20-$ClpK_{GI}$ decreased the number of cells with protein aggregates relative to sHsp20 with the $ClpK_{GI}$ mutant (Fig. 3.4). In summary, the presence

of the disaggregase activity as well as the expression of non-functional proteins increases sensitivity to desiccation which is correlated with the number of cells with protein aggregates.

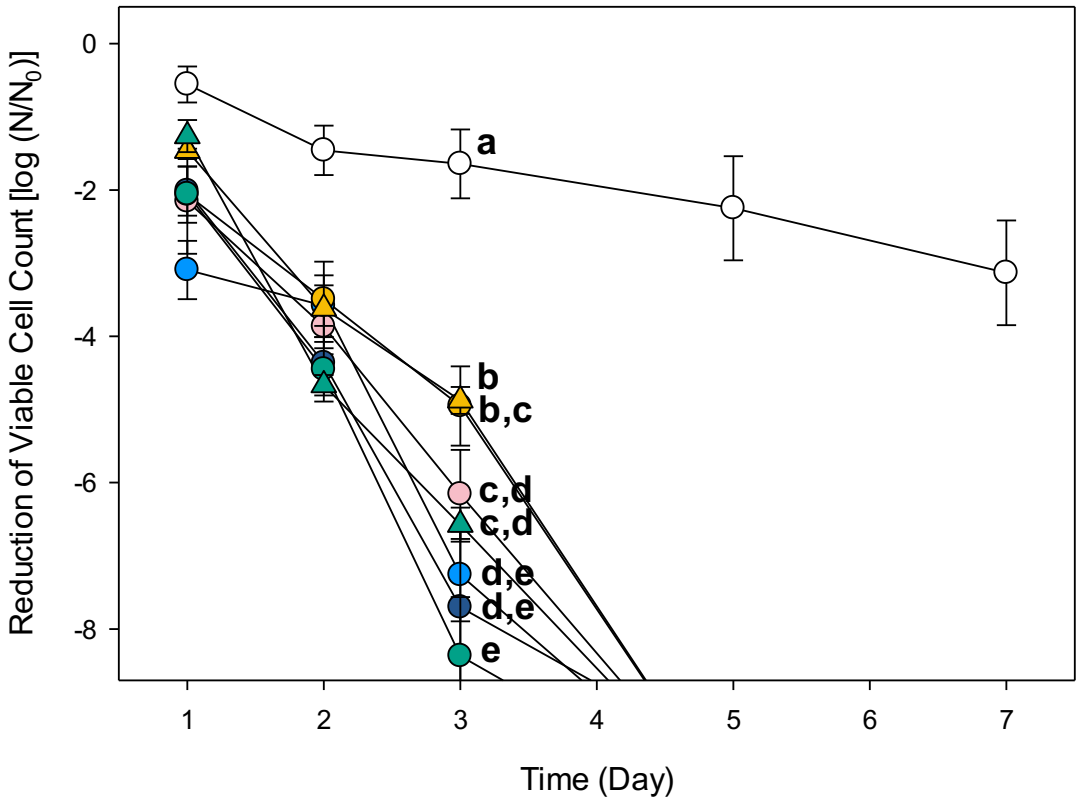

Figure 3.3. Influence of the genes of the protein homeostasis module-comprising genes on desiccation survival of *S.* Typhimurium ATCC 13311 after exposure to aw 0.75 at 21°C. The tested *S.* Typhimurium ATCC 13311 strains harbored plasmids including pRK767 (○), pRK*p2*::*orf1* (○), pRK*p2*::*shsp20* (●), pRK*p2*::*clpK*$_{GI}$ (●), pRK*p2*::*clpK*$_{GI}$-E383A/E723A (▲), pRK*p2*::*shsp20-clpK*$_{GI}$ (●), pRK*p2*::*shsp20-clpK*$_{GI}$-E383A/E723A (▲) and pRK*p2*::*shsp*$_{GI}$ (●). Data are mean ± standard deviation of three independent experiments. Means that do not have common superscripts are significantly different. Lines dropping below the x-axis refer to cell counts below the detection limit.

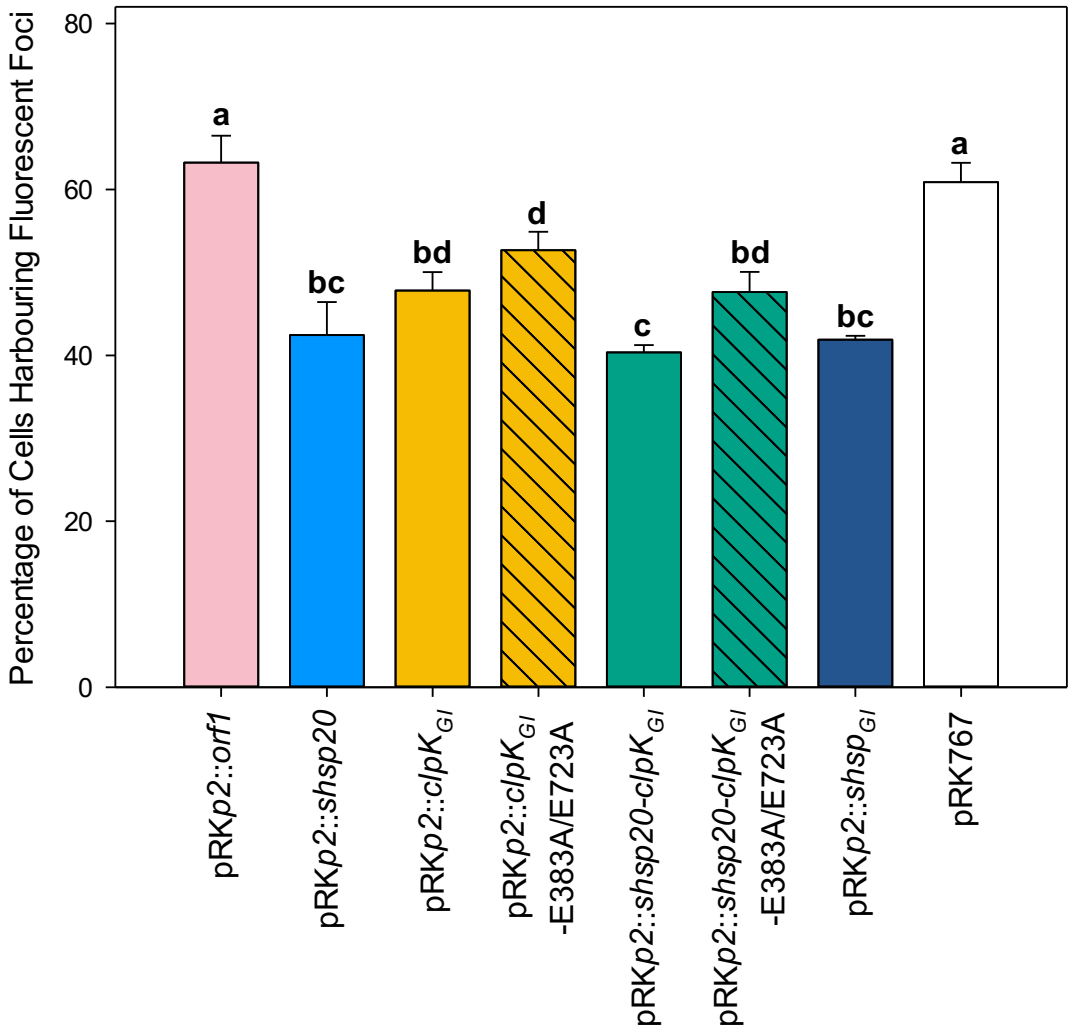

Figure 3.4. Percentage of the *S.* Typhimurium ATCC 13311 *ibpA-yfp* cells harbouring the genes of the protein homeostasis module-comprising genes with fluorescent foci representing protein aggregates.Data are mean ± standard deviation of three independent experiments. Bars that do not have common superscripts are significantly different.

3.3.5 Effect of energy waste on bacterial desiccation tolerance

To test if desiccation sensitivity is solely due to the expression of genes involved in protein homeostasis or if any form of extra resource usage by the cell can have the same effect, we tested several types of resource allocation in *S.* Typhimurium ATCC 13311. As all the experiments above

were conducted in strains carrying a plasmid, we investigated the effect of DNA replication, transcription and translation on desiccation tolerance through plasmid-based approaches. The pRK767 plasmid with *p2* promoter was used to assess the effect of transcription. The enhanced green fluorescent protein (eGFP) under the regulation of the *p2* promoter was used to determine the effect of protein production unrelated to protein homeostasis and pUCkan, a pUC19 derivative with both ampicillin and kanamycin resistance cassettes, was used to determine the effect of maintaining high copy plasmids and of growth in different antibiotics. Surprisingly, all strains with the pUC19 plasmids died rapidly relative to those carrying pRK767 derivatives. This was also the case for pUC*p2*ΔD::*egfp*, where protein expression was silenced by a promoter mutation (Table 3.5). Strains carrying pRK767 derivatives with the *p2* promoter and eGFP died more quickly than pRK767 without any insert (Table 3.5). Results indicated that the energy requirements of high copy plasmids, or of highly expressed proteins are also detrimental to bacterial survival under dry conditions.

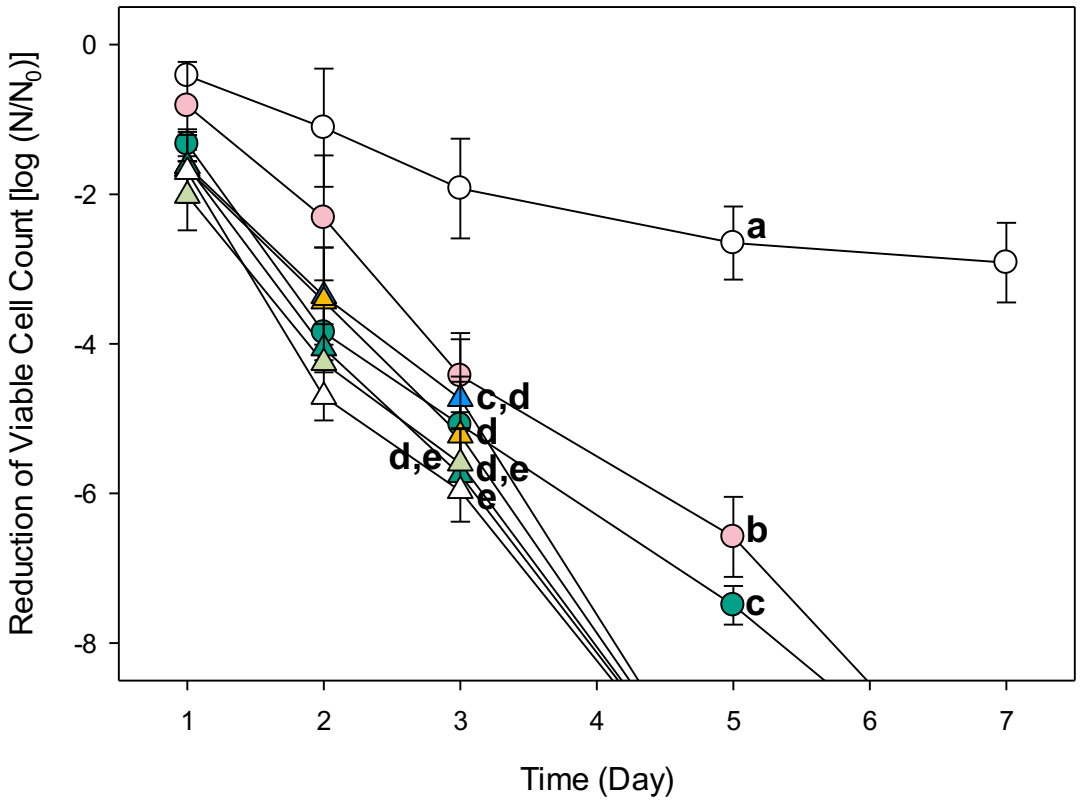

Figure 3.5. Influence of the non-tLST gene and high copy number plasmid on desiccation survival of *S.* Typhimurium ATCC 13311 after exposure to aw 0.75 at 21°C. All the strains were harvested from agar plate surface, washed, resuspended with 0.1% peptone water**,** air-dried for 5 hours and stored in air-tight containers for up to 21 days. The tested *S.* Typhimurium ATCC 13311 strains harboured plasmids including pRK767 (○), pRK*p2* (●), pRK*p2*::*egfp* (●), pUC*kan* harvested from kanamycin plate (▲), pUC*kan* harvested from ampicilin plate (▲), pUC*p2*::*egfp* (▲), pUC*p2*ΔD::*egfp* (▲) and pUC19 (△). Data are mean ± standard deviation of three independent experiments. Means that do not have common superscripts are significantly different. Lines dropping below the x-axis refer to cell counts below the detection limit.

3.3.6 Bacterial survival in PIF

The survival of *S.* Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 harbouring pLHR, pRF1 or pRK767 was also determined in PIF. Consistent with the results obtained in 0.1% peptone

water, the desiccation tolerance of *S.* Typhimurium decreased significantly in the presence of tLST or the protein homeostasis module compared to that of strains carrying the vector control (Fig. 3.6A). The reduction of cell counts was also observed in *C. sakazakii* pLHR and pRF1 compared to pRK767, but to a lesser extend (Fig. 3.6B). Taken together, the tLST and its protein homeostasis module also increased bacterial sensitivity to desiccation in a relevant food matrix.

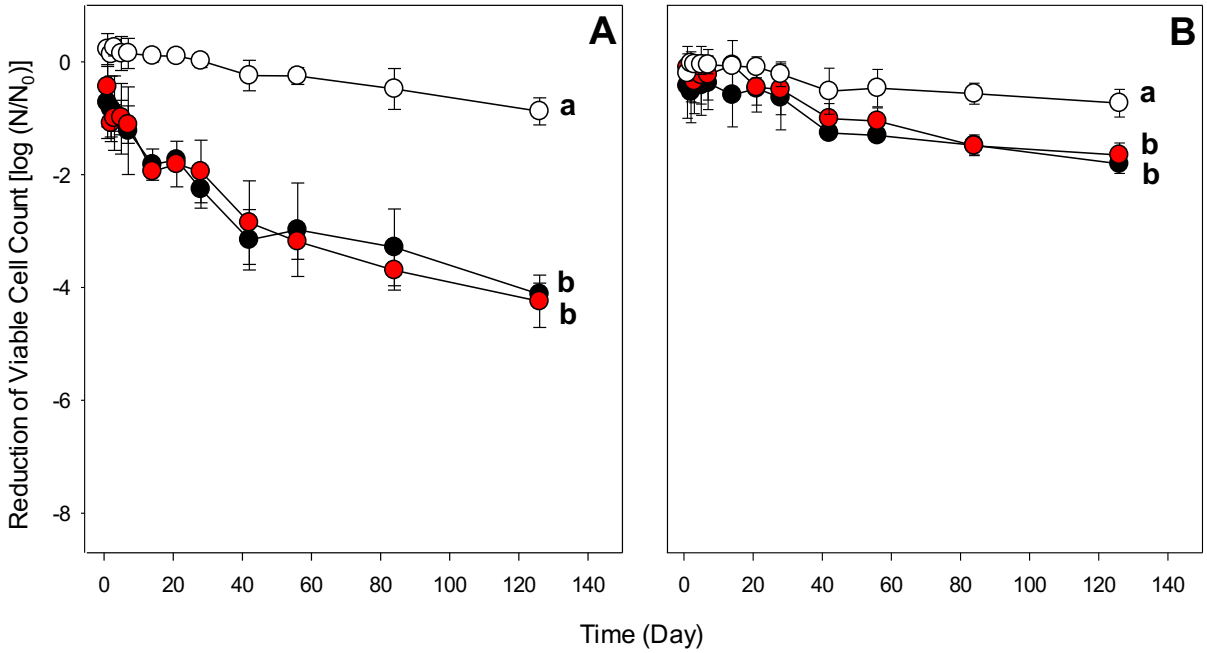

Figure 3.6. Influence of the tLST or protein homeostasis module on desiccation survival of *S.* Typhimurium ATCC 13311 and *C. sakazakii* FUA 10122 in infant formula after storage at a_W 0.33 and 21°C. (A) *S.* Typhimurium ATCC 13311 pRK767 (white), pLHR (black) and pRF1 (red). (B) *C. sakazakii* FUA 10122 pRK767 (white), pLHR (black) and pRF1 (red). Data are mean ± standard deviation of three independent experiments. In each panel, means that do not have common superscripts are significantly different.

3.4 Discussion

3.4.1 The impact of protein aggregate on bacterial desiccation tolerance

The presence of the tLST, specifically its protein homeostasis module, negatively affected bacterial desiccation tolerance in *S.* Typhimurium and *C. sakazakii* under laboratory conditions.

The use of a ClpK derivative with two amino acid substitutions that abrogate disaggregase activity but not the energy expenditure associated with protein expression allowed differentiation of the impact of protein homeostasis and energy expenditure on bacterial survival. Bacterial survival in the dry state is influenced by protein aggregates and the presence of energy intensive elements.

Protein aggregation is a prominent marker of cell aging. Protein aggregates are not segregated evenly into the progeny, but mother cells allocate the aggregates to the old pole in cell division; this segregation contributes to perpetuate the population (Lindner et al., 2008). The aggregates eventually accumulate within a small proportion of the cells which have slow metabolic and proliferation rates (Lindner et al., 2008). This genetically identical subpopulation has been identified as dormant cells that possess higher levels of tolerance to adverse circumstances and contribute to maintenance of the bacterial population (Pu et al., 2019). The phenomenon of genetically identical but phenotypically different populations was first described in 1944 by Joseph Bigger, who observed that a subpopulation of staphylococci was resistant to the effects of penicillin. The subpopulation was proposed as dormant non-dividing cells and termed “persisters” (Bigger, 1944). Although the concept of persister cells has only been applied in reference to antibiotic stress, our study warrants an expanded definition where the term persister is used to refer to dormant cells that have increased tolerance to other stressors, in this case the stress of desiccated environments. The viable but non-culturable (VBNC) state is also recognized as another type of cell dormancy. VBNC cells lost their cultivability on standard medium although they remain metabolically active and carry out respiration (Del et al., 2000). It has been hypothesized that persisters and VBNC cells reside in a continuum of cell dormancy state, from shallow to deep (Ayrapetyan et al., 2015, 2018; Bollen et al., 2021; Pu et al., 2019). It is possible that the disaggregase reduced the proportion of VNBC cells and the difference between the constructs with

disaggregase and without is greater than reported (Pu et al., 2019).

In strains of *Salmonella*, the tLST only presents in a few strains and the genomic island is differentially distributed in pathogenic and non-pathogenic strains of *E. coli* (Z. Wang et al., 2020b). Our *in silico* analysis of *C. sakazakii* revealed that the tLST is rare in isolates from dried sources relative to environmental sources or facilities that produce PIF. This suggests that either the PIF production process or survival during storage of PIF selects against strains of *C. sakazakii* harboring the tLST. The tLST encodes for a protein quality control machinery that reduced protein aggregates in *E. coli* and *Pseudomonas aeruginosa* (Kamal et al., 2019; C. Lee et al., 2018; C. Lee et al., 2015b; Li et al., 2020). We therefore hypothesized that the protein aggregate-carrying persister cells survive better in desiccated environments. This hypothesis was tested using the tLST as a tool to reduce protein aggregates. Interestingly, a high frequency of the tLST was found in strains of *C. sakazakii* isolated from humans, in fact it was the highest frequency observed for any environment, suggesting that the benefits of the tLST, such as heat resistance, outweigh its detrimental effect in *C. sakazakii* in a hospital environment.

The tLST was identified as a plasmid-encoded or chromosomal genomic island flanked by transmissible elements (Kamal et al., 2021). To determine whether the increased desiccation sensitivity of strains of *S.* Typhimurium and *C. sakazakii* that possess the tLST is attributed to the function of the tLST protein homeostasis module, the effect of the two small heat shock proteins, sHsp20 and $sHsp_{GI}$, and the disaggregase, $ClpK_{GI}$, on a low-copy number plasmid, pRK767 was examined (Gill & Warren, 1988; Kamal et al., 2021; Li et al., 2020; Mercer et al., 2017). sHsps co-aggregate with and sequester denatured proteins in an ATP-dependent manner, which keeps the proteins in a reversible state and facilitates efficient refolding or degradation by other chaperones (Ungelenk et al., 2016; Webster et al., 2019b). Disaggregases disaggregate insoluble protein

aggregates, allowing the proteins to be refolded into their functional form (Mogk et al., 2018; Mogk & Bukau, 2004). The disaggregase ClpB acts in collaboration with DnaK (Hsp70) to dissolve and clear cellular protein aggregates (Pu et al., 2019). In *P. aeruginosa* the ATP-dependent autonomous disaggregase $ClpK_{GI}$ functionally complements the DnaK-ClpB bichaperone complex (Katikaridis et al., 2021; Rosenzweig et al., 2013). The sHsps in the tLST impacted *Salmonella* survival more substantially than $ClpK_{GI}$ under dry conditions and eliminating the disaggregase activity of $ClpK_{GI}$ had no effect on sensitivity to desiccation. When both sHsp20 and $ClpK_{GI}$ were expressed together, the inactivation of the disaggregase resulted in as much as a 2 log(cfu/mL) difference in sensitivity to desiccation. Furthermore, fluorescent microscopy revealed that both sHsp20 and $ClpK_{GI}$ are able to reduce the number of cellular protein aggregates. However, only with the presence of sHsp20 can differences among a proportion of cells carrying protein aggregates be detected when the $ClpK_{GI}$ disaggregase activity is abolished. This suggested that sHsp20 and $ClpK_{GI}$ function independently and may have a synergistic effect in the same bacterial regulation network.

The tLST had a comparable effect on *S.* Typhimurium and *C. sakazakii* in PIF. The contamination of PIF with *Salmonella* and *C. sakazakii* has contributed to the burden of foodborne illness among infants (Koseki et al., 2015). Food matrices can affect the survival of desiccated bacteria, so it is crucial to reproduce, as closely as possible, the conditions that allow survival of the pathogen e.g., PIF (Dhaliwal et al., 2021; Doyle & Mazzotta, 2000; Finn et al., 2013a). The tLST reduced survival of both *Salmonella* and *Cronobacter* in PIF, affirming the BLAST analysis that showed that isolates from PIF sources are less likely to harbor the tLST than the facilities that produce PIF. *Salmonella* and *C. sakazakii* are well known for their outstanding ability to survive in low a_W environments. To date, desiccation survival of *Salmonella* and *C. sakazakii* was attributed to their

ability to form biofilms and to the synthesis or transport of compatible solutes (Du et al., 2018; Finn et al., 2013a). In this study, we correlated desiccation tolerance with the amount of naturally existing cellular protein aggregates in *S.* Typhimurium and *C. sakazakii*. Our study additionally revealed that the cellular protein aggregates also positively aid in this phenotype.

E. coli have generally been considered as less desiccation tolerant when compared to *S.* Typhimurium but Shiga toxin-producing *E. coli* (STEC) have also been associated with outbreaks of foodborne illness linked to dry foods (Deng et al., 1998; Hiramatsu et al., 2005; Miller et al., 2012; Ryu et al., 1999) The tLST only presents in a few strains in genomes of STEC (Z. Wang et al., 2020b) and *E. coli* utilizes protein aggregates for antibiotic stress tolerance (Pu et al., 2019). This may suggest that strains of *E. coli* use protein aggregates as a strategy for desiccation tolerance. Although tLST encoded proteins function similarly in *E. coli, Salmonella* and *Cronobacter* (Gajdosova et al., 2011; Mercer et al., 2015; Mercer, Walker, et al., 2017), the tLST did not reduce the number of proteins aggregates in *E. coli* cultures harvested from TSA, and expression of the tLST in strains of *E. coli* did not alter survival after desiccation. This suggests that the stress response of *Salmonella*, *Cronobacter* and *E. coli* prioritizes tolerance to different stressors, and that the interplay of accessory genes that complement the protein quality control machinery of the core genome with other aspects of the stress response differs among these species.

3.4.2 The impact of resources allocation on bacterial desiccation tolerance

The expression of ORF1 reduced desiccation tolerance despite the fact it is not a part of the protein quality control machinery, and it had no effect on reducing the cellular protein aggregates. This suggests other mechanisms for the reduced desiccation tolerance in presence of the tLST. All of the constructs used in the experiments were under the control of the native tLST *p2* promoter which constitutively expressed the downstream genes independent of the growth conditions (Mercer et

al., 2015; Mercer et al., 2017). In *E.* coli, the LST-encoded proteins are highly expressed (Li et al., 2020) and the substantial energy burden imposed by protein synthesis may affect bacterial fitness (Li et al., 2020; Z. Wang et al., 2021). Production of mRNA or protein under the *p2* promoter or the maintenance of high-copy pUC19 plasmids increased desiccation sensitivity, demonstrating that energy waste also negatively affects bacterial survival in desiccated environments. The high copy pUC19 plasmids had a much greater effect on desiccation survival compared to the low copy pRK767 plasmids. To confirm whether this was due to the maintenance of the plasmid or due to the stress of growth in ampicillin instead of tetracycline, *Salmonella* containing a pUC19 plasmid with both ampicillin and kanamycin was grown with either ampicillin or kanamycin. Both tetracycline and kanamycin target the ribosome, which may affect protein aggregation, but ampicillin targets peptidoglycan so likely has no effect on the protein homeostasis state (Wilson et al., 2020). The same levels of desiccation sensitivity were detected regardless of the applied antibiotics, excluding the impact of antibiotics under the conditions tested. The effect of maintaining the high copy plasmids obscured any effects from the elements inserted into those plasmids. This result contrasted with the results comparing the *p2* promoter and *egfp* in the low copy pRK767 and its empty vector control where there was approximately a 3 log difference after 3 d of storage. The desiccation sensitivity of tLST-carrying strains of *S.* Typhimurium and *C. sakazakii* is thus attributable to a combination of reduced protein aggregates and increased energy use.

3.5 Conclusion

In conclusion, the results of this study provide new insights into the underlying mechanisms of bacterial desiccation tolerance. The survival strategies of *S.* Typhimurium and *C. sakazakii* under desiccation include cell filamentation, the synthesis and accumulation of osmoprotectant

molecular and iron metabolism (Finn et al., 2013b; C. Yu et al., 2021). Our study correlated the ability of *S.* Typhimurium and *C. sakazakii* to survive in low a_W environments with their formation of protein aggregates and energy conservation. This phenomenon was reproducible in PIF, which emphasized the practical implications of this research. Further experiments can be conducted to directly investigate the relationship between desiccation tolerance and bacterial persister cells.

Chapter 4. Conclusion and general discussion

In conclusion, maintaining protein homeostasis contributes differently to bacterial stress tolerance. The tLST confers bacterial host with tolerance phenotypes towards elevated temperature, chlorine, oxidative chemicals and high hydrostatic pressure (C. Lee et al., 2018; Li et al., 2020; Mercer, Nguyen, et al., 2017; Z. Wang et al., 2020a). The protective effect of the tLST was largely attributed to its small heat shock proteins and disaggregase, which prevent the formation of protein aggregates, improve protein homeostasis and therefore aid in bacterial survival under stress (Li et al., 2020; Z. Wang et al., 2020a). However, maintenance of the tLST imposes a fitness cost to the bacterial host, which needs to be compensated with the frequently present lethal levels of selective pressure (Z. Wang et al., 2020a). The tLST also confers tolerance to *E. coli* to extreme alkaline pH but requires simultaneous chlorine stress as an activation signal. Instead of relying on the tLST-encoded protein quality control machinery, this phenotype attributes to the function of the Na^+/H^+ antiporter $KefB_{GI}$ which maintains membrane potential through acidification of the cytoplasm. The presence of tLST, specifically its protein homeostasis module, negatively impacts desiccation tolerance of *S.* Typhimurium and *C. sakazakii*. The additional protein quality control machinery enhances protein homeostasis by resolving the protein aggregates but also imposes additional fitness cost to the bacterial host which has been shown as detrimental to the host survival under desiccation. The tLST is postulated to originate from extreme habitats and it is widely spread among diverse members of beta- and gamma-Proteobacteria via horizontal transmission (Kamal et al., 2021). The maintenance of the tLST is promoted in human-made ecological niches including food processing facilities, wastewater treatment plants and nosocomial settings where its bacterial hosts need to withstand the lethal effects of frequent occurred high temperature, chlorine and oxidizing chemicals (Kamal et al., 2021; Z. Wang et al., 2020b, 2021). The tLST has been observed

at low frequency in the isolates from the dry sources or the bacterial species whose lifestyle largely depends on the desiccation. Thus, whether the presence of tLST benefits the bacterial host depends on the type, severity and frequency of the encountered stresses relative to the fitness cost of maintaining the tLST, which determines the prevalence and distribution of tLST in the different ecological niches.

Functional protein is the foundation for performing proper cellular activities, therefore protein quality control is essential to bacterial physiology. Perturbations in protein homeostasis can result in the generation of protein aggregates, which has been recognized as a hallmark of cellular aging, preventing bacterial proliferation. The powerful protein quality control machinery should be favored by evolution, but my research revealed that protein aggregates may also be an evolved strategy for bacterial stress adaptation. Further proteomic analysis of the bacterial aggresome and genome-wide comparison should be conducted to assess whether forming protein aggregates is a selectable trait through active processes and if there are genetic determinants present.

Taken together, this study improves the understanding of how protein homeostasis affects bacterial stress tolerance. Instead of being parts of the accessory genome protecting its host against multiple stresses, tLST can be a suitable tool to probe the role of protein aggregation in bacterial stress tolerance. Further studies may expand its application to investigate bacterial ecology and develop strategies to improve the microbial safety in food production under current industrial practice.

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