A screen for novel regulators of polyphosphate accumulation in *Escherichia coli* identifies genes involved in envelope stress, central metabolism, and phosphate homeostasis

Abby Simms

A thesis submitted to the University of Ottawa for the partial fulfillment of the requirements for the Master of Science in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa, Ottawa, Canada

ABSTRACT

Polyphosphates (polyP) are chains of inorganic phosphate that are present in a wide variety of organisms such as yeast, mammals, and bacteria. Despite the interesting and diverse functions of polyP across systems, bacterial polyP is of particular interest to researchers due to its contributions to bacterial survival. Across bacterial taxa, polyP accumulation promotes resistance to diverse environmental stressors, can act as a protein chaperone, and positively regulates pathogenicity, making polyP an interesting target to help treat bacterial infections. In the gram-negative bacterium, *Escherichia coli*, polyP is synthesized from ATP by a single polyphosphate kinase (PPK) enzyme, and is degraded into inorganic phosphate by an exopolyphosphatase. PolyP synthesis by PPK in *E. coli* is stress-dependent, as there is little to no detectable polyP present in the absence of stress, followed by the rapid accumulation of polyP upon exposure to stressors such as nutrient limitation. Despite this stress-specific activity, the exact trigger(s) of PPK activity at the onset of stress remain uncharacterized, as PPK's expression and *in vitro* activity do not correlate with polyP accumulation. To address this open question, this work aims to characterize novel regulators of polyP that can be leveraged to investigate the mechanism of PPK activation. To achieve this, we conducted a screen of single-gene deletion mutants targeting a highly conserved family of signalling proteins called two-component systems (TCS), which have existing links to polyP biology. In doing so we highlighted five polyP-regulators, three of which (*cpxA*, *arcB*, and *arcA*) have not yet been reported in the literature. Upon following up on one of our hits, the envelope stress-sensing histidine kinase CpxA, we found that activation of the CpxRA TCS promotes an increase in polyP accumulation. However, polyP regulation by this system appears to be independent of PPK or PPX expression, leaving the door open to future analyses of PPK regulation, possibly at the level of localization or oligomerization. Overall, this work contributes

to our fundamental knowledge surrounding the relationship between PPK activity, polyP accumulation, and cellular stress.

CONTRIBUTIONS AND ACKNOWLEDGEMENTS

I would like to first acknowledge Dr. Michael Downey for being a supportive mentor throughout this project, and for the conceptualization of the work. I would also like to acknowledge Maria Nikokavoura for her contribution to the screen for polyP-regulators (Table 1, the testing of 10 mutants and the creation of 6 mutants). Finally, I would like to acknowledge Dr. Sunayana Sarkar for her contribution to adapting the fractionation protocol (section 2.4, method used in Figure 8). Given these contributions, phrasings such as “we” or “our” are used within the results section.

I thank both current and past members of the Downey lab for their continuous support throughout my Master’s, especially Dr. Kanchi Baijal, who was an exceptional instructor and mentor. I would like to thank my thesis advisory committee members, Dr. Vanessa D’Costa and Dr. Laura Trinkle-Mulcahy, for their guidance and input on the project. Finally, I would also like to thank my family and friends for accommodating my “quick” trips to the lab, and for their endless encouragement.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

PolyP: Polyphosphate

Pi: Inorganic phosphate

VTC: Vacuolar transporter chaperone

PPK: Polyphosphate kinase

PPX: Exopolyphosphatase

(p)ppGpp: guanosine 5'-triphosphate 3'-diphosphate and guanosine 5'-diphosphate 3'-diphosphate

TCS: Two-component system(s)

HK: Histidine kinase

RR: Response regulator

LB: Lysogeny broth

MOPS: 3-(N-Morpholino)propanesulfonic acid

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

EDTA: Ethylenediaminetetraacetic acid

TBE: Tris, boric acid, EDTA

SDS: Sodium dodecyl sulfate

APS: Ammonium persulfate

TEMED: Tetramethylethylenediamine

DTT: Dithiothreitol

PAGE: Polyacrylamide gel electrophoresis

PVDF: Polyvinylidene difluoride

TBST: Tris-buffered saline with tween

WT: Wildtype

EV: Empty vector

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Polyphosphate is a highly conserved polymer

Chains of inorganic phosphate (Pi) residues joined by phosphoanhydride bonds, known as polyphosphates (polyP), are thought to be nearly universally conserved across kingdoms of life (*1*, *2*). These include microorganisms such as bacteria, fungi, algae, archaea, and protozoa, as well as metazoans such as mammals and *Drosophila melanogaster* (*3–6*). Despite this apparent universality, studying polyP in these diverse systems requires specific considerations, as polyP's chain lengths, functions, localization patterns, and metabolizing enzymes differ between organisms (*3*).

One microorganism that is frequently used to study polyP biology is the yeast, *Saccharomyces cerevisiae*. These single-celled eukaryotes store polyP within the vacuole, as the non-vacuolar accumulation of polyP is toxic (*7*). As such, the vacuolar storage of polyP is intrinsically linked with its synthesis, which is catalyzed by Vtc4 within the larger, multi-subunit VTC complex (*3*, *7*, *8*). These polyP stores can be mobilized through degradation by both endo- and exo- polyphosphatases, which cleave the internal and terminal bonds, respectively (*9*, *10*). This mobilization of polyP stores is important during cell cycle progression, DNA replication, and DNA damage repair, as the presence of polyP and its degrading enzymes contributes to the dNTP pool needed to facilitate these processes (*11*, *12*). PolyP is also instrumental in the cellular response to phosphate limitation in yeast, as polyP has been shown to act in a buffer system for both cytosolic Pi levels, and the sequential activation of phosphate starvation-response genes (*13*, *14*).

PolyP impacts countless other cellular functions in *S. cerevisiae*, and studying polyP in this model system can provide insights into polyP biology in mammalian cells. This translatable work is especially important since, while polyphosphatases have been identified in mammalian systems,

the enzyme(s) responsible for polyP synthesis *in vivo* in mammalian systems remains uncharacterized (*9*, *15*, *16*). One study demonstrated the ability of the F_1F_0 mitochondrial ATP synthase to synthesize polyP, however, the contribution of this activity to the *in vivo* polyP levels in various mammalian cell types remains unknown (*17*, *18*). Efforts to overcome the lack of a known mammalian polyP-synthase have used the expression of the polyP-synthesizing enzyme from *Escherichia coli* or the yeast exopolyphosphatase Ppx1 to analyze the effects of polyP accumulation or depletion, respectively, in metazoan cells (*6*, *12*, *19*). While these studies have been immensely informative, the identification of a polyP-synthesizing enzyme remains vital, particularly amongst the vast array of emerging polyP functions in mammalian cells such as promoting DNA damage repair (*12*), activating mTOR pathways (*20*), regulating blood clotting (*21*), and more (for a review, see (*15*) and references therein).

One prominent function of polyP across organisms is its ability to bind non-enzymatically to proteins, an activity recently defined as "polyP-binding", but formerly referred to as "polyphosphorylation" (*18*). The nature of polyP's binding to proteins has been heavily disputed in recent years, with different groups presenting contrasting findings that these interactions are either covalent or non-covalent (*22*, *23*). While the nature of this interaction does not retract from the importance of polyP-binding's functions, categorization as a non-covalent interaction would impact whether polyP-binding is classified as a post-translational modification (*18*). Screens for polyP-binding targets in both *S. cerevisiae* and *E. coli* have identified that polyP interacts with proteins involved in ribosome function and translation, hinting at a conserved role for polyP across both prokaryotes and eukaryotes (*24*, *25*). PolyP-binding has also been shown to target proteins involved in nuclear transport and vacuolar function in *S. cerevisiae* (*26*, *27*). Overall, polyP has

interesting roles across a range of systems, and understanding how this ubiquitous polymer exerts such a diverse array of functions is of interest to many researchers.

1.2. PolyP promotes survival and pathogenicity in bacteria

1.2.1. PolyP synthesis in bacteria

Bacteria are interesting systems to study polyP due the role it plays in survival and fitness. Bacteria synthesize polyP using polyphosphate kinase enzymes, PPK1 and/or PPK2, both of which are highly conserved throughout the bacterial kingdom (*28*, *29*). Some bacteria such as *E. coli* and *Salmonella typhi* possess only one PPK enzyme (PPK1), while other bacteria such as *Pseudomonas aeruginosa, Mycobacterium tuberculosis*, and *Myxococcus xanthus* possess both PPK1 and PPK2 (*28*). These enzymes use the terminal phosphate from nucleoside triphosphates to add to growing chains of polyP, each preferring different phospho-donors and generating different chain lengths (*28*, *30*). For example, in *P. aeruginosa*, the PPK1 enzyme uses exclusively ATP to generate long polyP chains, while PPK2 can use both ATP and GTP, and produces a wider range of chain lengths (*28*). Both PPK enzymes also possess nucleoside diphosphate kinase activity, generating nucleoside triphosphates from nucleoside diphosphates and polyP (e.g. making ATP from ADP) (*28*, *31*). While this reverse reaction has been shown in multiple bacteria *in vitro*, the contribution of this reverse reaction to the dynamics of polyP accumulation *in vivo* is yet to be investigated in depth (*28*, *31*, *32*). Deleting *ppk* genes inhibits polyP accumulation, which has allowed researchers to study the downstream effects of polyP by comparing wild-type and Δ*ppk* mutant phenotypes. Using this method, polyP has been found to impart resistance to a variety of stressors such as amino acid limitation (*33*), oxidative stress (*34*), heat stress (*35*), stationary phase growth (*36*), surface stress (*37*), and more. PolyP appears to promote resistance to this myriad of

stressors through multiple distinct mechanisms, many of which remain uncharacterized, implicating polyP in a complex network of regulatory processes across bacteria.

1.2.2. The mechanisms of polyP-mediated stress resistance

PolyP's ability to promote survival during amino acid starvation has been studied extensively, and the mechanisms by which this occurs are relatively well characterized in comparison to many other functions of polyP. During amino acid starvation, cellular responses direct the targeted degradation of proteins to release free amino acids, which promotes growth and survival (*33*, *38*). In *E. coli*, it was noted that Δ*ppk* mutants displayed a growth defect during amino acid starvation (*33*). The authors later found that polyP forms a complex with the Lon protease to facilitate the degradation of specific ribosomal proteins during growth in nutrient-limited media, presumably explaining the growth lag in polyP-deficient strains (*33*, *39*). The interaction between Lon and polyP also promotes the polyP-dependent proteolysis of DnaA, an outcome which inhibits DNA replication initiation during amino acid starvation and stationary phase growth (*3*, *40*). Interestingly, recent results have shown that polyP also inhibits DNA replication initiation during amino acid starvation by inhibiting the CobB deacetylase, leading to increased DnaA acetylation and decreased binding to the *oriC* replication initiation site (*41*). Together, these pathways likely all contribute to the polyP-dependent cellular adaptation to amino acid starvation and stationary phase growth.

In addition to modulating DNA replication and ribosomal subunit turnover during amino acid starvation, polyP has also been implicated in translational control in bacteria. Work from our lab identifying polyP-binding proteins in *E. coli* revealed that proteins involved in ribosome function were targets for polyP-binding, as were certain ribonucleases (*24*). These finding are complementary to one study which demonstrated that, during nitrogen starvation, polyP co-localizes with Hfq within cellular condensates that associate with the RNA degradosome (*24*, *42*).

These condensates promote the stability of transcripts involved in translational processes in a polyP-dependent manner, while also promoting the turnover of other diverse transcripts (*42*). Not just polyP, but PPK itself, has also been shown to associate with the RNA degradosome (*43*), emphasizing the involvement of polyP and its metabolizing enzymes in RNA stability. PolyP also binds to ribosomes, promoting both translational accuracy and inhibiting readthrough (*44*).

PolyP-binding is responsible for much more than just translational control, as polyP's interaction with proteins promotes resistance to both oxidative and heat stress (*34*, *45*). Several bacteria, such as *Mycobacterium smegmatis* (*37*), *Acinetobacter baumannii* (*35*), *Shigella flexerni, Salmonella typhimurium* (*46*), and *E. coli* (*34*, *45*) demonstrate sensitivity to oxidative or heat stress when *ppk* genes are deleted. However, mechanistic insights into how polyP is enacting this function, as is the case for much of bacterial polyP research, is primarily characterized in *E. coli*. PolyP can act as a protein chaperone to prevent protein aggregation during oxidative stress, as one study has shown that polyP-deficient mutants contained more insoluble proteins and displayed a higher induction of heat-shock response genes, while polyP was able to prevent protein aggregation *in vitro* (*34*). PolyP has also been shown to bind specifically to unfolding proteins during heat stress, keeping them in a soluble state that is primed for refolding (*45*). This function does not appear to be exclusive to proteins, since polyP has also been shown to act as a chelator of metal ions *in vivo*, with one study showing that polyP can sequester iron to inhibit the Fenton reaction and reduce cisplatin toxicity (*47*).

1.2.3. PolyP and PPK: promising targets to treat bacterial infections

Aside from countering various stressors, polyP also plays important roles in pathogenicity. Firstly, polyP has been shown to promote biofilm formation in several bacteria. Deleting *ppk* genes in *P. aeruginosa* (*48*) and *E. coli* (*49*) reduces biofilm formation, while one conflicting report in

Campylobacter jejuni found that Δ*ppk* mutants had improved biofilm formation (*50*). The same study also found that deleting *ppk* did not impact motility, while the presence of PPK has been shown to promote motility in *A. baumannii* and *P. aeruginosa* (*35*, *51*). Generally, polyP and PPK have been shown to promote virulence in several human pathogens including *E. coli* (*52*, *53*), *P. aeruginosa* (*48*), *M. tuberculosis* (*54*), *A. baumannii* (*35*), *C. jejuni* (*50*), *Klebsiella pneumoniae* (*55*) and more. While it is often difficult to distinguish between the effects of polyP and PPK in promoting virulence, some notable studies have used exogenously administered polyP to address this caveat. One study showed that long-chain polyP, rather than short-chain polyP, inhibits the expression of interferon-regulated genes, as well as the ability of macrophages to facilitate an appropriate inflammatory response (*53*). These results suggest not only that mammalian cells are affected specifically by long-chain microbial polyP, but also that *E. coli* polyP may exert some of its activity by being externalized; a process that has not yet been described in this bacterium (*53*, *56*). However, this effect was described in another study, which demonstrated the externalization of polyP in *M. smegmatis*, and the subsequent inhibitory effect of polyP on bacterial digestion by amoebas and macrophages (*56*).

PolyP's role in both virulence and survival during stress make PPK and polyP intriguing targets for novel antimicrobial treatments (*57*). As such, several groups have identified compounds that successfully inhibit PPK to interfere with pathogenic determinants. One group discovered that an ulcerative colitis-treating drug, mesalamine, inhibits PPK in *P. aeruginosa, E. coli*, and *Vibrio cholerae* (*49*). The inhibition of PPK by mesalamine was shown to decrease biofilm formation, stress resistance, and colonization of *Caenorhabditis elegans* (*49*). Additional screens for compounds that inhibit PPK have identified gallein and raloxifene as effective inhibitors of polyP accumulation and virulence, with gallein inhibiting both PPK1 and PPK2 enzymes in *P.*

aeruginosa (*54*, *58*). Inhibiting PPK can also increase susceptibility to existing antimicrobials, as our lab has shown that deleting *ppk* in polymyxin B-resistant *E. coli* restores susceptibility due to the downregulation of lipid A modifications (*59*). As a whole, polyP contributes to several stress-resistance mechanisms in various bacteria, and its additional role in pathogenicity underscores the importance of understanding the regulatory pathways both upstream and downstream of polyP accumulation.

1.3. PolyP metabolism in *E. coli*

1.3.1. PolyP synthesis and degradation in *E. coli*

The enzyme responsible for synthesizing polyP in *E. coli* is PPK (*30*), which shares homology with PPK1 enzymes (*28*, *29*), and uses ATP as the preferred substrate to generate polyP (*28*, *30*). In this reaction, PPK uses the terminal phosphate from ATP to autophosphorylate on a highly conserved histidine residue before commencing polyP synthesis (*30*, *60*, *61*). This phosphate group is then incorporated into polyP, and PPK proceeds to extend the chains to approximately 700-800 Pi residues in length (*28*, *60*). PPK does this in a processive manner, and is less competent at extending existing polyP chains (*60*, *62*). Studies conducted *in vitro* analyzing the oligomeric state of active PPK were consistent with the later-solved crystal structure, suggesting that PPK synthesizes polyP as a homodimer (*29*, *61*). However, the modulation of PPK's oligomeric state as a regulatory mechanism *in vivo* is yet to be thoroughly investigated. *In vitro* studies also show that *E. coli* PPK can catalyze the reverse reaction, synthesizing ATP from ADP and the terminal phosphate from polyP chains (*31*, *32*). However, evidence of the significance of this reaction *in vivo* is scarce.

On the contrary, one enzyme shown to facilitate polyP degradation *in vivo* is exopolyphosphatase (PPX), encoded immediately downstream of *ppk* under the control of the

same promoter (*34*, *63*). This enzyme hydrolyses the terminal phosphates from polyP chains to generate monomeric Pi (*63*). This polyP-hydrolysis is preferential of long chains and is also highly processive, almost exclusively generating orthophosphate as opposed to a range of smaller polyP chains (*63*, *64*). Selective polyP-degradation by PPX was once thought to be responsible for polyP accumulation during stress, after one group found that the stringent response signalling molecule(s), guanosine 5'-triphosphate 3'-diphosphate and guanosine 5'-diphosphate 3'-diphosphate (referred to as (p)ppGpp), could inhibit PPX and stimulate polyP accumulation *in vitro* (*65*). These results were later complemented by observations that exogenously expressing RelA, which is responsible for synthesizing (p)ppGpp, led to an increase in polyP accumulation (*66*). Many years later, Michael Gray published work revealing that results obtained using (p)ppGpp-deficient mutants were likely confounded by mutations in RNA polymerase subunits that commonly arise in these strains (*67*). He used these findings to support the idea that polyP accumulation is the result of PPK activity, rather than PPX inhibition, which he emphasized by showing that Δ*ppx* mutants do not accumulate polyP in the absence of stress (*67*).

1.3.2. The mechanism of PPK activation in response to stress is uncharacterized

Recent works have therefore focused on understanding how PPK regulates polyP accumulation in *E. coli*. PolyP accumulation in *E. coli* is stress-dependent, as there is no detectable polyP accumulation in the absence of stress (*67*). Stressors found to induce polyP accumulation seem to vary across studies, but some of these stressors include the limitation of specific nutrients such as phosphates or amino acids (*66*, *68*), osmotic stress (*42*, *68*), and oxidative stress (*34*). Considering the dependence of polyP accumulation on cellular stress, it is therefore puzzling that this sudden stress-induced accumulation of polyP is not accompanied by a similar increase in PPK expression, measured by both transcript levels and promoter activity (*67*, *69*). PPK isolated from the lysates

of cells starved for amino acids showed no significant differences in its ability to synthesize polyP from ATP *in vitro* (*65*). Additionally, PPK's *in vitro* activity was not notably different when isolated from either wild-type cells, or Δ*phoB* mutants that contain almost no polyP (*66*). This phenomenon does not appear to be consistent across bacteria, as both PPK transcript and protein levels have been reported to change in response to certain stressors and genetic mutations in other bacteria (*37*, *70*, *71*). One study aiming to address this question used random mutagenesis to isolate PPK variants (referred to as *ppk**) that accumulated increased polyP during nutrient starvation, but also synthesized polyP constitutively (*72*). Several of these *ppk** point mutations were found to potentially interfere with the dimeric interface of the enzyme, and a follow-up study showed that both oligomerization and activity were impacted by some, but not all, mutants *in vitro* (*72*, *73*). Based on these findings, the authors proposed that oligomerization or small molecules present only *in vivo* could be responsible for the regulation of PPK activity, however, more work is needed to establish a potential mechanism.

While the mechanism of PPK activation remains cryptic, many advances have been made in determining what signalling pathways or genes could be contributing to polyP metabolism during stress. Characterizing these polyP-regulators has the potential to provide insights into the mechanisms of both PPK and PPX activity. For example, the discovery of an RNA polymerase binding protein, DksA, as a polyP-regulator led authors to identify differences in transcript length from the *ppk* promoter between stress and non-stress conditions (*69*). While these differences in transcript length did not correlate with the increase in polyP accumulation at the onset of stress or with the decreased polyP observed in Δ*dksA* mutants, this experiment highlighted the differential regulation of *ppk* and *ppx* transcripts before and during stress (*69*). Alternatively, characterizing polyP-regulators can also highlight its functions. One prominent example of this is the discovery

that deleting *phoB*, the transcriptional regulator of the Pho regulon (*74*, *75*), causes polyP levels to drop drastically under low phosphate conditions, suggesting that activation of the Pho regulon is required for polyP accumulation (*66*). Coupled with reports that the overexpression of the phosphate exporter YjbB inhibits polyP accumulation, it has become clear that polyP plays an integral role in phosphate homeostasis in *E. coli* (*76*). Similarly, the identification of nitrogen-responsive proteins, GlnG and RpoN, as polyP-regulators emphasizes that polyP is involved in the cellular response to nitrogen limitation (*77*). All in all, discovering and characterizing regulators of polyP provides a promising strategy to investigate PPK activation and clarify the relationship between polyP and environmental stress.

1.4. Bacterial two-component systems regulate a broad array of cellular functions

1.4.1. Understanding two-component system signalling

Two-component systems (TCS) are one of the most prevalent groups of signalling proteins in prokaryotes, and their presence is relatively ubiquitous across bacterial taxa (*78*, *79*). While the number of TCS in each bacteria can range from tens to hundreds, the amount of TCS genes within a single bacterium seems to correlate with both the size of its genome, and the variability of its native environment (*78*, *79*). These systems were first identified through the observation that the nitrogen regulatory proteins in *Bradyrhizobium parasponiae*, NtrC and NtrB, shared distinct homology with signalling proteins with similar signal transduction mechanisms across various bacteria (*80*). This group therefore proposed the concept of TCS, where the first protein detects an environmental cue, and signals this to the second protein to activate a response (*80*). Since this initial description, the mechanism and structure of these systems have revealed to be far more complex. Members of this signalling family can be identified through sequence homology and the presence of distinct domains, as was done in *E. coli* in 1997 (*81*). The first component, known as

a sensor histidine kinase (HK), possesses a transmitter (or autokinase) domain, which contains a highly conserved histidine residue in a dimerization and histidine phosphotransfer (DHp) domain, as well as a catalytic domain (*81*, *82*). The second component is the response regulator (RR), which contains a receiver domain carrying a highly conserved aspartic acid residue, and an effector domain (*81*, *83*).

In line with their highly conserved structure, these systems also facilitate a well-documented series of signalling events. Typically, environmental signals or stressors are first detected by the HK, which will autophosphorylate on the highly conserved histidine residue using ATP as a substrate (*84*, *85*). This phosphate group is then transferred to the RR on its aspartic acid residue, which typically prompts the RR to either trigger a signalling cascade, or bind directly to DNA and act as a transcriptional regulator (*83*). While this is an example of a typical TCS, there are many instances of variation, particularly surrounding hybrid HKs which undergo several phospho-transfer events between domains before transferring the phosphate group to an RR (*81*, *86*, *87*). Another source of intricacy within these systems is the inherent phosphatase activity of numerous HKs. While often demonstrated *in vitro*, it is common for HKs to be able to dephosphorylate their cognate RRs in the absence of system-inducing cues, resulting in a balance between these kinase and phosphatase activities (*84*, *88*, *89*). Layering on another level of complexity, many systems display cross-regulation, with certain HK's demonstrating kinase activity towards the RR's of different systems (*85*).

TCS are involved in most aspects of bacterial life, such as the regulation of central metabolism, nutrient and ion homeostasis, motility, resistance to small molecules and antimicrobials, and more (*90–92*). The sizable impact of TCS on bacterial physiology, in addition to their predicable domain structure and signalling events, have made TCS targets for

bioengineering efforts in both the health and environmental sectors (*92*). Interest in these systems has warranted the creation of databases providing a comprehensive list of all TCS across a wide variety of bacteria, such as the "Prokaryotic 2-Component Systems" (P2CS) database (*93*, *94*). In *E. coli*, libraries of TCS deletion mutants have been subject to transcriptome analyses (*91*), phenotype microarrays (*90*), and infection assays (*95*) to broadly characterize the scope of TCS signalling in this bacterium. Such analyses have contributed to the growing understanding of the importance of these systems to prokaryotic physiology, and importantly, pathogenicity (*90*, *91*, *95*).

1.4.2. Existing connections between TCS and polyP prompt further investigation

Given their wide scope across most cellular processes in bacteria, it is unsurprising that certain TCS are involved in polyP biology. A notable example of this is the regulation of polyP accumulation by the PhoBR TCS in *E. coli,* but also by phosphate-regulating TCS in other bacteria (*66*, *70*, *96*). The PhoBR system appears to be essential for polyP accumulation in *E. coli* since, as previously mentioned, cells are almost completely devoid of polyP when *phoB* is deleted (*66*). In *M. tuberculosis* on the other hand*, ppk* transcription was shown to be regulated by the RR RegX3 during phosphate limitation through direct binding to its promoter (*70*). Another example of polyP-regulation by TCS involves the NtrCB system, as deleting the RR NtrC (*glnG*) causes polyP levels to decrease, but this effect appears to be dependent on the media composition and nitrogen availability (*67*, *68*, *77*). Equally interesting is the regulation of TCS by polyP, which has been demonstrated for multiple systems. PolyP has been shown to promote the expression of the BasRS system, which contributes to the expression of proteins responsible for generating lipid A modifications in *E. coli* (*59*). PolyP has also been shown to regulate the MprAB system in *M. smegmatis*, possibly through direct phosphorylation of MprB (*37*).

1.5. Project hypotheses and aims

As discussed, one critical open question surrounding *E. coli* polyP metabolism is as follows: What is triggering PPK activation, and subsequent polyP synthesis, exclusively at the onset of stress? Investigations aiming to address this question have targeted polyP, as polyP-regulating genes can provide insights into both possible levels of regulation of PPK, as well as the cellular pathways contributing to polyP accumulation. While several known polyP-regulators are members of TCS, no comprehensive analysis of the relationship between polyP and TCS has been conducted to date. Such analyses could be particularly insightful given the stress-responsive nature of both TCS and polyP. Additionally, while it is known that polyP functions to combat various stressors in bacteria, investigating the possible effects of diverse TCS on polyP metabolism could provide important knowledge surrounding how polyP responds to changes in the environment and within the cell.

I therefore hypothesized that polyP-regulators could be found amongst TCS genes, and that identified regulators could be further leveraged to improve our understanding of PPK activation. To investigate this, I conceptualized three aims:

Aim 1: Conduct a screen of all TCS genes in *E. coli* in search of mutants that accumulate altered polyP levels.

Aim 2: Further characterize the mechanisms of polyP-regulation by TCS.

Aim 3: Use polyP-regulating mutants to investigate the mechanism of PPK activation in response to cellular stress.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and growth conditions

2.1.1. Strain and plasmid construction

Unless otherwise specified, the strain background used throughout this work is *E. coli* MG1655 (wildtype and Δ*ppk* strains received from Michael Gray). Strains originating from the Keio knockout collection (Horizon Discovery) (*97*) are from the *E. coli* strain background BW25113, and strains generated in this background within this work originate from the BW25113 Keio parent strain (Horizon Discovery).

All deletions generated in this work were created using the λ red recombinase system described by Datsenko and Wanner in 2000 (*98*). In brief, inserts containing a kanamycin resistance gene surrounded on both sides by FLP recognition target (FRT) sites were amplified from the pKD4 plasmid with primers containing homology to the genomic region of interest. Strains to be modified were transformed beforehand to contain pSIM6 for the inducible expression of the λ red genes at 42˚C for 12.5 minutes (*99*). Following growth and induction, cells were made electrocompetent, treated with the appropriate insert, and later plated on LB supplemented with kanamycin to select for mutants. All deletions were verified by PCR to assure absence of the open reading frame and insertion of the kanamycin resistance cassette at the correct location. Where relevant, the kanamycin resistance gene was removed using the FLP-recombinase activity of the pCP20 plasmid (*100*). Tagged strains were generated using the same method, but the insert was amplified from the pSUB11 plasmid containing a 3Flag epitope tag followed by the kanamycin resistance gene (*101*). Tagged strains were verified by PCR for the presence of the kanamycin resistance cassette at the correct location. All strains used in this work are described in **Table S1.**

Plasmids created for this work were cloned using the Gibson Assembly Cloning Kit (New England Biolabs). Both pCpxA and pNlpE vectors contain the *cpxA* and *nlpE* open reading frames, respectively, cloned into a pBAD18 backbone under the arabinose-inducible pBAD promoter (*102*).

2.1.2. Growth conditions and nutrient downshift

Prior to growth experiments, cells were grown on LB agar plates and in LB overnight cultures supplemented with the appropriate antibiotic. Nutrient limitation was conducted using MOPS minimal media (Teknova) supplemented with 0.4% glucose (or 0.5% glycerol in strains containing pBAD18-based vectors), 0.1 mM K_2HPO_4, and the appropriate antibiotic. Antibiotic concentrations are as follows: kanamycin 50 μg/mL, ampicillin 100 μg/mL.

When growing cells for polyP detection or western blotting, the optical density at a wavelength of 600 nm (OD_{600}) of liquid overnight cultures was taken using a spectrophotometer. Cultures were then diluted to an OD_{600} of 0.1 in fresh LB and grown at 37ºC with shaking (~210 rpm) until mid-exponential phase (~0.6-0.8 OD_{600}). Cells were pelleted and washed one or two times with 1xPBS prior to being transferred to an equal volume of MOPS minimal media. Cells were then grown in MOPS at 37ºC with shaking (~210 rpm) for 3 or 4 hours before an equal amount of cells was harvested from each sample, and pellets were frozen at -80˚C.

2.2. PolyP detection

2.2.1. PolyP extraction

PolyP was extracted using a modified version of the phenol-chloroform extraction protocol developed by Bru and colleagues in 2016 (*103*), which has been described in (*59*). This protocol is written with similar wording to (*59*) to fully communicate the utilized methods.

Starting with 5 OD_{600} equivalents of frozen cells, cell pellets were resuspended in LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.4, 0.2% SDS) for cell lysis and immediately transferred to a solution of 600 µL neutral phenol and 150 µL sterile water. Samples were mixed by vortexing for 20 seconds and then heated at 65ºC for 5 minutes before being incubated on ice for 1 minute. 600 µL of chloroform was added, samples were mixed by vortexing for 20 seconds, and phases were separated by centrifugation for 2 minutes at 13, 000 g. The top phase was carefully transferred to new tubes already containing 600 µL of chloroform, and phases were separated once again as described. The top phase was then carefully transferred to clean tubes, and each sample was treated with 2 µL of 2 U/µL DNAse I (Thermo Fisher Scientific) and 2 µL of 10 mg/mL RNAse A (Bio Basic) and incubated at 37˚C for 1 hour. PolyP was precipitated for 3 hours or overnight at -20ºC in 1 mL of 100% ethanol and 40 µL of 3 M sodium acetate, and then pelleted by centrifugation for 20 minutes at 13, 000 g (4ºC). Pellets were washed once with 70% ethanol and centrifuged for 5 minutes at 13,000 g (4ºC) before being resuspended in 30 µL H_2O. PolyP extracts were preserved at -80ºC.

2.2.2. PolyP detection by gel electrophoresis

PolyP extracts were visualized by gel electrophoresis using 15.8% TBE-urea gels (5.25 g urea, 7.9 mL 30% acrylamide, 3 mL 5xTBE, 150 µL 10% APS, 15 µL TEMED). Extracts were mixed in a 1:1 ratio with loading dye (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 30% glycerol, bromophenol blue) and loaded alongside polyP chain length standards (Wako Chemicals USA) of 60 (6.5 mM) and 130 (1.25 mM) Pi residues in length before the gel was run at 100V for 1 hour and 45 minutes in 1xTBE buffer. The gel was then stained in staining solution (25% methanol, 5% glycerol, and 0.05% toluidine blue O) for 15-25 minutes to visualize polyP. The gel was destained using the

same solution without toluidine blue O. Gels were scanned and images were cropped for the construction of figures. Changes in brightness, if any, were applied equally across the image.

2.2.3. PolyP quantification

PolyP was quantified using the Phosphinity Total Polyphosphate Quantification Kit (Aminoverse) (*104*). *E. coli* polyP extracts were diluted by a factor of 15-40 in nuclease-free water prior to usage in the assay. In brief, the kit uses a purified *S. cerevisiae* Ppx1 enzyme to specifically hydrolyze polyP to orthophosphate. Pi is then quantified in a colorimetric assay where Pi-molybdate complexes react with ascorbic acid, turning the solution varying shades of blue (*104*). Absorbance of samples in a clear 96-well plate were read at 882 nm using a BioTek Synergy H1 Multi-mode plate reader (Agilent). Standards of 20 µM, 65 µM, 110 µM, 155 µM, and 200 µM Pi were used to create a calibration curve to calculate absorbance as a measure of Pi. Extracts not treated with the enzyme were used as blanks for residual Pi in the sample. PolyP levels were reported as µM Pi, calculated as follows (*104*):

$$\text{PolyP (µM Pi)} = \big((\text{Pi from treated sample}) - (\text{Pi from blank sample})\big)$$

Quantifications were conducted for at least three biological replicates of an experiment as one singular assay to ensure consistent performance of reagents.

2.3. Western blotting

2.3.1. Protein preparation

Western blotting was conducted using the same methods described in (*59*) and (*24*), and is therefore explained similarly below. First, 3 OD_{600} equivalents of cells were resuspended in 100 µL of a sample buffer solution containing 800 µL of 3x sample buffer (240 mM Tris-HCl pH 6.8, 30% glycerol, 6% SDS, bromophenol blue), 100 µL DTT, and 100 µL Tris-HCl pH 8.8 (1.5 M).

Once resuspended, samples were boiled at 100˚C for 10 minutes before being centrifuged at max speed for 2 minutes. Supernatants were transferred to clean tubes and stored at -20˚C.

2.3.2. Gel electrophoresis and western blotting

Samples were run via SDS-PAGE using 10-15% acrylamide gels. Samples were then transferred to a PVDF membrane and kept in TBST. Membranes were blocked with 5% powdered milk in TBST, and then probed with either αFlag-HRP (Sigma-Aldrich), or αZipA (*105*) followed by incubation with an anti-rabbit secondary antibody. Membranes were washed in TBST before being treated with a 1:1 solution of Immobilon HRP substrate peroxide solution and luminol reagent (Millipore). Membranes were developed using autoradiography film and a Konica SRX-101A film developer. Membranes were stained with Ponceau S to ensure equal protein loading. Films and membranes stained with Ponceau S were scanned and cropped for figure construction. Changes in brightness, if any, were applied equally across the image.

2.4. Fractionation assays

Fractionation analyses were carried out using a modified version of the protocols described in Malherbe et al., 2019 (*106*). Cells were grown under the indicated conditions and 8 OD_{600} equivalents of cells were pelleted by centrifugation at 4ºC and 4200 rpm for 15 minutes. At this point, cells could be frozen at -80ºC. To first extract the periplasm by cold osmotic shock, cell pellets were resuspended in 850 µL of 1xPBS and centrifuged at 20,627- 20,800 g for 3 minutes at 4ºC. All subsequent steps were performed on ice, and centrifugations at 4ºC. The supernatants were discarded and pellets were resuspended in 900 µL of buffer #1 (100 mM Tris-HCl pH 8.0, 500 mM sucrose, 0.5 mM EDTA pH 8.0, 1x protease inhibitor cocktail tablet (Sigma-Aldrich)) and incubated on ice for ~5 minutes before being centrifuged again with the same settings. Supernatants were discarded again, pellets were resuspended in 400 µL of a solution of 1 mM

$MgCl_2$ in sterile water, and then incubated on ice for 2 minutes. Samples were centrifuged again, and the resulting supernatants were transferred to clean tubes and kept at -20ºC as the periplasmic fraction. The remaining pellets were kept at -20ºC as the cytoplasmic and insoluble/membrane fractions.

To separate the cytoplasmic and insoluble fractions, the pellets were first resuspended in 900 µL of buffer #2 (50 mM Tris-HCl pH 8.0, 250 mM sucrose, 10 mM $MgSO_4$, 1x protease inhibitor cocktail tablet). 0.9 µL of a 235 µg/µL lysozyme solution was added, and each sample was diluted in 900 µL of sterile water. Samples were transferred to screw-capped tubes and incubated on ice for 5 minutes. Samples were then centrifuged using the previously described settings, and pellets were resuspended in 1.2 mL of 10 mM Tris-HCl pH 8.0. Using a cannister of liquid nitrogen and a water bath heated to 37ºC, samples were then freeze-thaw cycled as follows to disrupt cells: 2 minutes in liquid nitrogen, 3 minutes at 37ºC, repeated 4 times. Samples were then placed back on ice and treated with 3.2 µL of 2 U/µL DNAse I (Thermo Fisher Scientific), 3.2 µL of 10 mg/mL RNase A (Bio Basic), and 20 µL of 1 M $MgSO_4$, and then incubated on ice for 10 minutes. Samples were then centrifuged for 25 minutes using the described settings, and the supernatants were carefully transferred to clean tubes and kept at -20ºC as the cytoplasmic fraction. Samples were centrifuged again briefly, and excess supernatant was discarded to prevent contamination between fractions. The resulting pellet was resuspended in 1 mL of buffer #3 (50 mM Tris-HCl pH 8.0, 2.5 mM EDTA pH 8.0, 1x protease inhibitor cocktail tablet) and kept at -20ºC as the insoluble/membrane fraction.

Fractionated protein samples were mixed in a 1:1 ratio with 2x sample buffer (as described in 2.3.1. protein preparation) and loaded directly onto an SDS polyacrylamide gel for western blot analysis (see section 2.3).

2.5. Statistical analyses

Statistical analyses were carried out for polyP quantification assays in Figures 3B and 4B using the GraphPad Prism10 software. Means for at least three biological replicates of each strain were compared using a one-way ANOVA followed by a post-hoc Tukey's multiple comparisons test to determine significant differences between groups.

3. RESULTS

3.1. Conducting a targeted genetic screen for novel regulators of polyP

3.1.1. The Keio collection is not a suitable tool for analyzing polyP levels

To discover polyP-regulators, we conducted a screen of single-gene deletion mutants in search of mutants that accumulated altered polyP levels. During this screen, mutants were grown alongside wild-type *E. coli* in nutrient-rich LB media until mid-exponential phase before being shifted to nutrient-poor MOPS minimal media (supplemented with 0.4% glucose and 0.1 mM K_2HPO_4 unless specified otherwise) to induce polyP accumulation (*68*). After 3 hours, polyP was extracted and visualized by gel electrophoresis (**Figure 1**). Each mutant was tested in at least two independent experiments, and mutants that reproducibly accumulated altered levels of polyP compared to the wildtype were classified as "hits." While quantifying polyP levels is possible, visualizing differences using PAGE provides a criteria for mutants warranting further investigation.

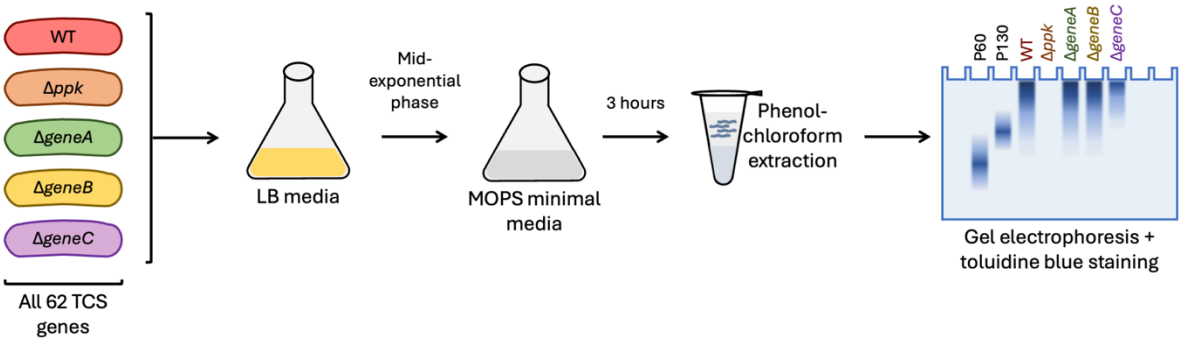

Figure 1: Workflow while screening for regulators of polyP. Schematic depicting the experimental workflow used while conducting the screen for polyP-regulators.

To increase the capacity of the screen, we sought to take advantage of a commercially available collection of 3985 *E. coli* single-gene knockout strains (supplied in duplicate) called the

Keio knockout collection (*97*). Upon commencing the screen using this collection set, we were immediately concerned by the high quantity of hits that were arising, as well as differences between different isolates of the same deletion (**Figure 2A**). To investigate this, we recreated four of the initial hits (Δ*htpG,* Δ*cpxA*, Δ*hslO*, and Δ*rcsC*) in both MG1655 and BW25113 backgrounds. We observed that across both backgrounds only one mutant from this selection, Δ*cpxA*, had reproducible changes in polyP levels compared to the wildtype (**Figure 2B**). These results led us to conclude that the Keio collection was not a suitable tool to conduct this screen. Going forward, all mutants used in this screen were created in the lab.

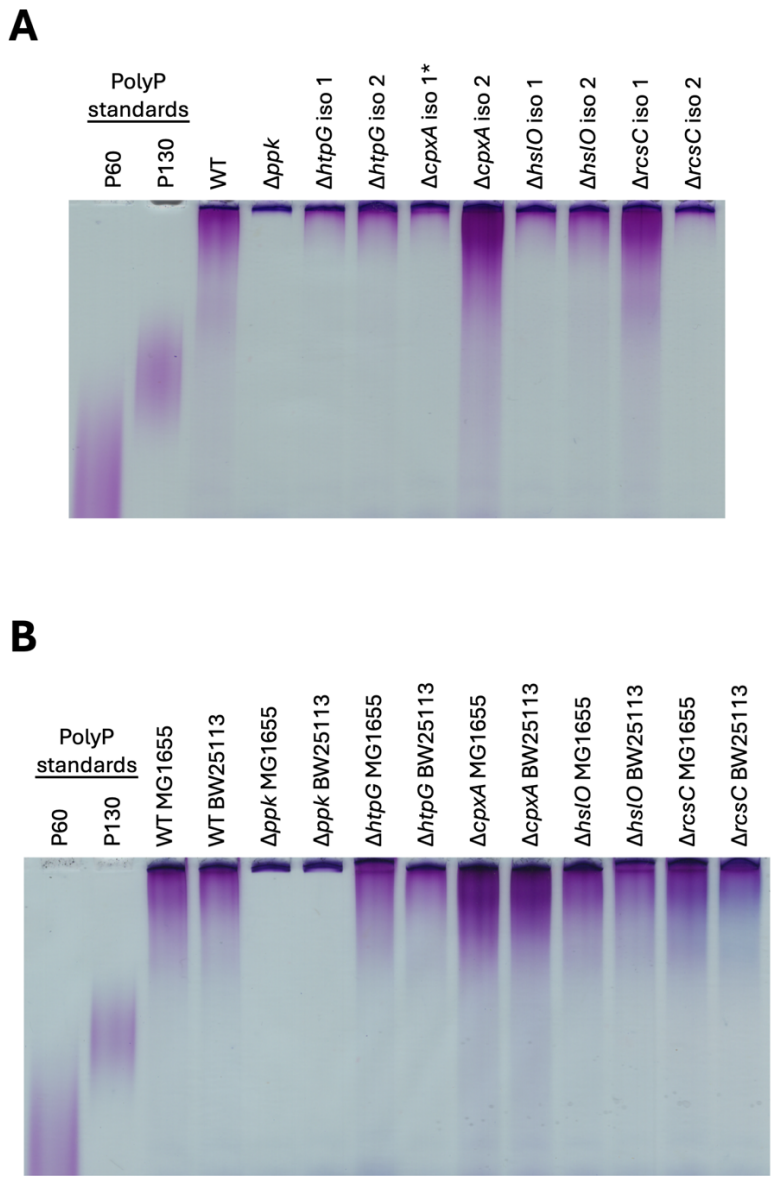

Figure 2: The Keio collection is not a suitable tool for analyzing polyP accumulation. A) Two isolates of four different deletions from the Keio knockout collection were grown in LB alongside wild-type and Δ*ppk* mutants (*E.* coli BW25113) until mid-exponential phase before being switched into MOPS minimal media. After 3 hours, cells were harvested and polyP was extracted by phenol-chloroform extraction. Isolated polyP was visualized by gel electrophoresis on a 15.8% TBE-urea polyacrylamide gel followed by staining with toluidine blue. PolyP standards of 60 and 130 Pi residues in length were included. * Whole genome sequencing later revealed that this is not a Δ*cpxA* mutant. minimum n=2. B) Mutants from the Keio collection were re-created in two different *E. coli* K-12 strain backgrounds (MG1655 and BW25113) and grown alongside wild-type and Δ*ppk* mutants from each strain background. Cells were grown and polyP was extracted using the same protocol as described for panel A. minimum n=2.

3.1.2. A targeted screen of all *E. coli* TCS genes identifies 5 polyP-regulators

There are 62 TCS genes in *E. coli* (*81*, *93*), all of which were individually deleted and screened for polyP accumulation following a nutrient downshift into MOPS minimal media. A comprehensive summary of the findings are presented in **Table 1**. Overall, the screen identified 5 polyP-regulators: *cpxA, arcB, arcA, phoR,* and *phoB*. The former three genes, to my knowledge, have never been reported in the literature as regulators of polyP in *E. coli*. On the other hand, Δ*phoR* and Δ*phoB* mutants have been previously shown to have increased and decreased polyP levels, respectively (*66*). In addition to the recapitulation of Δ*phoR* and Δ*phoB* phenotypes, this screen shows general consistency with results obtained by several groups over recent years. For example, the lack of polyP regulation by NtrC (*glnG*) following a nutrient downshift to MOPS was shown by Gray in 2019, but was contradicted by a later study that revealed a decrease in polyP accumulation in Δ*glnG* mutants that was subsequently rescued when glutamine was administered prior to the nutrient downshift (*77*). The same study also found no polyP regulation by Δ*atoC* and Δ*zraR* mutants, which is consistent with our findings. Comparisons between the results of this screen and previous works looking at the effects of specific TCS genes on polyP accumulation will be dissected in the discussion.

Table 1: TCS genes screened for polyP accumulation.

Sensor Histidine Kinases				**Response Regulators**			
Deletion (Δ)	**Uniprot ID**	**Hit?**	**PolyP phenotype**	**Deletion (Δ)**	**Uniprot ID**	**Hit?**	**PolyP phenotype**
arcB	P0AEC3	Yes	Decrease	*arcA*	P0A9Q1	Yes	Decrease
atoS	Q06067	No	N/A	*atoC*	Q06065	No	N/A
baeS	P30847	No	N/A	*baeR*	P69228	No	N/A
barA	P0AEC5	No	N/A	*basR*	P30843	No	N/A
basS	P30844	No	N/A	*btsR*	P0AFT5	No	N/A
btsS	P0AD14	No	N/A	*cheB*	P07330	No	N/A
cheA	P07363	No	N/A	*cheY*	P0AE67	No	N/A

cpxA	P0AE82	Yes	Increase	*cpxR*	P0AE88	No	N/A
creC	P08401	No	N/A	*creB*	P08368	No	N/A
cusS	P77485	No	N/A	*cusR*	P0ACZ8	No	N/A
dcuS	P0AEC8	No	N/A	*dcuR*	P0AD01	No	N/A
dpiB	P77510	No	N/A	*dpiA*	P0AEF4	No	N/A
envZ	P0AEJ4	No	N/A	*evgA*	P0ACZ4	No	N/A
evgS	P30855	No	N/A	*fimZ*	P0AEL8	No	N/A
glnL	P0AFB5	No	N/A	*glnG*	P0AFB8	No	N/A
glrK	P52101	No	N/A	*glrR*	P0AFU4	No	N/A
hprS	P76339	No	N/A	*hprR*	P76340	No	N/A
kdpD	P21865	No	N/A	*kdpE*	P21866	No	N/A
narQ	P27896	No	N/A	*narL*	P0AF28	No	N/A
narX	P0AFA2	No	N/A	*narP*	P31802	No	N/A
phoQ	P23837	No	N/A	*ompR*	P0AA16	No	N/A
phoR	P08400	Yes	Increase	*phoB*	P0AFJ5	Yes	Decrease
qseC	P40719	No	N/A	*phoP*	P23836	No	N/A
rcsC	P0DMC5	No	N/A	*qseB*	P52076	No	N/A
rcsD	P39838	No	N/A	*rcsB*	P0DMC7	No	N/A
rstB	P18392	No	N/A	*rssB*	P0AEV1	No	N/A
torS	P39453	No	N/A	*rstA*	P52108	No	N/A
uhpB	P09835	No	N/A	*torR*	P38684	No	N/A
ypdA	P0AA93	No	N/A	*uhpA*	P0AGA6	No	N/A
zraS	P14377	No	N/A	*uvrY*	P0AED5	No	N/A
				ypdB	P0AE39	No	N/A
				zraR	P14375	No	N/A

3.2. The regulation of polyP by the CpxRA system

3.2.1. The envelope stress-responsive HK, CpxA, regulates polyP through a CpxR-dependent mechanism

We found that Δ*cpxA* mutants had increased polyP accumulation in two different *E. coli* K-12 strain backgrounds (**Figure 2B**). In MG1655, this was demonstrated by both visualization on a gel, and by using an enzymatic polyP quantification assay (**Figure 3B**). We were able to rescue this phenotype by exogenously expressing CpxA from an arabinose-inducible promoter (pCpxA)

in Δ*cpxA* mutants, indicating that CpxA itself is responsible for the observed changes in polyP accumulation (**Figure 3A**). However, expressing CpxA in wild-type cells did not reverse the phenotype to decrease polyP levels (**Figure 3A**).

CpxA is the HK from the CpxRA system, and it autophosphorylates in response to various signals including alkaline pH (*107*), alcohols (*108*), increased copper concentrations (*109*), treatment with certain antimicrobial peptides (*110*), and protein aggregation or mislocalization in the periplasm (*111*). Therefore, the CpxRA TCS is considered to be an envelope stress-responsive system, as system-inducing signals are largely thought to contribute to envelope stress (*109*, *111–113*). Once autophosphorylated, CpxA then transfers this phosphate group to its RR, CpxR, activating the system (*89*). Phospho-CpxR acts as a transcriptional regulator, controlling the expression of genes encoding for various chaperones and proteases, metabolism-related proteins, proteins involved in the acid stress response, membrane proteins, motility-related proteins, and more (*110*, *114–116*).

Interestingly, when we deleted *cpxR*, we did not observe an increase in polyP levels (**Figure 3B)**. This suggested that the canonical function of this TCS is not repressing polyP accumulation, but rather that an alternate function of CpxA is likely responsible for the observed phenotype. Interestingly, in the absence of activating signals, CpxA can act as a CpxR-phosphatase rather than a kinase (*89*). The current understanding of this mechanism is that CpxA's phosphatase activity keeps CpxR in its inactive form to counteract phosphorylation by the metabolic intermediate, acetyl phosphate (AcP) (*89*, *107*, *117*). In fact, Δ*cpxA* mutants grown in glucose-supplemented media display an increase in both CpxR phosphorylation and system activation (*107*, *117*, *118*). We therefore suspected that this mechanism was responsible for the pattern of polyP regulation observed in Δ*cpxA* and Δ*cpxR* mutants grown in MOPS media. To investigate this, we

conducted an epistasis experiment where we visualized polyP levels in a mutant strain lacking both *cpxA* and *cpxR*. As observed in **Figure 3B**, Δ*cpxA* Δ*cpxR* double mutants accumulated a similar level of polyP to the wildtype, and significantly less polyP than Δ*cpxA* single mutants. This indicates that CpxA regulates polyP through a CpxR-dependent mechanism.

This result sparked the emergence of a working model where Δ*cpxA* mutants promote polyP accumulation by activating the CpxRA system (**Figure 3C**). In this model, the conditions in MOPS media would promote CpxA-independent CpxR phosphorylation, which would normally be counteracted by CpxA's phosphatase activity. However, in Δ*cpxA* mutants, the build-up of phosphorylated CpxR would lead to an increase in system activation, and the phospho-CpxR-mediated transcriptional control of genes involved in polyP regulation by an unknown mechanism. To gain a better understanding of CpxA's regulation of polyP dynamics, rather than solely at the height of polyP accumulation, we visualized polyP levels in wild-type and Δ*cpxA* mutants after both 3 and 4 hours of growth in MOPS. While there was an increase in polyP accumulation in Δ*cpxA* mutants at 3 hours as shown previously, this phenotype was even more pronounced at 4 hours (**Figure 3D**). As reported in the literature, polyP levels in wild-type cells decreased after 3 hours following the switch into MOPS media (*68*). PolyP levels in Δ*cpxA* mutants also showed a decrease between 3 and 4 hours, but less so than in the wildtype. This result suggests that CpxA may regulate polyP by inhibiting its degradation or prolonging its synthesis, but further investigations are required (see discussion).

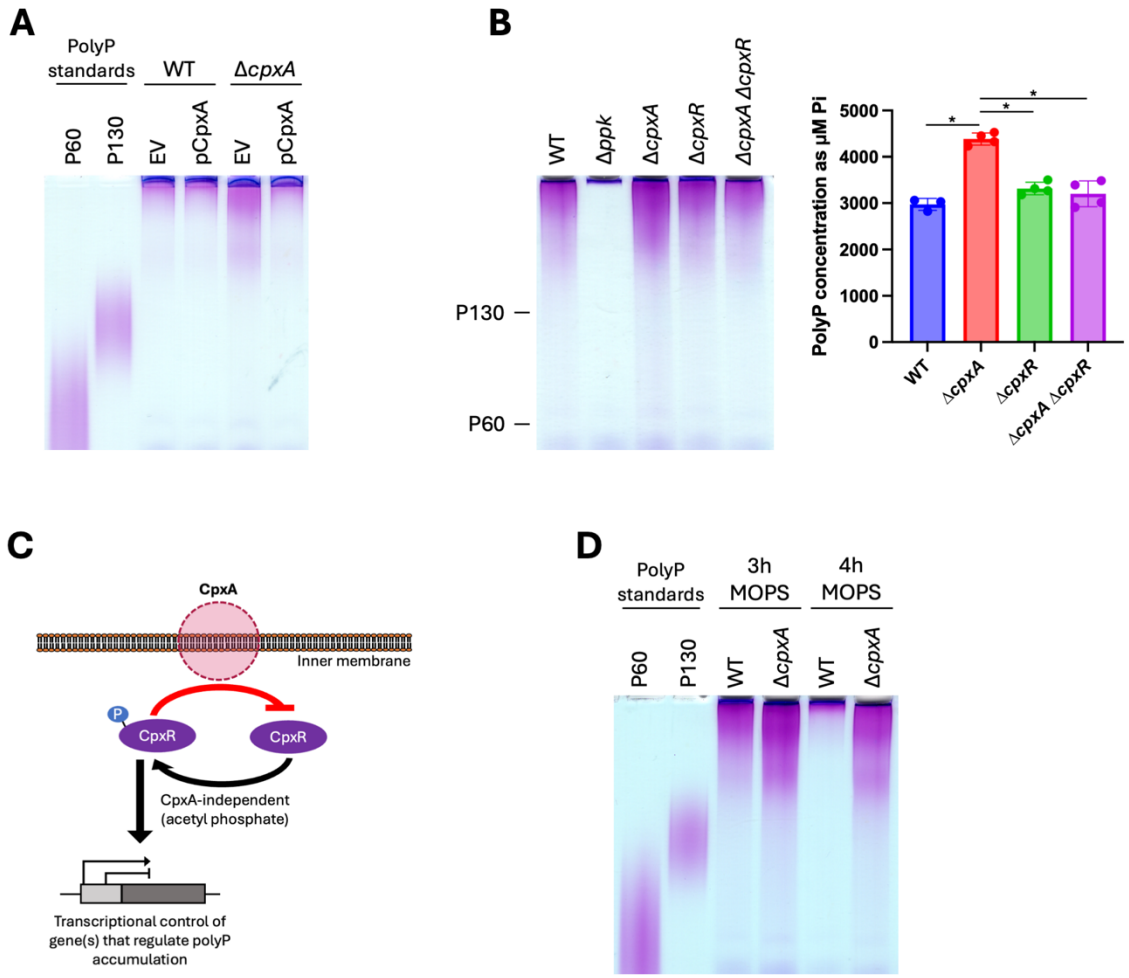

Figure 3: CpxA regulates polyP accumulation through a CpxR-dependent mechanism. A) Wild-type and Δ*cpxA* mutants were transformed to contain either the pBAD18 empty vector (EV), or a vector containing the *cpxA* gene cloned into the pBAD18 backbone (pCpxA). Cells were grown in LB containing 0.5% arabinose until mid-exponential phase, and then switched into MOPS minimal media supplemented with 0.5% glycerol instead of glucose. After 3 hours, cells were harvested and polyP was extracted by phenol-chloroform extraction. Isolated polyP was visualized by gel electrophoresis on a 15.8% TBE-urea polyacrylamide gel followed by staining with toluidine blue. PolyP standards of 60 and 130 Pi residues in length were included. n=3. B) Left: The indicated strains were grown in LB until mid-exponential phase and then switched into MOPS media for 3 hours. PolyP was extracted and visualized as described in panel A. PolyP standards, P60 and P130, are the same as those depicted in Figure 4B, and are therefore represented as written size markers. n=3. Right: PolyP extracts from three or four biological replicates of the representative experiment shown on the left were quantified using the Phosphinity polyP quantification kit, depicting polyP levels as µM of Pi. Dots represent individual data points and error bars represent the standard deviation. * represents $p<0.05$ based on the results of a one-way ANOVA followed by a post-hoc Tukey's multiple comparisons test. C) Schematic for the working model of the mechanism of polyP regulation in Δ*cpxA* mutants. D) Wild-type and Δ*cpxA* mutants were grown as described in panel B, and cells were harvested at 3 and 4 hours after the switch into MOPS media. PolyP was extracted and visualized as described in panel A. n=3.

3.2.2. Activation of the CpxRA system promotes polyP accumulation

To follow up on our working model, the next step in our investigation was to determine if Δ*cpxA* mutants grown in MOPS media display an increase in CpxRA system activation. This idea is supported by the literature, as the system has been shown to be active in Δ*cpxA* mutants grown in various types of media supplemented with glucose (*107*, *117*). To test for system activation, we used western blotting to observe the expression of one of the most highly inducible members of CpxR's regulon, CpxP (*116*, *119*). CpxP is a periplasmic protein whose expression levels are specifically regulated by the CpxRA system (*120*). It can also play a regulatory role in the system by inhibiting CpxA's autokinase activity in the absence of envelope stress (*121*, *122*). In fact, examining *cpxP* expression using a *cpxP-lacZ* fusion is commonly used to investigate CpxRA system activation throughout the field (*89*). Western blots analyzing the expression of C-terminally epitope-tagged CpxP showed that, while CpxP levels were difficult to detect in wild-type cells, Δ*cpxA* mutants had robust CpxP expression (**Figure 4A**). Similar to the wildtype, expression was below the level of detection in Δ*cpxA* Δ*cpxR* double mutants, following the same pattern of regulation observed for polyP levels (**Figures 4A**). The inability to consistently detect CpxP in wild-type and double mutants is coherent with one study that found that the CpxP protein could only be visualized when the system was active (*123*). Furthermore, CpxP expression in Δ*cpxA* mutants was higher during growth in MOPS media compared to LB media, suggesting that growth in MOPS promotes system activation in Δ*cpxA* mutants (**Figure 4A**).

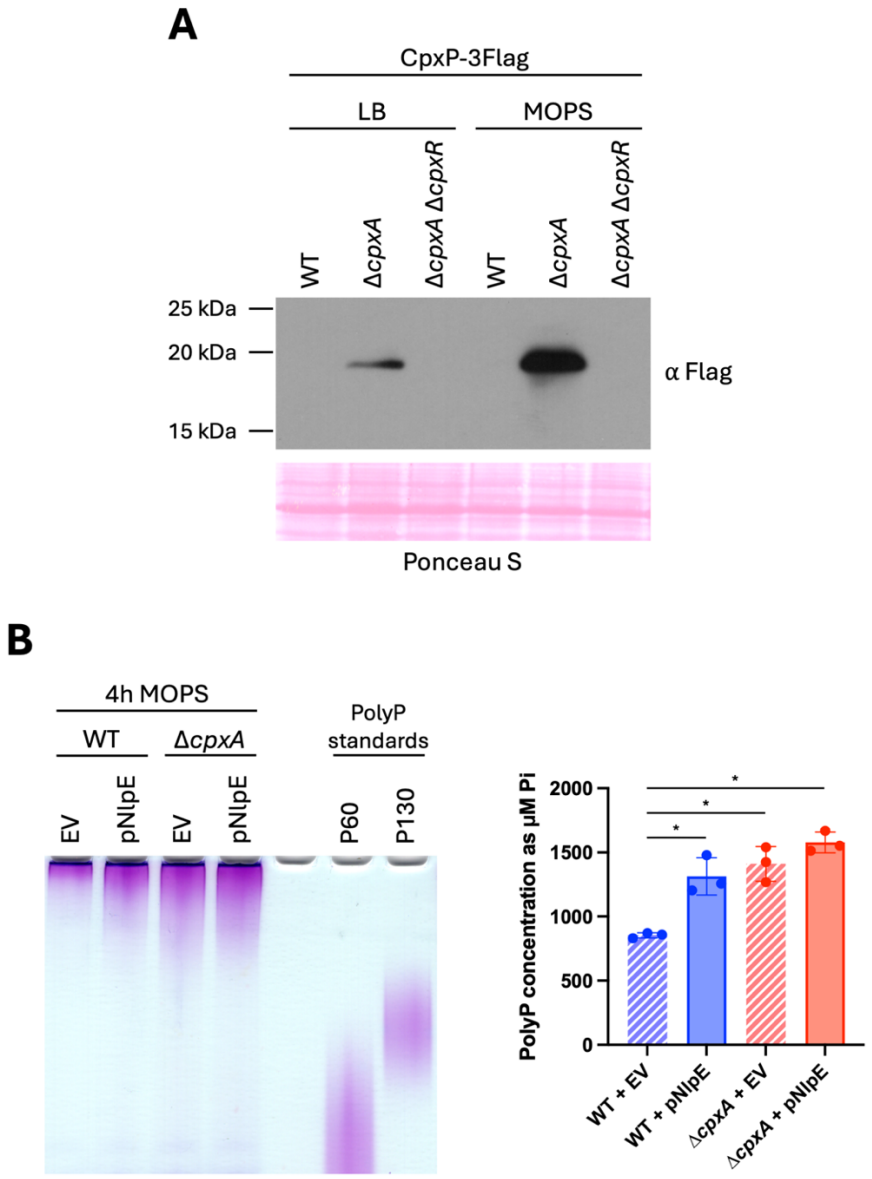

Figure 4: CpxRA system activation promotes increased polyP accumulation. A) The indicated strains were grown in LB until mid-exponential phase, and then switched into MOPS media for 3 hours. Cells were harvested at mid-exponential phase and after growth in MOPS. Proteins were isolated as described in the methods section (2.3.1). Samples were run using SDS PAGE on a 15% polyacrylamide gel. Proteins were transferred to a PVDF membrane that was blocked in 5% milk and then probed with an αFlag-HRP antibody. The membrane was stained with Ponceau S to ensure equal loading of proteins. n=3. B) Left: Strains containing either the pBAD18 empty vector (EV) or a vector with the *nlpE* gene cloned into the pBAD18 backbone (pNlpE) were grown in LB supplemented with 0.5% arabinose until mid-exponential phase. Samples were then switched into MOPS media supplemented with 0.5% glycerol instead of glucose. Cells were harvested after 4 hours of growth in MOPS media and polyP was extracted using phenol-chloroform extraction. PolyP was visualized on a 15.8% TBE-urea polyacrylamide gel and stained with toluidine blue. PolyP standards of 60 and 130 Pi residues in length were included. n=3. Right: PolyP extracts from three biological replicates of the representative experiment shown on the left were quantified using the Phosphinity polyP quantification kit, depicting polyP levels as µM of Pi. Dots represent individual data

points and error bars represent the standard deviation. * represents $p<0.05$ based on the results of a one-way ANOVA followed by a post-hoc Tukey's multiple comparisons test.

Next, to investigate if activation of the CpxRA system, rather than an unknown function of the Cpx genes, was promoting polyP accumulation during nutrient limitation, we sought to activate the CpxRA system using a different mechanism. As discussed, Δ*cpxA* mutants activate the system due to the absence of its phosphatase activity, however, activating the system by inducing the phosphorylation of CpxR by CpxA should also be capable of promoting increased polyP levels. To achieve this, we cloned the outer membrane lipoprotein, NlpE, into a pBAD18 vector backbone under the control of an arabinose-inducible promoter (pNlpE). NlpE overexpression has been shown to activate the CpxRA system (*109*, *111*). System induction by NlpE rescues the toxicity associated with lipoprotein trafficking defects, which is reportedly due to a physical interaction between NlpE and CpxA (*109*, *111*). After 1.5 hours of growth and induction in LB followed by 4 hours of growth in MOPS, polyP levels in wild-type cells containing pNlpE were significantly higher than in strains containing only the empty vector (**Figure 4B**). We chose to monitor this phenotype at 4 hours following the discovery that the CpxRA system maybe have a more prominent role in modulating polyP accumulation after 3 hours of growth in MOPS (**Figure 3D**). PolyP levels in wild-type cells containing pNlpE were similar to those in Δ*cpxA* mutants containing either vector (**Figure 4B)**. The lack of a significant additive effect on polyP accumulation in Δ*cpxA* mutants containing pNlpE is unsurprising, since NlpE was proposed to signal through CpxA to activate the system (*111*). This demonstrated that CpxRA system activation by NlpE could also promote an increase in polyP accumulation, and supports the hypothesis that CpxRA system activation is a positive regulator of polyP accumulation. A model for the regulation of polyP by the CpxRA system is depicted in **Figure 5**.

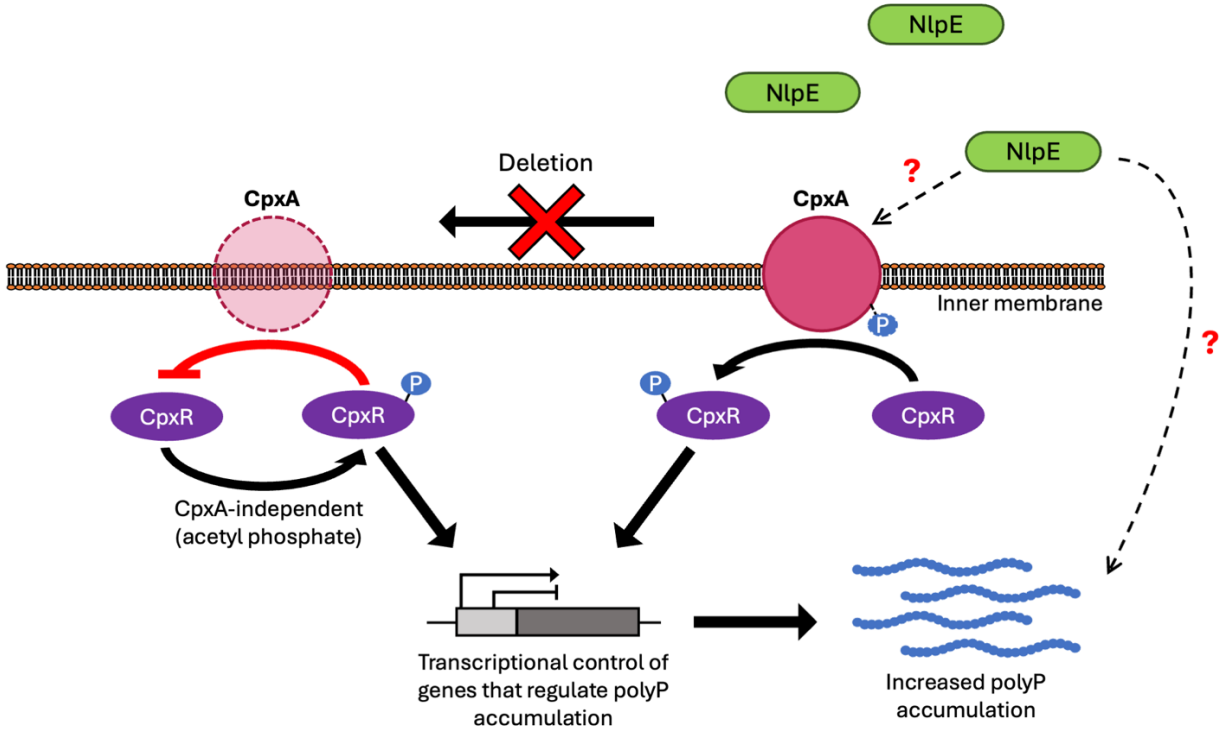

Figure 5: Working model for the regulation of polyP accumulation by the CpxRA system. A schematic depicting the working model for polyP regulation by the CpxRA system based on findings in this work. During growth in MOPS in the absence of CpxA, CpxR is phosphorylated independently from the system, and keeps the system activated due to the lack of CpxA's phosphatase activity. In a wild-type cell where NlpE is being overexpressed, NlpE signals to activate the CpxRA system. Both mechanisms contribute to phosphorylated CpxR regulating the transcription of gene(s) that, through an uncharacterized mechanism, promote an increase in polyP accumulation.

3.3. PolyP regulation by the ArcAB system

3.3.1. The ArcAB system regulates polyP through an unknown mechanism that is likely independent of RpoS

The two other novel polyP-regulators identified by this screen were *arcB* and *arcA*, as deleting either of these genes from the ArcAB TCS lead to similar decreases in polyP accumulation (**Figure 6**). While we consistently observed a decrease in polyP levels in these mutants, the intensity of said decrease was slightly variable across replications. PolyP levels in an Δ*arcB* Δ*arcA* double mutant resembled those in single mutants, suggesting that both ArcB and ArcA regulate polyP

through the same pathway, and that the ArcAB system promotes polyP accumulation during nutrient limitation (**Figure 6**). The hybrid HK, ArcB, autophosphorylates in response to oxygen limitation signalled by the quinone pool (*87*, *124*, *125*). Oxidized quinones, which accumulate under aerobic conditions, inhibit the system by preventing ArcB autophosphorylation (*125*). Thus, the reduction of these quinones during anaerobiosis removes this inhibition, allowing ArcB to autophosphorylate (*125*). Then, after a series of phospho-transfer steps between its own domains, ArcB phosphorylates its RR, ArcA (*87*). Phosphorylated ArcA acts primarily as a transcriptional repressor targeting genes involved in aerobic metabolism, while overall directly or indirectly regulating upwards of 100 genes (*126*).

Although the mechanism of polyP regulation by the ArcAB system has yet to be investigated in depth, our data suggests that the observed decrease in polyP levels in ArcAB system mutants are independent of the stress-responsive alternative sigma factor, RpoS. There are mixed results surrounding the relationship between RpoS and polyP accumulation. The ability of RpoS to regulate polyP levels, as well as the direction of said regulation, appears to depend heavily on the tested growth conditions (*67*, *68*, *127*). Meanwhile, ArcB has been shown to phosphorylate the orphan response regulator, RssB, which then promotes the degradation of RpoS (*128*). ArcA is also capable of binding to the *rpoS* promoter to inhibit its transcription, meaning that the ArcAB system contributes to RpoS protein levels, in part through RssB (*128*). By deleting *rssB* in both wild-type and Δ*arcB* backgrounds, we observed that the deletion of *rssB* alone did not affect polyP levels, while Δ*arcB* Δ*rssB* double mutants had polyP levels comparable to those in Δ*arcB* single mutants (**Figure 6**). This preliminarily suggests that the regulation of polyP by the ArcAB system likely does not involve RpoS.

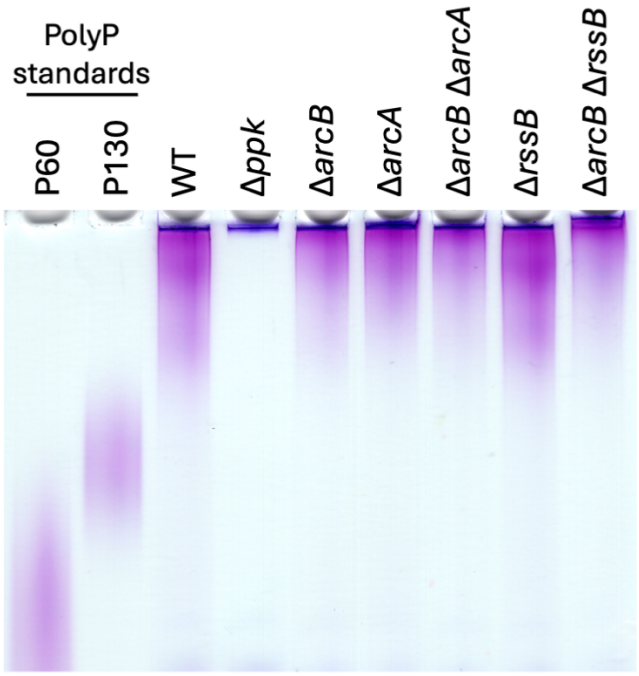

Figure 6: The ArcAB system promotes polyP accumulation. The indicated strains were grown in LB until mid-exponential phase and then switched into MOPS media. After 3 hours, cells were harvested and polyP was extracted by phenol-chloroform extraction. Isolated polyP was visualized by gel electrophoresis on a 15.8% TBE-urea polyacrylamide gel followed by staining with toluidine blue. PolyP standards of 60 and 130 Pi residues in length were included. n=3.

3.4. Investigating the mechanism of PPK activation

3.4.1. PPK and PPX expression do not correlate with changes in polyP accumulation

One of the aims of this work was to use novel polyP-regulators to further investigate the mechanism of PPK activation and subsequent polyP accumulation during stress. Since polyP-regulation by the CpxRA system was investigated extensively in this work, we wanted to visualize the expression of polyP-metabolizing enzymes during growth in LB and MOPS in these mutants. Both PPK and PPX were C-terminally tagged with a 3Flag epitope tag in wild-type and Δ*cpxA* mutants. Consistent with the literature, the expression of PPK and PPX were not notably different between growth in LB and MOPS (**Figure 7**), recapitulating the idea that polyP accumulation at

the onset of stress is not due to changes in the expression of either of these proteins. Additionally, no changes in PPK or PPX expression were observed between wild-type and Δ*cpxA* mutants, showing that CpxA does not regulate polyP by altering the expression of polyP-metabolizing enzymes, and emphasizing that the expression of these enzymes does not correlate with fluctuations in polyP levels.

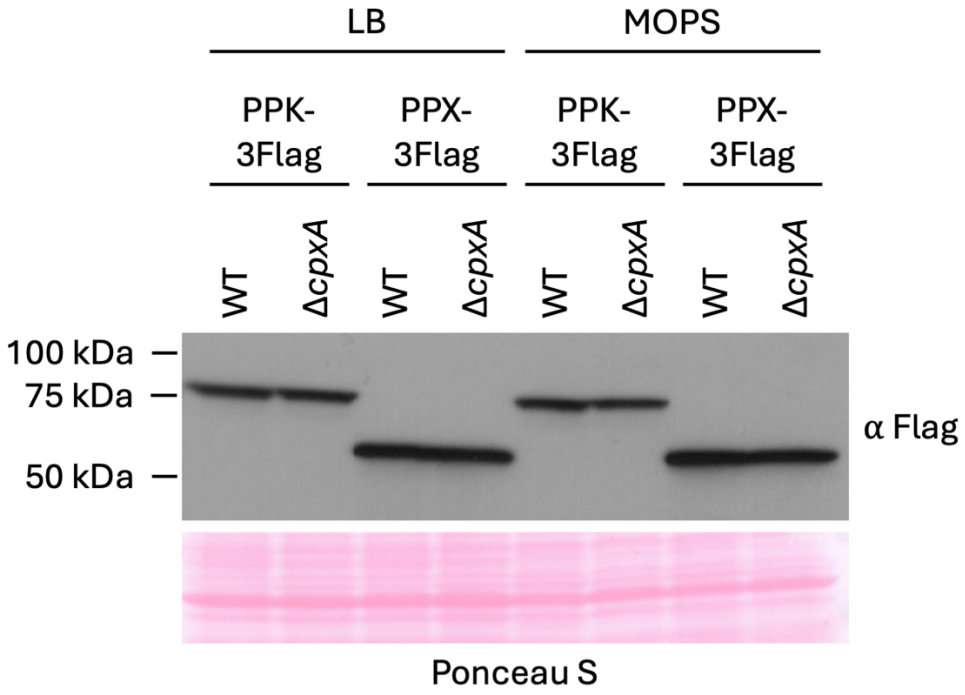

Figure 7: PPK and PPX expression do not correlate with polyP levels. The indicated strains were grown in LB until mid-exponential phase, and then switched into MOPS media for 3 hours. Cells were harvested at mid-exponential phase and after growth in MOPS. Proteins were isolated as described in the methods section (2.3.1). Samples were run using SDS PAGE on a 12% polyacrylamide gel. Proteins were transferred to a PVDF membrane that was blocked in 5% milk and then probed with an αFlag-HRP antibody. The membrane was stained with Ponceau S to ensure equal loading of proteins. n=3.

3.4.2. PPK's localization remains a promising and underexplored regulatory mechanism

PPK has been isolated from the insoluble/membrane fractions in several targeted studies (*62*, *129*). We are therefore currently optimizing a fractionation protocol to analyze PPK's localization under different conditions (e.g. LB vs. MOPS) and in polyP-regulating mutants (e.g. Δ*cpxA*, Δ*phoB*) (*106*). Preliminarily, we have thus far been able to detect PPK in all fractions (cytoplasm,

membrane, and periplasm), despite notable variability in protein levels in each fraction between repetitions (**Figure 8**). We have also noted the appearance of a slow-migrating upper band in the membrane fractions, while the lower band aligns with the established size of monomeric PPK (~80 kDa) (*61*, *129*). Importantly, the inner membrane protein, ZipA (~50 kDa) (*105*), was localized to the insoluble/membrane fraction using this protocol, indicating minimal contamination of cytoplasmic and periplasmic fractions by membrane proteins. Consistent with western blots from whole-cell extracts (**Figure 7**), there do not appear to be substantial changes in PPK's protein levels within each fraction between stress and non-stress conditions in either wild-type or Δ*cpxA* mutants (**Figure 8**). However, more work is needed to optimize this protocol in order to interpret changes in relative expression between fractions and across conditions.

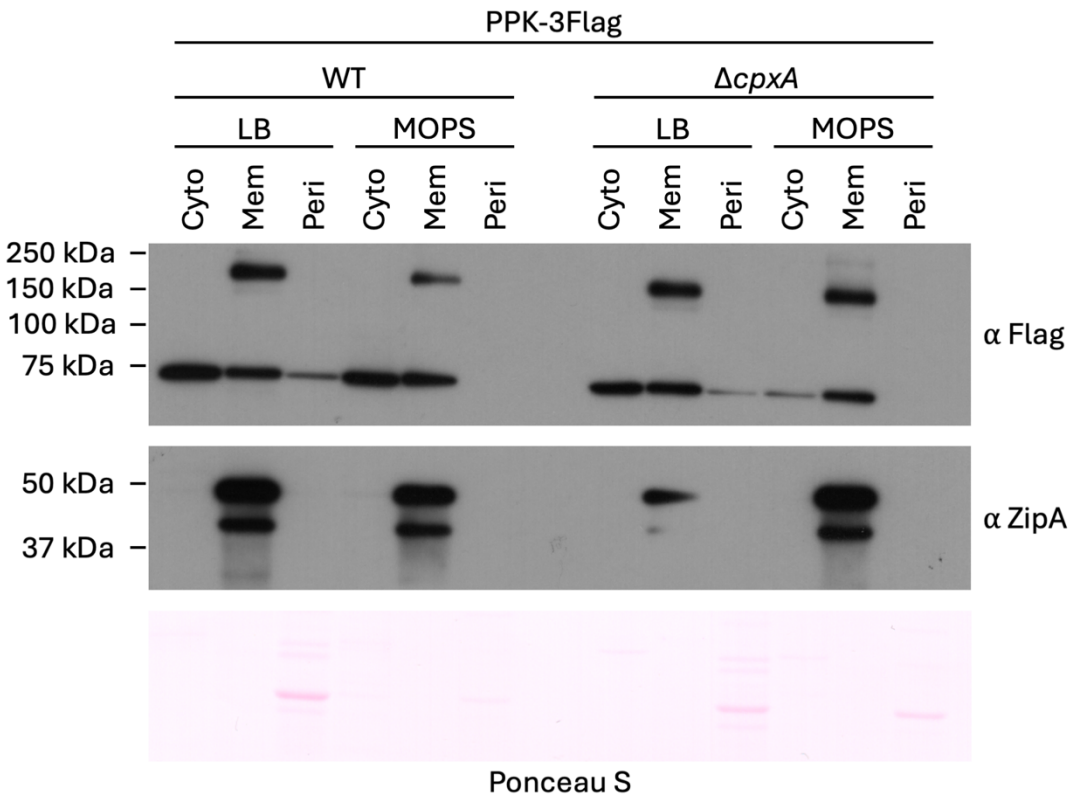

Figure 8: PPK can be detected in the cytoplasm, membrane, and periplasm. The indicated strains were grown in LB until mid-exponential phase, and then switched into MOPS media for 3 hours. Cells were harvested at mid-exponential phase and after growth in MOPS. Samples were separated into periplasmic (Peri), cytoplasmic (Cyto), and membrane (Mem) fractions using a cold-osmotic shock followed by cell lysis by freeze-thaw cycling, as described in the methods section (2.4). Samples were run via SDS PAGE on a 10% polyacrylamide gel and then proteins were transferred to a PVDF membrane. The membrane was blocked in 5% milk and then probed with the indicated antibody. The membrane was stained with Ponceau S to ensure equal loading of proteins. n=2.

4. DISCUSSION

4.1. Five polyP-regulators were identified amongst *E. coli* TCS genes

This work describes a comprehensive genetic screen of all TCS genes in *E. coli*, where I highlight five polyP-regulators. Three of these five regulators, to my knowledge, have not yet been reported in the literature. While using a commercially available collection set such as the Keio knockout collection would have been ideal for a screen of this size, strains within this collection set appear to have changes in polyP levels that are independent of the desired deletion, making it unsuitable for the purpose of this study, and likely for other works surrounding polyP biology. Although there are many possibilities as to why polyP is altered in these strains, I surmise that it could be due to suppressor mutations that arise throughout the construction and duplication process of this high-throughput collection set. This is supported by one study that identified several extraneous mutations in a selection of strains from the Keio collection, with Δ*rcsC* and Δ*cpxA* being included in their analysis (*130*). Moreover, unpublished work from our lab found that a Δ*ppk* isolate from the Keio collection set contained a point mutation in the *pitA* gene that rescued the characteristic growth defect of Δ*ppk* mutants on MOPS media (Brianna Kore, unpublished honours thesis). Creating deletions in-house in a low-throughput manner should mitigate issues that arise in such large-scale collections. However, it remains important to consider polar effects (especially within operons (*131*)) and the appearance of suppressor mutations when working with any mutant. These findings highlight the importance of validating results obtained while using commercially available strain collections, whether that be by recreating key strains or complementing observed phenotypes.

In creating all strains used throughout the screen, I have generated a small library of single-gene deletion mutants for all TCS genes in *E. coli* (**Table 1 and S1**). This resource could be

exploited in future works analyzing polyP accumulation in response to diverse environmental stressors. As discussed previously, certain genes targeted by this screen have been tested for polyP accumulation in the literature, and while my screen shows consistency with several studies, there are also cases of inconsistency. The reason for inconsistent polyP phenotypes in the same mutants across different studies remains unclear, but one explanation could be that polyP levels are sensitive to conditions that have not yet been characterized. Bowlin and colleagues suggested in 2022 that the composition of the "non-stress" nutrient-rich LB plays a stronger role in polyP accumulation than previously believed. After identifying nitrogen regulatory proteins RpoN and GlnG, as well as GlrR, as regulators of polyP, they found that the addition of glutamine to LB prior to the switch to MOPS abolished polyP-regulation by RpoN and GlnG (*77*). Notably, I did not find altered polyP levels in Δ*glnG* or Δ*glrR* mutants (**Table 1**). The exact conditions and timing of "nutrient starvation" could also be contributing to differences between studies. While puzzling, detecting changes in polyP accumulation in different mutants across studies offers a unique opportunity to narrow down candidates for the elusive factor "X", a protein or molecule believed to integrate signals from these diverse pathways to ultimately modulate polyP accumulation (*68*). Perhaps future studies could examine overlapping downstream effectors of polyP-regulating genes in search of common proteins or small molecules.

Using a single environmental condition to induce polyP accumulation in this screen not only provides a possible explanation for variance, but is also a notable limitation of this work. While MOPS minimal media is commonly used to induce polyP accumulation due to its deficiency in amino acids and phosphate, it is unclear whether the concentrations of other nutrients in MOPS could induce certain TCS. Keeping this in mind, it is possible that polyP-regulators could be missed upon initial screening. Alternatively, hits from this screen could lack the ability to regulate

polyP if tested under different conditions. For example, I did not find that either protein in the zinc-responsive ZraRS system regulated polyP accumulation during growth in MOPS, however, it is possible that growth in a minimal media with a different zinc concentration would yield different results (*132*). The same applies to other stressors such as osmotic, oxidative, or heat stress, which could result in polyP regulation by a different set of TCS than identified in this screen.

4.2. Elucidating the relationship between the CpxRA system and polyP accumulation

From the 5 polyP-regulators identified after the nutrient downshift to MOPS media, I focused mostly on the polyP-promoting Δ*cpxA* mutant. I have demonstrated this phenotype in two strain backgrounds (**Figure 2B**), and have complemented it by re-introducing CpxA from an inducible vector (**Figure 3A**). I also found that CpxA regulates polyP using a mechanism dependent on CpxR, since Δ*cpxA* Δ*cpxR* double mutants do not display the same increase in polyP as Δ*cpxA* single mutants (**Figure 3B**). Based on these results, I proposed that Δ*cpxA* mutants are regulating polyP through CpxRA system activation, which would explain the dependence on CpxR for the observed phenotype (**Figure 3C**). However, inhibition of the CpxRA system does not appear to hinder polyP accumulation, as Δ*cpxR* mutants and strains overexpressing CpxA do not display a decrease in polyP accumulation (**Figures 3A and 3B**).

Countless studies have shown that Δ*cpxA* mutants activate the CpxRA system, and various *in vitro* and *in vivo* data support that this is due to the absence of its CpxR-phosphatase activity (*89*, *107*, *117*). These reports typically grow cells in media containing glucose as a carbon source, as glucose precedes the formation of AcP through the Pta-AckA pathway which contributes to CpxR-phosphorylation, as is the case for several other response regulators (*133*, *134*). Therefore, there are generally two described mechanisms for CpxRA system activation: 1) conditions which require both CpxA and CpxR to activate the system, such as alkaline conditions or protein

trafficking defects (*107*, *111*), and 2) conditions that promote the CpxA-independent phosphorylation of CpxR (*107*, *117*). My model first describes polyP regulation by the CpxA-independent activation mechanism, which I support by showing that CpxP is highly expressed in Δ*cpxA* mutants during growth in MOPS (**Figure 4A**). Interestingly, this experiment also showed that the system is more highly active in Δ*cpxA* mutants in LB than in wild-type cells grown in MOPS. Despite this result, I did not observe polyP accumulation in Δ*cpxA* mutants grown in LB (data not shown), suggesting that system activation alone is not sufficient to induce polyP accumulation in the absence of nutrient limitation. One caveat to using CpxP-3Flag protein expression to look at system activation is that this small protein is also involved in a feedback mechanism with CpxA, as CpxP overexpression can inhibit the system by interacting with CpxA (*121*, *135*). However, the deletion of *cpxP* does not substantially activate the system (*119*), mitigating concerns that the tag could interfere with this interaction to falsely activate the system.

Since my results supported that system activation occurs in the same strains and conditions as the increase in polyP, I next wanted to determine if the system activation mechanism dependent on both CpxA and CpxR would achieve the same phenotype. I tested this by overexpressing NlpE, which signals defects in protein trafficking to the outer membrane and activates the system (*109*, *111*) (**Figure 4B**). These experiments were conducted in wild-type and Δ*cpxA* mutant backgrounds, showing the relatively epistatic nature of overexpressing NlpE in the absence of CpxA (**Figure 4B**). However, to conclude that NlpE is promoting increased polyP levels through the CpxRA system, I should repeat these experiments in a Δ*cpxR* mutant background, which I would expect to abolish the observed increase in polyP levels when NlpE is overexpressed. Nonetheless, my results support that envelope stress signals originating from the periplasm

promote polyP accumulation, a finding that to my knowledge, has not been described before in *E. coli*.

4.3. The cellular pathways connecting the CpxRA system and polyP-metabolizing enzymes remains unclear

These results implicate polyP in a conserved envelope stress-responsive pathway, a function coherent with polyP's role as a protein chaperone that stabilizes unfolding proteins (*34*, *45*). Importantly, PPK and PPX are not known members of CpxR's regulon as described using transcriptomics (*115*, *136*) and proteomics (*137*). Therefore, it is unlikely that CpxR directly regulates *ppk* or *ppx* transcription. I indirectly support this in **Figure 7**, showing no difference in PPK or PPX protein levels between wild-type and Δ*cpxA* mutants at the peak of polyP accumulation. It is therefore more likely that another member of CpxR's regulon participates in polyP regulation. While analyzing polyP accumulation in members of CpxR's regulon could address this, the large size (likely over 100 genes (*114*)) of this regulon makes this strategy impractical. Interestingly however, previous work has shown that deleting the envelope stress-responsive alternative sigma factor, *rpoE*, results in a decrease in polyP levels, although the significance or mechanism of this has not yet been characterized (*69*). This is especially notable due to the overlap between the CpxR and RpoE regulons (*116*), introducing the possibility that a gene contained within both regulons could be responsible for promoting polyP accumulation. Another possibility is that the CpxRA system is regulating polyP through RpoE, an interpretation confounded by mixed results surrounding RpoE-regulation by the CpxRA system, with some studies showing that it represses *rpoE* transcription, and one transcriptomics analysis showing increased expression (*116*, *136*). A similar contradiction surrounding the regulation of polyP by RpoE was observed with *glrR,* whose deletion promotes polyP accumulation, a phenotype that

does not align with GlrR's promotion of RpoE expression (*77*). Proteomics analyses in UPEC also indicated that CpxRA system activation downregulates members of the Pho regulon, notably PhoB (*115*). Since Δ*phoB* mutants have very low levels of polyP (*66*), it is unlikely that the CpxRA system's influence on the Pho regulon is responsible for the observed polyP phenotype.

A more suitable strategy to investigate how the CpxRA system (or my other TCS hits) influences polyP metabolism could be to directly analyze PPK and PPX biology. As discussed briefly, I was first able to visualize PPK expression using western blotting, where I saw no notable changes in protein levels between cells grown in LB and MOPS, or in Δ*cpxA* mutants (**Figure 7**). This is consistent with existing literature demonstrating that PPK expression does not correlate with the onset of polyP accumulation, or in polyP-regulating mutants such as Δ*dksA* (*67*, *69*). However, the comparison of polyP levels between 3 and 4 hours of growth in MOPS suggests that more investigations surrounding PPK and PPX expression could be insightful (**Figure 3D**). These results suggest that inhibiting polyP-degradation, or prolonging its synthesis, could explain the increased polyP in Δ*cpxA* mutants (**Figure 3D**). I would therefore like to repeat these experiments by looking at PPK and PPX expression beyond 3 hours of growth in MOPS. It is worth noting that the increase in polyP levels in Δ*cpxA* mutants roughly resembles that of Δ*ppx* mutants (*67*), contributing to the idea that polyP-degradation could be the target of the CpxRA system. Considering this, it would also be interesting to investigate PPX activity in Δ*cpxA* mutants, perhaps by measuring *ppx* transcription or the redox state of the enzyme, which has been shown to modulate its activity (*34*, *69*).

4.4. PPK's mechanism of activation remains elusive

While I did not see any substantial changes in PPK levels in western blots between LB and MOPS (**Figure 7**), one caveat to these results is that PPK has been found within insoluble membrane

fractions throughout the literature (*62*, *129*, *138*). Meanwhile, the protein isolations used in these western blots had been boiled, which should lead to the aggregation of membrane proteins (*139*). The ability to visualize PPK under these conditions suggests that PPK may not reside exclusively in the cytoplasm or membrane, a hypothesis supported by one high-throughput study characterizing PPK as a "peripheral inner membrane protein" (*138*). I am therefore keenly interested in PPK's localization, particularly as a possible mechanism of regulation. While the fractionation protocol requires more refinement before any conclusions about changes in localization can be made, I was able detect PPK in the cytoplasmic, periplasmic, and insoluble (membrane) fractions. Meanwhile, the control protein ZipA was localized primarily to its designated compartment (*140*) as visualized by a ZipA-specific antibody (*105*) (**Figure 8**). This supports the idea that there could be more than one population of PPK within the cell, which opens the door for future investigations into if different PPK populations possess different enzymatic activities. I intend to optimize this protocol to allow for the comparison of protein levels between conditions and strain backgrounds. Notably, the identity of the higher molecular weight PPK product in the membrane fractions remains unknown, and the optimization of this protocol could help to facilitate its identification. Since one early study claimed that PPK was localized to the outer membrane (*129*), it could also be important to further separate the insoluble fraction into inner and outer membrane fractions (*141*). Overall, the literature surrounding PPK's localization remains complex, and studying its localization as a possible regulatory mechanism is a thus far underexplored avenue.

On top of analyzing PPK's localization, it could also be important to expand investigations surrounding PPK expression. Aside from analyzing protein levels, it would be interesting to complement my western blots with RT-qPCR analyses. Michael Gray showed in 2020 that *ppk*

transcript levels decreased abruptly after the switch from LB to MOPS media, and later recovered to initial levels (*69*). He also demonstrated a slight decrease in *ppk* mRNA in the polyP-inhibited Δ*dksA* mutant, and that elongation of the *ppk-ppx* transcript was inhibited following the switch to MOPS (*69*). These results suggest that while protein levels appear to remain consistent (**Figure 7**), that does not necessarily dismiss the possible involvement of temporal transcriptional regulation. It is therefore important to consistently consider different approaches to understand PPK regulation.

4.5. The PhoBR and ArcAB systems regulate polyP accumulation

In addition to my investigation of the CpxRA system and PPK dynamics, we also conducted some more preliminary follow-up analyses for the other hits identified in the screen: *arcB*, *arcA*, *phoR*, and *phoB*. PhoR and PhoB, the HK and RR of the PhoBR TCS (respectively) (*74*, *75*), have already been shown to regulate polyP, and investigations into the modulation of polyP accumulation by phosphate-regulating genes are fairly extensive (*66*). Studies suggest that the activation of the PhoBR TCS is necessary for polyP accumulation, as polyP levels are extremely low when *phoB* is deleted, or in Δ*phoR* Δ*creC* double mutants where the non-cognate HK, CreC, is unable to phosphorylate PhoB (*66*, *142*). The PhoBR system is activated in response to phosphate starvation, and PhoB promotes the expression of several genes involved in phosphate transport and usage, which has been coined the "Pho regulon" (*75*). This implicates phosphate homeostasis in the control of polyP accumulation, a similar scenario as observed in *S. cerevisiae* (*13*, *14*). Since the goal of this study was to uncover novel regulators of polyP, follow-up studies on the PhoBR TCS were not prioritized. However, our lab is actively working to uncover the intricacies of polyP metabolism and phosphate homeostasis.

I demonstrated that both ArcB and ArcA promote polyP accumulation, as their deletion led to a modest, but slightly variable, decrease in polyP levels (**Figure 6**). Additionally, the lack of an additive effect in Δ*arcB* Δ*arcA* double mutants suggests that ArcB and ArcA are promoting polyP through their canonical TCS pathway. To my knowledge, this is the first time that the regulation of polyP levels has been reported for the ArcAB system in *E. coli*, however, ArcA has been previously identified downstream of polyP accumulation. One study that combined transcriptomic, proteomic, and phenotypic microarray data to analyze cellular changes in Δ*ppk*, Δ*ppx*, and Δ*ppk* Δ*ppx* mutants demonstrated that ArcA was downregulated in polyP-deficient mutants, and upregulated in Δ*ppx* mutants which contain increased polyP (*143*). They also observed expression changes in a series of ArcA-regulated metabolism and respiration genes between Δ*ppk* and Δ*ppx* mutants, leading the authors to propose that polyP somehow promotes anaerobic metabolism (*143*). While this suggests a possibly circular mechanism of regulation between the ArcAB system and polyP, it is important to note that this study used cells grown in LB, meaning that cellular changes could be due to alternative functions of PPK and PPX, rather than polyP itself. Additionally, proteomics analyses conducted in our lab looking for differentially expressed genes between wild-type and Δ*ppk* mutants grown in MOPS did not identify ArcA (*59*). Nonetheless, the results presented in this work further implicate polyP in central metabolism and cellular respiration processes.

The relationship between polyP and the stationary phase alternative sigma factor, RpoS, appears to be complex. Early studies found that RpoS could promote or abolish polyP accumulation during a downshift to nitrogen limited media, or osmotic shock, respectively (*68*). More recent studies suggest that RpoS is not involved in polyP regulation during nutrient limitation in MOPS media, contrasted by one report that Δ*rpoS* mutants have less polyP under this condition

(*67*, *127*). The ArcAB system represses RpoS expression, as ArcB can phosphorylate the orphan RR RssB, which promotes RpoS degradation, while phosphorylated ArcA can also repress RpoS transcription (*128*). Given that RssB was already to be targeted by this screen, I wanted to investigate if deleting *rssB* in a wild-type or Δ*arcB* background would similarly decrease polyP levels, or cause further polyP deficiency in these strains, respectively. Δ*rssB* mutants accumulated similar levels of polyP to the wildtype, while Δ*arcB* Δ*rssB* double mutants mimicked Δ*arcB* single mutants (**Figure 6)**, suggesting that ArcB signalling through RssB was not responsible for the observed polyP phenotype.

4.6. What is the significance of polyP regulation by identified hits?

Aside from the regulation of polyP by the phosphate-regulating PhoBR system, it is thus far unclear why polyP modulation would be a target of envelope stress and anaerobic growth-regulating systems. In *M. smegmatis*, Δ*ppk* mutants are sensitive to hypoxia and surface stress by SDS treatment (*37*). However, not only are envelope stress and hypoxia not known polyP-inducing stressors in *E. coli*, but it is also unclear how polyP accumulation would contribute to these processes. Firstly, it is important to recall that the CpxRA system responds to diverse stressors and enacts a growing list of functions, some intersecting with known functions of polyP. Since system activation promotes the expression of several periplasmic protein folding chaperones, it is interesting to consider if polyP's chaperone activity is contributing to this adaptive response (*34*, *116*, *137*). However, since the immature or aggregated proteins reported to activate the system reside in the periplasm (*109*, *111*, *123*), it is unclear if polyP could interact with these proteins. The CpxRA system also promotes resistance to copper (*109*), a stressor that one group has shown is mitigated by polyP accumulation and subsequent degradation (*144*). Finally, considering the results from Varas and colleagues in 2017, it is also possible that polyP plays a role in regulating

cellular respiration and central metabolism, highlighting a possible connection to my discovered hits, as both the CpxRA and ArcAB systems negatively regulate respiratory chain proteins such as *sdhC*, *nuoA*, and *cyoA* (*126*, *143*, *145*, *146*).

Overall, this work presents an essential foundation for understanding the mechanism of polyP accumulation, both directly by PPK, and indirectly through upstream environmental and cellular signals. More work will be needed to characterize the mechanism of PPK regulation both in identified hits, and during various stress conditions. Also to be uncovered is how exactly polyP regulation is targeted by these three TCS that are responsive to immensely diverse signals.

5. FUTURE DIRECTIONS

5.1. Evaluate polyP metabolism under a variety of conditions

As discussed, the screen for novel polyP-regulators used a nutrient downshift as the sole polyP-inducing stressor. Therefore, not only would it be interesting to re-analyze polyP levels in our library of mutants under different polyP-inducing stress conditions, but I could also use clues from the newly discovered polyP-regulating genes to identify novel stressors. While slightly intuitive, one example of this is the PhoBR system, which is activated in response to low phosphate, a condition that was also found to trigger polyP accumulation (*66*). Following this example, I could employ CpxRA- and ArcAB- inducing stressors to test if polyP accumulates under a variety of novel conditions. These conditions could include envelope stress through treatment with drugs such as globomycin (*111*) or other antimicrobials (*110*), ethanol (*108*), alkaline conditions (*107*), and anaerobic growth (*147*). Investigations as such are particularly important considering the incredibly diverse range of stressors that have been shown to trigger polyP accumulation, making it difficult to reconcile what molecular pathways are involved.

5.2. Explore the biological significance of polyP regulation by TCS

TCS function to facilitate cellular adaptation to specific stressors or conditions, therefore, mutants devoid of certain TCS frequently possess specific growth phenotypes (*90*, *91*). PolyP regulation by any particular system suggests that polyP may play a role in adapting to that system's target signal, which opens the door to exploring the biological functions of polyP. By comparing both growth and molecular phenotypes between TCS mutants and mutants lacking *ppk*, I could investigate if polyP is involved in mediating known phenotypes associated with each hit. This is especially interesting in Δ*cpxA* mutants, which possess a range of phenotypes including resistance to several antimicrobials such as amikacin, gentamicin, and hydroxyurea (*148*). By activating the

CpxRA TCS in polyP-deficient strains (Δ*ppk*), I could use spot test assays to observe if polyP is involved in CpxRA's resistance to these, and other, stressors. This strategy can also be employed for the ArcAB system, which has an unsurprisingly extensive repertoire of associated growth phenotypes (*90*). Most intriguing is the sensitivity of both polyP-deficient mutants and Δ*arcA* to hydrogen peroxide, providing an exciting opportunity to investigate if these phenotypes are linked (*149*, *150*). Somewhat similarly, regulation by certain TCS could point to stress conditions where the bacteria benefit from the presence of polyP. For example, studies in *Mycobacterium* spp. have found that Δ*ppk* mutants are more susceptible to surface stress by SDS treatment, a phenotype which is coherent with the involvement of *sigE* both upstream and downstream of polyP accumulation, since *sigE* was reportedly upregulated in response to this stressor (*37*, *70*). It would therefore be interesting to test if Δ*ppk* mutants alone are more susceptible to stressors such as hypoxia (*147*), envelope stress (*111*), pH fluctuations (*107*), and treatment with aminoglycosides (*148*) .

Equally important would be to test if fluctuations in polyP levels in the hits can recapitulate the molecular phenotypes associated with polyP accumulation. For example, our lab has shown that polyP promotes the expression of proteins involved in generating lipid A modifications, however, a similar decrease in protein expression was not observed in Δ*phoB* mutants, which have a significant lack of polyP (*59*). It would therefore be intriguing to test if mutating other polyP-regulating genes could mimic these molecular changes.

5.3. Observe PPK's oligomeric state as a possible regulatory mechanism

There is a sizeable body of evidence to suggest that PPK's oligomeric state is involved in its regulation. One study by Tzeng and Kornberg used radiation target analysis to study PPK's oligomerization during its various enzymatic activities, and they report that while PPK

autophosphorylation can occur as a dimer or tetramer, polyP synthesis occurs primarily when PPK is a dimer (*29*). More recently, two hyperactive *ppk** mutants showed an increase in the dimeric and monomeric forms of the enzyme, as opposed to the predominantly tetrameric form of wild-type PPK observed in these *in vitro* experiments (*72*, *73*). The PPK1 enzyme from *M. tuberculosis* also adopts a dimeric conformation in its autophosphorylated form (*37*). To investigate if this mode of regulation is relevant *in vivo*, I could use native PAGE to resolve intact PPK, followed by western blot analysis (*151*, *152*). Products visualized by western blotting that are various magnitudes larger than the expected protein size would represent specific oligomeric states. By comparing the size of the detected products in wild-type cells grown in either LB or MOPS media, or in polyP-regulating mutants, I could determine if changes in oligomerization contribute to PPK activity *in vivo*. Establishing if PPK's oligomeric state impacts polyP dynamics would allow for the improved targeting of PPK; identifying compounds that either block or enhance oligomeric interactions to modulate polyP levels.

5.4 Investigate polyP regulation by TCS in pathogenic strains

Blood stream infections caused by *E. coli* pose a significant threat to the population, as described by both world-wide and nation-wide analyses (*153*, *154*). Many of these infections appear to originate from urinary tract infections (UTIs) (*154*), and one study conducted in Isreal showed that close to half of *E. coli*-driven blood stream infections were multi-drug resistant (*153*). Both polyP and TCS are involved in virulence and antibiotic resistance in *E. coli*, emphasizing the importance of characterizing these pathways further (*53*, *95*). This prompts several exciting possibilities for future analyses. First, one high-priority set of experiments would be to determine if polyP levels are still altered in each hit in pathogenic *E. coli* strains, such as UPEC. Since inhibiting PPK in *E. coli* K-12 was shown to promote antibiotic susceptibility (*59*), and inhibiting it in UPEC decreased

resistance to oxidative stress and interfered with biofilm formation (*49*), it would be intriguing to test if targeting polyP accumulation through adjacent cellular pathways would yield similar phenotypic patterns. This investigation would be particularly important considering that both the CpxRA and ArcAB systems have also been shown to influence virulence in UPEC (*95*, *115*, *155*). Second, I could expand the screen into TCS that are specific to UPEC, such as the KguRS and GrpQP TCS, which influence growth on alpha-ketoglutarate and type 1 fimbria expression, respectively (*156*, *157*). Measuring polyP levels in mutants targeting these systems could provide additional insights into the roles of pathogen-associated genes in polyP regulation.

5.5. Manipulating TCS to fine-tune polyP levels

Aside from its possible uses in fighting bacterial infections, polyP accumulation has also been of interest to researchers due to its uses for enhanced biological phosphorus removal (EBPR) (*158*, *159*). EBPR involves the removal of phosphate from wastewater using diverse bacteria (termed polyphosphate-accumulating organisms) that can store Pi as polyP (*158*). Studies aiming to engineer bacteria for this purpose have investigated ways to optimize polyP production, as well as ways to limit and control its degradation (*159*, *160*). Therefore, describing methods to fine-tune polyP accumulation is of interest to the environmental sector. As such, I could compound polyP-regulating mutations, including those identified in this study, in an effort to deliberately control polyP accumulation. For example, could overexpressing ArcA or ArcB in a Δ*cpxA* mutant increase polyP levels even further? Could overexpressing PhoB in a Δ*phoR* mutant achieve the same phenotype? These investigations could provide a useful foundation for researchers seeking to better understand how to tightly control polyP accumulation dynamics for EBPR.

6. CONCLUSIONS

In this work, I describe a screen of single-gene deletion mutants targeted towards *E. coli* TCS in search of novel regulators of polyP accumulation. Addressing my first aim, the screen highlighted five TCS genes able to alter the accumulation of polyP during nutrient limitation: *cpxA*, *arcB*, *arcA*, *phoB*, and *phoR*. To my knowledge three of these genes (*cpxA*, *arcB*, and *arcA*) have not yet been described as polyP-regulators in the literature. Next, in addressing my second aim, I discovered that deleting *cpxA* promotes polyP accumulation through the activation of its envelope stress-responsive TCS, the CpxRA system. It is thus far unclear how the CpxRA system regulates polyP at the level of its metabolizing enzymes, but so far, I've shown that system activation is not modulating the protein levels of either PPK or PPX. Finally, in investigating my third aim, I demonstrated that nutrient limitation, which triggers polyP accumulation, also does not seem to alter PPK or PPX expression, nor did it robustly alter PPK's localization patterns. At present, immediate next steps would be to clarify the significance of polyP regulation by these diverse TCS and conduct more in-depth analyses surrounding PPK's regulation. Overall, the work presented in this thesis presents an extensive foundation for better understanding how environmental stress contributes to polyP accumulation. I have also begun to characterize novel mechanisms regulating polyP metabolism in *E. coli*, and have initiated investigations to characterize the trigger for PPK activation in response to stress. As a whole, this work will not only contribute to our fundamental knowledge surrounding polyP and PPK biology, but also provides useful insights into possible pathways to target polyP accumulation to exploit for antimicrobial treatments.

7. BIBLIOGRAPHY

1. I. S. Kulaev, V. M. Vagabov, T. V. Kulakovskaya, "The Occurrence of Polyphosphates in living organisms" in *The Biochemistry of Inorganic Polyphosphates: Second Edition* (John Wiley & Sons, Ltd, 2005), pp. 37-44.
2. A. Kornberg, N. N. Rao, D. Ault-Riché, Inorganic polyphosphate: A molecule of many functions. *Annu. Rev. Biochem.* **68**, 89-125 (1999).
3. A. Denoncourt, M. Downey, Model systems for studying polyphosphate biology: a focus on microorganisms. *Curr. Genet.* **67**, 331-346 (2021).
4. B. Lázaro, A. Sarrias, F. J. Tadeo, J. Marc Martínez-Láinez, A. Fernández, E. Quandt, B. Depares, T. Dürr-Mayer, H. Jessen, J. Jiménez, J. Clotet, S. Bru, Optimized biochemical method for human Polyphosphate quantification. *Methods* **234**, 211-222 (2025).
5. D. F. Soto, A. Recalde, A. Orell, S. V. Albers, A. Paradela, C. A. Navarro, C. A. Jerez, Global effect of the lack of inorganic polyphosphate in the extremophilic archaeon *Sulfolobus solfataricus*: A proteomic approach. *J. Proteomics* **191**, 143-152 (2019).
6. S. Sarkar, H. Sharma, S. Y. Hosen, J. S. Ladke, S. Moser, D. Balasubramanian, S. Raran-Kurussi, H. J. Jessen, R. Bhandari, M. Jaiswal, Probing metazoan polyphosphate biology using *Drosophila* reveals novel and conserved polyP functions. *Elife* **14**, RP104841 (2026).
7. R. Gerasimaite, S. Sharma, Y. Desfougères, A. Schmidt, A. Mayer, Coupled synthesis and translocation restrains polyphosphate to acidocalcisome-like vacuoles and prevents its toxicity. *J. Cell Sci.* **127**, 5093-104 (2014).
8. M. Hothorn, H. Neumann, E. D. Lenherr, M. Wehner, V. Rybin, P. O. Hassa, A. Uttenweiler, M. Reinhard, A. Schmidt, J. Seiler, A. G. Ladurner, C. Herrmann, K. Scheffzek, A. Mayer, Catalytic core of amembrane-associated eukaryotic polyphosphate polymerase. *Science* **324**, 513-6 (2009).
9. L. McCarthy, M. Downey, The emerging landscape of eukaryotic polyphosphatases. *FEBS Lett.* **597**, 1447-1461 (2023).
10. N. Andreeva, L. Ledova, L. Ryazanova, A. Tomashevsky, T. Kulakovskaya, M. Eldarov, Ppn2 endopolyphosphatase overexpressed in *Saccharomyces cerevisiae*: Comparison with Ppn1, Ppx1, and Ddp1 polyphosphatases. *Biochimie* **163**, 101-107 (2019).

11. S. Bru, J. M. Martínez-Laínez, S. Hernández-Ortega, E. Quandt, J. Torres-Torronteras, R. Martí, D. Canadell, J. Ariño, S. Sharma, J. Jiménez, J. Clotet, Polyphosphate is involved in cell cycle progression and genomic stability in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **101**, 367-80 (2016).
12. S. Bru, B. Samper-Martín, E. Quandt, S. Hernández-Ortega, J. M. Martínez-Laínez, E. Garí, M. Rafel, J. Torres-Torronteras, R. Martí, M. P. C. Ribeiro, J. Jiménez, J. Clotet, Polyphosphate is a key factor for cell survival after DNA damage in eukaryotic cells. *DNA Repair (Amst).* **57**, 171-178 (2017).
13. S. Bru, L. Michaillat Mayer, G.-D. Kim, D. Qiu, H. J. Jessen, A. Mayer, Acidocalcisome-like vacuoles constitute a feedback-controlled phosphate buffering system for the cytosol. *Elife* **14**, RP108181 (2025).
14. M. R. Thomas, E. K. O'Shea, An intracellular phosphate buffer filters transient fluctuations in extracellular phosphate levels. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 9565-70 (2005).
15. Y. Desfougères, A. Saiardi, C. Azevedo, Inorganic polyphosphate in mammals: Where's Wally? *Biochem. Soc. Trans.* **48**, 95-101 (2020).
16. K. D. Kumble, A. Kornberg, Endopolyphosphatases for long chain inorganic polyphosphate in yeast and mammals. *J. Biol. Chem.* **271**, 27146-51 (1996).
17. A. Y. Baev, P. R. Angelova, A. Y. Abramov, Inorganic polyphosphate is produced and hydrolyzed in F0F1-ATP synthase of mammalian mitochondria. *Biochem. J.* **477**, 151-1524 (2020).
18. L. McCarthy, K. Baijal, M. Downey, A framework for understanding and investigating polyphosphate-protein interactions. *Biochem. Soc. Trans.* **53**, 1-12 (2025).
19. E. Bondy-Chorney, I. Abramchuk, R. Nasser, C. Holinier, A. Denoncourt, K. Baijal, L. McCarthy, M. Khacho, M. Lavallée-Adam, M. Downey, A Broad Response to Intracellular Long-Chain Polyphosphate in Human Cells. *Cell Rep.* **33**, 108318 (2020).
20. S. M. Hassanian, P. Dinarvand, S. A. Smith, A. R. Rezaie, Inorganic polyphosphate elicits pro-inflammatory responses through activation of the mammalian target of rapamycin complexes 1 and 2 in vascular endothelial cells. *J. Thromb. Haemost.* **13**, 860-71 (2015).

21. F. Müller, N. J. Mutch, W. A. Schenk, S. A. Smith, L. Esterl, H. M. Spronk, S. Schmidbauer, W. A. Gahl, J. H. Morrissey, T. Renné, Platelet Polyphosphates Are Proinflammatory and Procoagulant Mediators In Vivo. *Cell* **139**, 1143-56 (2009).
22. C. Azevedo, F. Borghi, X. B. Su, A. Saiardi, On the covalent nature of lysine polyphosphorylation. *Mol. Cell* **84**, 1811-1815 (2024).
23. N. Neville, K. Lehotsky, K. A. Klupt, M. Downey, Z. Jia, Polyphosphate attachment to lysine repeats is a non-covalent protein modification. *Mol. Cell* **84**, 1802-1810 (2024).
24. K. Baijal, B. Kore, I. Abramchuk, A. Denoncourt, S. Han, A. Simms, A. Dagenais, A. R. Long, A. D. Rudner, M. Lavallée-Adam, M. J. Gray, M. Downey, Identification of polyphosphate-binding proteins in *Escherichia coli* uncovers targets involved in translation control and ribosome biogenesis. *mBio* **16**, e0050025 (2025).
25. A. Bentley-DeSousa, C. Holinier, H. Moteshareie, Y. C. Tseng, S. Kajjo, C. Nwosu, G. F. Amodeo, E. Bondy-Chorney, Y. Sai, A. Rudner, A. Golshani, N. E. Davey, M. Downey, A Screen for Candidate Targets of Lysine Polyphosphorylation Uncovers a Conserved Network Implicated in Ribosome Biogenesis. *Cell Rep.* **22**, 3427-3439 (2018).
26. C. Azevedo, T. Livermore, A. Saiardi, Protein polyphosphorylation of lysine residues by inorganic polyphosphate. *Mol. Cell* **58**, 71-82 (2015).
27. L. McCarthy, A. Bentley-DeSousa, A. Denoncourt, Y. C. Tseng, M. Gabriel, M. Downey, Proteins required for vacuolar function are targets of lysine polyphosphorylation in yeast. *FEBS Lett.* **594**, 21-30 (2020).
28. H. Zhang, K. Ishige, A. Kornberg, A polyphosphate kinase (PPK2) widely conserved in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 16678-83 (2002).
29. C. M. Tzeng, A. Kornberg, The multiple activities of polyphosphate kinase of *Escherichia coli* and their subunit structure determined by radiation target analysis. *J. Biol. Chem.* **275**, 3977-83 (2000).
30. A. Kornberg, S. R. Kornberg, E. S. Simms, Metaphosphate synthesis by an enzyme from *Escherichia coli*. *Biochim. Biophys, Acta* **20**, 215-27 (1956).
31. A. Kuroda, A. Kornberg, Polyphosphate kinase as a nucleoside diphosphate kinase in *Escherichia coli* and *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 439-42 (1997).

32. S. R. Kornberg, Adenosine triphosphate synthesis from polyphosphate by an enzyme from *Escherichia coli*. *Biochim. Biophys. Acta* **26**, 294-300 (1957).
33. A. Kuroda, S. Tanaka, T. Ikeda, J. Kato, N. Takiguchi, H. Ohtake, Inorganic polyphosphate kinase is required to stimulate protein degradation and for adaptation to amino acid starvation in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14264-14269 (1999).
34. M. J. Gray, W. Y. Wholey, N. O. Wagner, C. M. Cremers, A. Mueller-Schickert, N. T. Hock, A. G. Krieger, E. M. Smith, R. A. Bender, J. C. A. Bardwell, U. Jakob, Polyphosphate Is a Primordial Chaperone. *Mol. Cell* **53**, 689-99 (2014).
35. H. Lv, Y. Zhou, B. Liu, J. Guan, P. Zhang, X. Deng, D. Li, J. Wang, Polyphosphate Kinase Is Required for the Processes of Virulence and Persistence in *Acinetobacter baumannii*. *Microbiol. Spectr.* **10**, e0123022 (2022).
36. N. N. Rao, A. Kornberg, Inorganic polyphosphate supports resistance and survival of stationary-phase *Escherichia coli*. *J. Bacteriol.* **178**, 1394-400 (1996).
37. K. Sureka, S. Dey, P. Datta, A. K. Singh, A. Dasgupta, S. Rodrigue, J. Basu, M. Kundu, Polyphosphate kinase is involved in stress-induced mprAB-sigE-rel signalling in mycobacteria. *Mol. Microbiol.* **65**, 261-76 (2007).
38. A. J. Sussman, C. Gilvarg, Protein turnover in amino acid-starved strains of *Escherichia coli* K-12 differing in their ribonucleic acid control. *J. Biol. Chem.* **244**, 6304-6 (1969).
39. A. Kuroda, K. Nomura, R. Ohtomo, J. Kato, T. Ikeda, N. Takiguchi, H. Ohtake, A. Kornberg, Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* **293**, 705-8 (2001).
40. M. H. Gross, I. Konieczny, Polyphosphate induces the proteolysis of ADP-bound fraction of initiator to inhibit DNA replication initiation upon stress in *Escherichia coli*. *Nucleic Acids Res.* **48**, 5457-5466 (2020).
41. E. Boguszewska, Z. Hirsz, M. Sroka, K. Bury, W. Chmura, A. Strzałka, M. Kołodziej, J. Zakrzewska-Czerwińska, I. Konieczny, PolyP inhibits CobB deacetylase triggering a regulatory coupling to prevent DNA replication initiation. *Nucleic Acids Res.* **54**, gkag144 (2026).
42. J. Guan, R. L. Hurto, A. Rai, J. Bhattrai, C. A. Azaldegui, L. A. Ortiz-Rodríguez, Q. Liu, J. S. Biteen, L. Freddolino, U. Jakob, Polyphosphate modulates the stress-responsive

formation of functional RNA-protein condensates in bacteria and mammalian cells. *PLoS Biol.* **24**, e3003775 (2026).

43. E. Blum, B. Py, A. J. Carpousis, C. F. Higgins, Polyphosphate kinase is a component of the *Escherichia coli* RNA degradosome. *Mol. Microbiol.* **26**, 387-98 (1997).
44. P. McInerney, T. Mizutani, T. Shiba, Inorganic polyphosphate interacts with ribosomes and promotes translation fidelity in vitro and in vivo. *Mol. Microbiol.* **60**, 438-47 (2006).
45. N. G. Yoo, S. Dogra, B. A. Meinen, E. Tse, J. Haefliger, D. R. Southworth, M. J. Gray, J. U. Dahl, U. Jakob, Polyphosphate Stabilizes Protein Unfolding Intermediates as Soluble Amyloid-like Oligomers. *J. Mol. Biol.* **430**, 4195-4208 (2018).
46. K. S. Kim, N. N. Rao, C. D. Fraley, A. Kornberg, Inorganic polyphosphate is essential for long-term survival and virulence factors in *Shigella* and *Salmonella* spp. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 7675-80 (2002).
47. F. Beaufay, E. Quarles, A. Franz, O. Katamanin, W. Y. Wholey, U. Jakob, Polyphosphate functions in vivo as an iron chelator and fenton reaction inhibitor. *mBio* **11**, e01017-20 (2020).
48. M. H. Rashid, K. Rumbaugh, L. Passador, D. G. Davies, A. N. Hamood, B. H. Iglewski, A. Kornberg, Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 9636-41 (2000).
49. J. U. Dahl, M. J. Gray, D. Bazopoulou, F. Beaufay, J. Lempart, M. J. Koenigsknecht, Y. Wang, J. R. Baker, W. L. Hasler, V. B. Young, D. Sun, U. Jakob, The anti-inflammatory drug mesalamine targets bacterial polyphosphate accumulation. *Nat. Microbiol.* **2**, 16267 (2017).
50. H. L. Candon, B. J. Allan, C. D. Fraley, E. C. Gaynor, Polyphosphate kinase 1 is a pathogenesis determinant in *Campylobacter jejuni*. *J. Bacteriol.* **189**, 8099-108 (2007).
51. M. H. Rashid, A. Kornberg, Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4885-90 (2000).
52. Y. Du, X. Wang, Z. Han, Y. Hua, K. Yan, B. Zhang, W. Zhao, C. Wan, Polyphosphate Kinase 1 Is a Pathogenesis Determinant in Enterohemorrhagic *Escherichia coli* O157:H7. *Front. Microbiol.* **12**, 762171 (2021).

53. J. Roewe, G. Stavrides, M. Strueve, A. Sharma, F. Marini, A. Mann, S. A. Smith, Z. Kaya, B. Strobl, M. Mueller, C. Reinhardt, J. H. Morrissey, M. Bosmann, Bacterial polyphosphates interfere with the innate host defense to infection. *Nat. Commun.* **11**, 4035 (2020).

54. S. Chugh, P. Tiwari, C. Suri, S. K. Gupta, P. Singh, R. Bouzeyen, S. Kidwai, M. Srivastava, N. R. Rameshwaram, Y. Kumar, S. Asthana, R. Singh, Polyphosphate kinase-1 regulates bacterial and host metabolic pathways involved in pathogenesis of *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* **121**, e2309664121 (2024).

55. D. Rojas, A. E. Marcoleta, M. Gálvez-Silva, M. A. Varas, M. Díaz, M. Hernández, C. Vargas, G. Nourdin-Galindo, E. Koch, P. Saldivia, J. Vielma, Y. H. Gan, Y. Chen, N. Guiliani, F. P. Chávez, Inorganic Polyphosphate Affects Biofilm Assembly, Capsule Formation, and Virulence of Hypervirulent ST23 *Klebsiella pneumoniae*. *ACS Infect. Dis.* **10**, 606-623 (2024).

56. R. Rijal, L. A. Cadena, M. R. Smith, J. F. Carr, R. H. Gomer, Polyphosphate is an extracellular signal that can facilitate bacterial survival in eukaryotic cells. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 31923-31934 (2020).

57. K. Baijal, M. Downey, Targeting polyphosphate kinases in the fight against *Pseudomonas aeruginosa*. *mBio* **12**, e0147721 (2021).

58. N. Neville, N. Roberge, X. Ji, P. Stephen, J. L. Lu, Z. Jia, A dual-specificity inhibitor targets polyphosphate kinase 1 and 2 enzymes to attenuate virulence of *Pseudomonas aeruginosa*. *mBio* **12**, e0059221 (2021).

59. K. Baijal, I. Abramchuk, C. M. Herrera, T. F. Mah, M. S. Trent, M. Lavallée-Adam, M. Downey, Polyphosphate kinase regulates LPS structure and polymyxin resistance during starvation in *E. coli*. *PLoS Biol.* **22**, e3002558 (2024).

60. K. D. Kumble, K. Ahn, A. Kornberg, Phosphohistidyl active sites in polyphosphate kinase of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14391-5 (1996).

61. Y. Zhu, W. Huang, S. S. K. Lee, W. Xu, Crystal structure of a polyphosphate kinase and its implications for polyphosphate synthesis. *EMBO Rep.* **6**, 681-7 (2005).

62. K. Ahn, A. Kornberg, Polyphosphate kinase from *Escherichia coli*: Purification and demonstration of a phosphoenzyme intermediate. *J. Biol. Chem.* **265**, 11734-9 (1990).

63. M. Akiyama, E. Crooke, A. Kornberg, An exopolyphosphatase of *Escherichia coli*: The enzyme and its ppx gene in a polyphosphate operon. *J. Biol. Chem.* **268**, 633-9 (1993).
64. J. Alvarado, A. Ghosh, T. Janovitz, A. Jauregui, M. S. Hasson, D. A. Sanders, Origin of Exopolyphosphatase Processivity: Fusion of an ASKHA Phosphotransferase and a Cyclic Nucleotide Phosphodiesterase Homolog. *Structure* **14**, 1263-1272 (2006).
65. A. Kuroda, H. Murphy, M. Cashel, A. Kornberg, Guanosine tetra- and pentaphosphate promote accumulation of inorganic polyphosphate in *Escherichia coli*. *J. Biol. Chem.* **272**, 21240-3 (1997).
66. N. N. Rao, S. Liu, A. Kornberg, Inorganic polyphosphate in *Escherichia coli*: The phosphate regulon and the stringent response. *J. Bacteriol.* **180**, 2186-93 (1998).
67. M. J. Gray, Inorganic polyphosphate accumulation in *Escherichia coli* is regulated by DksA but not by (p)ppGpp. *J. Bacteriol.* **201**, e00664-18 (2019).
68. D. Ault-Riché, C. D. Fraley, C. M. Tzeng, A. Kornberg, Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. *J. Bacteriol.* **180**, 1841-7 (1998).
69. M. J. Gray, Interactions between DksA and stress-responsive alternative sigma factors control inorganic polyphosphate accumulation in *Escherichia coli*. *J. Bacteriol.* **202**, e00133-20 (2020).
70. S. Sanyal, S. K. Banerjee, R. Banerjee, J. Mukhopadhyay, M. Kundu, Polyphosphate kinase 1, a central node in the stress response network of *Mycobacterium tuberculosis*, connects the two-component systems MprAB and SenX3-RegX3 and the extracytoplasmic function sigma factor, sigma E. *Microbiology (United Kingdom)* **159**, 2074-2086 (2013).
71. S. Ghorbel, A. Smirnov, H. Chouayekh, B. Sperandio, C. Esnault, J. Kormanec, M. J. Virolle, Regulation of ppk expression and in vivo function of Ppk in *Streptomyces lividans* TK24. *J. Bacteriol.* **188**, 6269-76 (2006).
72. A. K. Rudat, A. Pokhrel, T. J. Green, M. J. Gray, Mutations in *Escherichia coli* polyphosphate kinase that lead to dramatically increased in vivo polyphosphate levels. *J. Bacteriol.* **200**, e00697-17 (2018).

73. M. Q. Bowlin, A. D. Lieber, A. R. Long, M. J. Gray, C-terminal Poly-histidine Tags Alter *Escherichia coli* Polyphosphate Kinase Activity and Susceptibility to Inhibition. *J. Mol. Biol.* **436**, 168651 (2024).
74. K. Makino, H. Shinagawa, M. Amemura, T. Kawamoto, M. Yamada, A. Nakata, Signal transduction in the phosphate regulon of *Escherichia coli* involves phosphotransfer between PhoR and PhoB proteins. *J. Mol. Biol.* **210**, 551-9 (1989).
75. S. G. Gardner, W. R. McCleary, Control of the phoBR Regulon in *Escherichia coli*. *EcoSal Plus* **8**, 10.1128/ecosalplus.ESP-0006-2019 (2019).
76. K. Motomura, R. Hirota, N. Ohnaka, M. Okada, T. Ikeda, T. Morohoshi, H. Ohtake, A. Kuroda, Overproduction of YjbB reduces the level of polyphosphate in *Escherichia coli*: A hypothetical role of YjbB in phosphate export and polyphosphate accumulation. *FEMS Microbiol. Lett.* **320**, 25-32 (2011).
77. M. Q. Bowlin, A. R. Long, J. T. Huffines, M. J. Gray, The role of nitrogen-responsive regulators in controlling inorganic polyphosphate synthesis in *Escherichia coli*. *Microbiology (United Kingdom)* **168**, 001185 (2022).
78. E. J. Capra, M. T. Laub, Evolution of two-component signal transduction systems. *Annu. Rev. Microbiol.* **66**, 325-47 (2012).
79. M. Y. Galperin, A census of membrane-bound and intracellular signal transduction proteins in bacteria: Bacterial IQ, extroverts and introverts. *BMC Microbiol.* **5** (2005).
80. B. T. Nixon, C. W. Ronson, F. M. Ausubel, Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes ntrB and ntrC. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 7850-4 (1986).
81. T. Mizuno, Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. *DNA Res.* **4**, 161-8 (1997).
82. T. Krell, J. Lacal, A. Busch, H. Silva-Jiménez, M. E. Guazzaroni, J. L. Ramos, Bacterial sensor kinases: Diversity in the recognition of environmental signals. *Annu. Rev. Microbiol.* **64**, 539-59 (2010).
83. R. Gao, T. R. Mack, A. M. Stock, Bacterial response regulators: versatile regulatory strategies from common domains. *Trends Biochem. Sci.* **32**, 225-234 (2007).

84. P. Casino, V. Rubio, A. Marina, The mechanism of signal transduction by two-component systems. *Curr. Opin. Struct. Biol.* **20**, 763-71 (2010).
85. K. Yamamoto, K. Hirao, T. Oshima, H. Aiba, R. Utsumi, A. Ishihama, Functional characterization in vitro of all two-component signal transduction systems from *Escherichia coli*. *J. Biol. Chem.* **280**, 1448-56 (2005).
86. K. Ishige, S. Nagasawa, S. Tokishita, T. Mizuno, A novel device of bacterial signal transducers. *EMBO J.* **13**, 5195-202 (1994).
87. D. Georgellis, A. S. Lynch, E. C. Lin, In vitro phosphorylation study of the Arc two-component signal transduction system of *Escherichia coli*. *J. Bacteriol.* **179**, 5429-35 (1997).
88. R. Gao, A. M. Stock, Probing kinase and phosphatase activities of two-component systems in vivo with concentration-dependent phosphorylation profiling. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 672-7 (2012).
89. T. L. Raivio, T. J. Silhavy, Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *J. Bacteriol.* **179**, 7724-33 (1997).
90. L. Zhou, X. H. Lei, B. R. Bochner, B. L. Wanner, Phenotype MicroArray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J. Bacteriol.* **185** (2003).
91. T. Oshima, H. Aiba, Y. Masuda, S. Kanaya, M. Sugiura, B. L. Wanner, H. Mori, T. Mizuno, Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol. Microbiol.* **46**, 281-91 (2002).
92. J. T. Lazar, J. J. Tabor, Bacterial two-component systems as sensors for synthetic biology applications. *Curr. Opin. Syst. Biol.* **28**, 100398 (2021).
93. P. Ortet, D. E. Whitworth, C. Santaella, W. Achouak, M. Barakat, P2CS: Updates of the prokaryotic two-component systems database. *Nucleic Acids Res.* **43**, D536-41 (2015).
94. M. Barakat, P. Ortet, C. Jourlin-Castelli, M. Ansaldi, V. Méjean, D. E. Whitworth, P2CS: A two-component system resource for prokaryotic signal transduction research. *BMC Genomics* **10**, 315 (2009).
95. J. R. Brannon, S. A. Reasoner, T. A. Bermudez, S. L. Comer, M. A. Wiebe, T. L. Dunigan, C. J. Beebout, T. Ross, A. Bamidele, M. Hadjifrangiskou, Mapping niche-

specific two-component system requirements in uropathogenic *Escherichia coli*. *Microbiol. Spectr.* **12**, e02236-23 (2024).

96. C. Zhong, P. Zhang, C. Liu, M. Liu, W. Chen, J. Fu, X. Qi, G. Cao, The PolS-PolR Two-Component System Regulates Genes Involved in Poly-P Metabolism and Phosphate Transport in *Microlunatus phosphovorus*. *Front. Microbiol.* **10**, 2127 (2019).
97. T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, H. Mori, Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol.* **2**, MSB4100050 (2006).
98. K. A. Datsenko, B. L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 6640-5 (2000).
99. S. Datta, N. Costantino, D. L. Court, A set of recombineering plasmids for gram-negative bacteria. *Gene* **379**, 109-15 (2006).
100. P. P. Cherepanov, W. Wackernagel, Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**, 9-14 (1995).
101. S. Uzzau, N. Figueroa-Bossi, S. Rubino, L. Bossi, Epitope tagging of chromosomal genes in *Salmonella*. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 15264-9 (2001).
102. L. M. Guzman, D. Belin, M. J. Carson, J. Beckwith, Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* **177**, 4121-30 (1995).
103. S. Bru, J. Jiménez, D. Canadell, J. Ariño, J. Clotet, Improvement of biochemical methods of polyP quantification. *Microb. Cell.* **4**, 6-15 (2016).
104. J. J. Christ, L. M. Blank, Enzymatic quantification and length determination of polyphosphate down to a chain length of two. *Anal. Biochem.* **548**, 82-90 (2018).
105. B. Geissler, D. Elraheb, W. Margolin, A gain-of-function mutation in *ftsA* bypasses the requirement for the essential cell division gene *zipA* in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 4197-202 (2003).
106. G. Malherbe, D. P. Humphreys, E. Davé, A robust fractionation method for protein subcellular localization studies in *Escherichia coli*. *Biotechniques* **66**, 171-178 (2019).

107. A. J. Wolfe, N. Parikh, B. P. Lima, B. Zemaitaitis, Signal integration by the two-component signal transduction response regulator CpxR. *J. Bacteriol.* **190**, 2314-22 (2008).
108. E. J. Clarke, C. A. Voigt, Characterization of combinatorial patterns generated by multiple two-component sensors in *E. coli* that respond to many stimuli. *Biotechnol. Bioeng.* **108**, 666-75 (2011).
109. K. L. May, K. M. Lehman, A. M. Mitchell, M. Grabowicz, A stress response monitoring lipoprotein trafficking to the outer membrane. *mBio* **10**, e00618-19 (2019).
110. Z. Zhao, Y. Xu, B. Jiang, Q. Qi, Y.J. Tang, M. Xian, J. Wang, G. Zhao, Systematic Identification of CpxRA-Regulated Genes and Their Roles in *Escherichia coli* Stress Response. *mSystems* **7**, e0041922 (2022).
111. A. Delhaye, G. Laloux, J. F. Collet, The lipoprotein NlpE is a cpx sensor that serves as a sentinel for protein sorting and folding defects in the *Escherichia coli* envelope. *J. Bacteriol.* **201**, e00611-18 (2019).
112. T. L. Raivio, Everything old is new again: An update on current research on the Cpx envelope stress response. *Biochim. Biophys. Acta Mol. Cell Res.* **1843**, 1529-1541 (2014).
113. A. Delhaye, J. F. Collet, G. Laloux, Fine-tuning of the Cpx envelope stress response is required for cell wall homeostasis in *Escherichia coli*. *mBio* **7**, e00047-16 (2016).
114. P. De Wulf, A. M. McGuire, X. Liu, E. C. C. Lin, Genome-wide profiling of promoter recognition by the two-component response regulator CpxR-P in *Escherichia coli*. *J. Biol. Chem.* **277**, 26652-61 (2002).
115. L. Dbeibo, J. J. van Rensburg, S. N. Smith, K. R. Fortney, D. Gangaiah, H. Gao, J. Marzoa, Y. Liu, H. L. T. Mobley, S. M. Spinola, Evaluation of CpxRA as a therapeutic target for uropathogenic *Escherichia coli* infections. *Infect. Immun.* **86**, e00798-17 (2018).
116. N. L. Price, T. L. Raivio, Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *J. Bacteriol.* **191**, 1798-815 (2009).
117. B. P. Lima, T. T. T. Huyen, K. Bäsell, D. Becher, H. Antelmann, A. J. Wolfe, Inhibition of acetyl phosphate-dependent transcription by an acetylatable lysine on RNA polymerase. *J. Biol. Chem.* **287**, 32147-60 (2012).

118. B. P. Lima, H. Antelmann, K. Gronau, B. K. Chi, D. Becher, S. R. Brinsmade, A. J. Wolfe, Involvement of protein acetylation in glucose-induced transcription of a stress-responsive promoter. *Mol. Microbiol.* **81**, 1190-204 (2011).
119. P. A. DiGiuseppe, T. J. Silhavy, Signal detection and target gene induction by the CpxRA two-component system. *J. Bacteriol.* **185**, 2432-40 (2003).
120. P. N. Danese, T. J. Silhavy, CpxP, a stress-combative member of the Cpx regulon. *J. Bacteriol.* **180**, 831-9 (1998).
121. T. L. Raivio, D. L. Popkin, T. J. Silhavy, The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J. Bacteriol.* **181**, 5263-72 (1999).
122. R. Fleischer, R. Heermann, K. Jung, S. Hunke, Purification, reconstitution, and characterization of the CpxRAP envelope stress system of *Escherichia coli*. *J. Biol. Chem.* **282**, 8583-93 (2007).
123. D. D. Isaac, J. S. Pinkner, S. J. Hultgren, T. J. Silhavy, The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 17775-17779 (2005).
124. D. Georgellis, O. Kwon, E. C. C. Lin, Quinones as the redox signal for the Arc two-component system of bacteria. *Science* **292**, 2314-2316 (2001).
125. R. Malpica, B. Franco, C. Rodriguez, O. Kwon, D. Georgellis, Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 13318-23 (2004).
126. D. M. Park, M. S. Akhtar, A. Z. Ansari, R. Landick, P. J. Kiley, The Bacterial Response Regulator ArcA Uses a Diverse Binding Site Architecture to Regulate Carbon Oxidation Globally. *PLoS Genet.* **9**, e1003839 (2013).
127. H. Jiang, M. Milanov, G. Jüngert, L. Angebauer, C. Flender, E. Smudde, F. Gather, T. Vogel, H. J. Jessen, H.-G. Koch, Control of a chemical chaperone by a universally conserved ATPase. *iScience* **27**, 110215 (2024).
128. F. Mika, R. Hengge, A two-component phosphotransfer network involving ArcB, ArcA, and RssB coordinates synthesis and proteolysis of σS (RpoS) in *E. coli*. *Genes Dev.* **19**, 2770-81 (2005).

129. M. Akiyama, E. Crooke, A. Kornberg, The polyphosphate kinase gene of *Escherichia coli*: Isolation and sequence of the ppk gene and membrane location of the protein. *J. Biol. Chem.* **267**, 22556-61 (1992).
130. O. Sen, X. Liu, S. Kjelleberg, S. A. Rice, T. Seviour, Potential confounding mutations in Keio knockout strains: implications for research accuracy. *Microbiol. Spectr.* **13**, e0203624 (2025).
131. A. Mateus, M. Shah, J. Hevler, N. Kurzawa, J. Bobonis, A. Typas, M. M. Savitski, Transcriptional and Post-Transcriptional Polar Effects in Bacterial Gene Deletion Libraries. *mSystems* **6**, e00813-21 (2021).
132. S. Leonhartsberger, A. Huber, F. Lottspeich, A. Böck, The hydH/G genes from *Escherichia coli* code for a zinc and lead responsive two-component regulatory system. *J. Mol. Biol.* **307**, 93-105 (2001).
133. A. J. Wolfe, Physiologically relevant small phosphodonors link metabolism to signal transduction. *Curr. Opin. Microbiol.* **13**, 204-9 (2010).
134. B. T. Weinert, V. Iesmantavicius, S. A. Wagner, C. Schölz, B. Gummesson, P. Beli, T. Nyström, C. Choudhary, Acetyl-Phosphate Is a Critical Determinant of Lysine Acetylation in *E. coli*. *Mol. Cell* **51**, 265–272 (2013).
135. K. Tschauner, P. Hörnschemeyer, V. S. Müller, S. Hunke, Dynamic Interaction between the CpxA sensor kinase and the periplasmic accessory protein CpxP mediates signal recognition in *E. coli*. *PLoS One* **9**, e107383 (2014).
136. T. L. Raivio, S. K. D. Leblanc, N. L. Price, The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *J. Bacteriol.* **195**, 2755-67 (2013).
137. K. Surmann, E. Ćudić, E. Hammer, S. Hunke, Molecular and proteome analyses highlight the importance of the Cpx envelope stress system for acid stress and cell wall stability in *Escherichia coli*. *Microbiologyopen* **5**, 582-96 (2016).
138. M. Papanastasiou, G. Orfanoudaki, M. Koukaki, N. Kountourakis, M. F. Sardis, M. Aivaliotis, S. Karamanou, A. Economou, The *Escherichia coli* peripheral inner membrane proteome. *Mol. Cell. Proteomics* **12**, 599-610 (2013).
139. A. Karginov, M. Agaphonov, A simple enrichment procedure improves detection of membrane proteins by immunoblotting. *Biotechniques* **61**, 260-261 (2016).

140. C. A. Hale, P. A. J. de Boer, Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli*. *Cell* **88**, 175-85 (1997).
141. S. Shu, W. Mi, Separating Inner and Outer Membranes of *Escherichia coli* by EDTA-free Sucrose Gradient Centrifugation. *Bio. Protoc.* **13**, e4638 (2023).
142. M. Amemura, K. Makino, H. Shinagawa, A. Nakata, Cross talk to the phosphate regulon of *Escherichia coli* by PhoM protein: PhoM is a histidine protein kinase and catalyzes phosphorylation of PhoB and PhoM-open reading frame 2. *J. Bacteriol.* **172**, 6300-7 (1990).
143. M. Varas, C. Valdivieso, C. Mauriaca, J. Ortíz-Severín, A. Paradela, I. Poblete-Castro, R. Cabrera, F. P. Chávez, Multi-level evaluation of *Escherichia coli* polyphosphate related mutants using global transcriptomic, proteomic and phenomic analyses. *Biochim. Biophys. Acta Gen. Subj.* **1861**, 871-883 (2017).
144. M. Grillo-Puertas, L. A. Schurig-Briccio, L. Rodríguez-Montelongo, M. R. Rintoul, V. A. Rapisarda, Copper tolerance mediated by polyphosphate degradation and low-affinity inorganic phosphate transport system in *Escherichia coli*. *BMC Microbiol.* **14**, 72 (2014).
145. R. L. Guest, J. Wang, J. L. Wong, T. L. Raivio, A bacterial stress response regulates respiratory protein complexes to control envelope stress adaptation. *J. Bacteriol.* **199**, e00153-17 (2017).
146. V. Tsviklist, R. L. Guest, T. L. Raivio, The Cpx Stress Response Regulates Turnover of Respiratory Chain Proteins at the Inner Membrane of *Escherichia coli*. *Front. Microbiol.* **12**, 732288 (2022).
147. M. Bekker, S. Alexeeva, W. Laan, G. Sawers, J. T. de Mattos, K. Hellingwerf, The ArcBA two-component system of *Escherichia coli* is regulated by the redox state of both the ubiquinone and the menaquinone pool. *J. Bacteriol.* **192**, 746-54 (2010).
148. T. F. Mahoney, T. J. Silhavy, The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics. *J. Bacteriol.* **195**, 1869-74 (2013).
149. C. Loui, A. C. Chang, S. Lu, Role of the ArcAB two-component system in the resistance of *Escherichia coli* to reactive oxygen stress. *BMC Microbiol.* **9**, 183 (2009).
150. T. Shiba, K. Tsutsumi, H. Yano, Y. Ihara, A. Kameda, K. Tanaka, H. Takahashi, M. Munekata, N. N. Rao, A. Kornberg, Inorganic polyphosphate and the induction of rpoS expression. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11210-5 (1997).

151. C. Arndt, S. Koristka, H. Bartsch, M. Bachmann, Native polyacrylamide gels. *Methods Mol. Biol.* **869**, 49-53 (2012).
152. I. Wittig, H. P. Braun, H. Schägger, Blue native PAGE. *Nat. Protoc.* **1**, 418-28 (2006).
153. S. F. Feldman, E. Temkin, L. Wullfhart, A. Nutman, V. Schechner, P. Shitrit, R. Shvartz, M. J. Schwaber, A. Andremont, Y. Carmeli, A nationwide population-based study of *Escherichia coli* bloodstream infections: incidence, antimicrobial resistance and mortality. *Clin. Microbiol. Infect.* **28**, 879.e1-879.e7 (2022).
154. M. Bonten, J. R. Johnson, A. H. J. van den Biggelaar, L. Georgalis, J. Geurtsen, P. I. de Palacios, S. Gravenstein, T. Verstraeten, P. Hermans, J. T. Poolman, Epidemiology of *Escherichia coli* Bacteremia: A Systematic Literature Review. *Clin. Infect. Dis.* **72**, 1211-1219 (2021).
155. A. N. Brown, M. T. Anderson, M. A. Bachman, H. L. T. Mobley, The ArcAB Two-Component System: Function in Metabolism, Redox Control, and Infection. *Microbiol. Mol. Biol. Rev.* **86**, e0011021 (2022).
156. W. Cai, Y. Wannemuehler, G. Dell'Anna, B. Nicholson, N. L. Barbieri, S. Kariyawasam, Y. Feng, C. M. Logue, L. K. Nolan, G. Li, A Novel Two-Component Signaling System Facilitates Uropathogenic *Escherichia coli*'s Ability to Exploit Abundant Host Metabolites. *PLoS Pathog.* **9**, e1003428 (2013).
157. X. Li, Y. Pang, L. Jiang, L. Liu, J. Zhou, C. Jin, Q. Wang, H. Sun, Q. Li, Z. Chen, J. Qin, J. Mu, B. Liu, Q. Zhang, Y. Liu, L. Feng, L. Wang, Two-component system GrpP/GrpQ promotes pathogenicity of uropathogenic *Escherichia coli* CFT073 by upregulating type 1 fimbria. *Nat. Commun.* **16**, 607 (2025).
158. Y. Zhang, X. Qiu, J. Luo, H. Li, S. W. How, D. Wu, J. He, Z. Cheng, Y. Gao, H. Lu, A review of the phosphorus removal of polyphosphate-accumulating organisms in natural and engineered systems. *Sci. Total Environ.* **912**, 169103 (2024).
159. M. Liang, S. Frank, H. Lünsdorf, M. J. Warren, M. B. Prentice, Bacterial microcompartment-directed polyphosphate kinase promotes stable polyphosphate accumulation in *E. coli*. *Biotechnol. J.* **12**, 1600415 (2017).
160. J. Kato, K. Yamada, A. Muramatsu, Hardoyo, H. Ohtake, Genetic improvement of *Escherichia coli* for enhanced biological removal of phosphate from wastewater. *Appl. Environ. Microbiol.* **59**, 3744-9 (1993).

8. APPENDIX

Table S1: List of strains used in this work.

Internal reference number	Strain background	Genotype	Marker	Figure or table #
BMD541	MG1655	WT (F^- λ^- *rph*-1 *ilvG*$^-$ *rfb*-50)		Figures 2B, 3B, 3D, 6
BMD542	MG1655	Δ*ppk* (KanR excised)		Figures 2B, 3B, 6
BMD577	BW25113	WT (*rrnB*3 Δ*lacZ*4787 *hsdR*514 Δ(*araBAD*)567 Δ(*rhaBAD*)568 *rph*-1		Figures 2A and 2B
BMD590	BW25113	Δ*ppk* (KanR excised)		Figures 2A and 2B
Keio plate 45	BW25113	Δ*htpG*::KanR	Kan+	Figure 2A
Keio plate 46	BW25113	Δ*htpG*::KanR	Kan+	Figure 2A
Keio plate 5	BW25113	Δ*cpxA*::KanR *	Kan+	Figure 2A
Keio plate 6	BW25113	Δ*cpxA*::KanR	Kan+	Figure 2A
Keio plate 71	BW25113	Δ*hslO*::KanR	Kan+	Figure 2A
Keio plate 72	BW25113	Δ*hslO*::KanR	Kan+	Figure 2A
Keio plate 1	BW25113	Δ*rcsC*::KanR	Kan+	Figure 2A
Keio plate 2	BW25113	Δ*rcsC*::KanR	Kan+	Figure 2A
BMD685	MG1655	Δ*htpG*::KanR	Kan+	Figure 2B
BMD687	MG1655	Δ*cpxA*::KanR	Kan+	Figure 2B and Table 1
BMD690	MG1655	Δ*hslO*::KanR	Kan+	Figure 2B
BMD691	MG1655	Δ*rcsC*::KanR	Kan+	Figure 2B and Table 1
BMD693	BW25113	Δ*htpG*::KanR	Kan+	Figure 2B
BMD695	BW25113	Δ*cpxA*::KanR	Kan+	Figure 2B
BMD698	BW25113	Δ*hslO*::KanR	Kan+	Figure 2B
BMD699	BW25113	Δ*rcsC*::KanR	Kan+	Figure 2B
BMD732	MG1655	Δ*cpxR*::KanR	Kan+	Table 1
BMD764	MG1655	Δ*envZ*::KanR	Kan+	Table 1
BMD766	MG1655	Δ*hprS*::KanR	Kan+	Table 1
BMD768	MG1655	Δ*zraS*::KanR	Kan+	Table 1
BMD805	MG1655	Δ*kdpD*::KanR	Kan+	Table 1
BMD808	MG1655	Δ*phoQ*::KanR	Kan+	Table 1
BMD810	MG1655	Δ*barA*::KanR	Kan+	Table 1
BMD820	MG1655	Δ*creC*::KanR	Kan+	Table 1

BMD822	MG1655	Δ*glnL*::KanR	Kan+	Table 1
BMD878	MG1655	Δ*btsS*::KanR	Kan+	Table 1
BMD880	MG1655	Δ*ypdA*::KanR	Kan+	Table 1
BMD886	MG1655	Δ*uhpB*::KanR	Kan+	Table 1
BMD888	MG1655	Δ*baeS*::KanR	Kan+	Table 1
BMD894	MG1655	Δ*phoB*::KanR	Kan+	Table 1
BMD954	MG1655	Δ*arcB*::KanR	Kan+	Table 1
BMD956	MG1655	Δ*cusS*::KanR	Kan+	Table 1
BMD973	MG1655	Δ*narX*::KanR	Kan+	Table 1
BMD975	MG1655	Δ*atoS*::KanR	Kan+	Table 1
BMD986	MG1655	Δ*dcuS*::KanR	Kan+	Table 1
BMD988	MG1655	Δ*basS*::KanR	Kan+	Table 1
BMD990	MG1655	Δ*dpiB*::KanR	Kan+	Table 1
BMD1005	MG1655	Δ*arcA*::KanR	Kan+	Table 1
BMD1009	MG1655	Δ*rssB*::KanR	Kan+	Table 1
BMD1025	MG1655	Δ*evgS*::KanR	Kan+	Table 1
BMD1027	MG1655	Δ*torS*::KanR	Kan+	Table 1
BMD1029	MG1655	Δ*ompR*::KanR	Kan+	Table 1
BMD1031	MG1655	Δ*phoP*::KanR	Kan+	Table 1
BMD1033	MG1655	Δ*kdpE*::KanR	Kan+	Table 1
BMD1036	MG1655	Δ*qseE*::KanR	Kan+	Table 1
BMD1038	MG1655	Δ*cheA*::KanR	Kan+	Table 1
BMD1040	MG1655	Δ*rcsB*::KanR	Kan+	Table 1
BMD1042	MG1655	Δ*narQ*::KanR	Kan+	Table 1
BMD1044	MG1655	Δ*ypdB*::KanR	Kan+	Table 1
BMD1046	MG1655	Δ*qseC*::KanR	Kan+	Table 1
BMD1048	MG1655	Δ*rstB*::KanR	Kan+	Table 1
BMD1057	MG1655	Δ*uhpA*::KanR	Kan+	Table 1
BMD1059	MG1655	Δ*baeR*::KanR	Kan+	Table 1
BMD1061	MG1655	Δ*phoR*::KanR	Kan+	Table 1
BMD1075	MG1655	Δ*creB*::KanR	Kan+	Table 1
BMD1077	MG1655	Δ*basR*::KanR	Kan+	Table 1
BMD1079	MG1655	Δ*btsR*::KanR	Kan+	Table 1
BMD1081	MG1655	Δ*uvrY*::KanR	Kan+	Table 1
BMD1113	MG1655	Δ*zraR*::KanR	Kan+	Table 1
BMD1117	MG1655	Δ*cusR*::KanR	Kan+	Table 1
BMD1119	MG1655	Δ*narL*::KanR	Kan+	Table 1
BMD1121	MG1655	Δ*glnG*::KanR	Kan+	Table 1
BMD1123	MG1655	Δ*hprR*::KanR	Kan+	Table 1

BMD1135	MG1655	Δ*rcsD*::KanR	Kan+	Table 1
BMD1137	MG1655	Δ*cheY*::KanR	Kan+	Table 1
BMD1139	MG1655	Δ*dcuR*::KanR	Kan+	Table 1
BMD1141	MG1655	Δ*dpiA*::KanR	Kan+	Table 1
BMD1143	MG1655	Δ*torR*::KanR	Kan+	Table 1
BMD1145	MG1655	Δ*evgA*::KanR	Kan+	Table 1
BMD1161	MG1655	Δ*glrR*::KanR	Kan+	Table 1
BMD1162	MG1655	Δ*atoC*::KanR	Kan+	Table 1
BMD1164	MG1655	Δ*rstA*::KanR	Kan+	Table 1
BMD1166	MG1655	Δ*narP*::KanR	Kan+	Table 1
BMD1168	MG1655	Δ*qseB*::KanR	Kan+	Table 1
BMD1170	MG1655	Δ*cheB*::KanR	Kan+	Table 1
BMD1172	MG1655	Δ*fimZ*::KanR	Kan+	Table 1
BMD993	MG1655	WT pBAD18 (AmpR)	Amp+	Figure 3A
BMD995	MG1655	WT pBAD18-CpxA (AmpR)	Amp+	Figure 3A
BMD997	MG1655	Δ*cpxA* (KanR excised) pBAD18 (AmpR)	Amp+	Figure 3A
BMD999	MG1655	Δ*cpxA* (KanR excised) pBAD18-CpxA (AmpR)	Amp+	Figure 3A
BMD772	MG1655	Δ*cpxA* (KanR excised)		Figure 3B (gel)
BMD978	MG1655	Δ*cpxR* (KanR excised)		Figure 3B (gel)
BMD980	MG1655	Δ*cpxA* (KanR excised) Δ*cpxR* (KanR excised)		Figure 3B (gel)
BMD773	MG1655	Δ*cpxA* (KanR excised)		Figure 3D
BMD1013	MG1655	CpxP-3xFLAG-KanR	Kan+	Figure 4A
BMD1015	MG1655	Δ*cpxA* (KanR excised) CpxP-3xFLAG-KanR	Kan+	Figure 4A
BMD1019	MG1655	Δ*cpxA* (KanR excised) Δ*cpxR* (KanR excised) CpxP-3xFLAG-KanR	Kan+	Figure 4A
BMD994	MG1655	WT pBAD18 (AmpR)	Amp+	Figure 4B
BMD998	MG1655	Δ*cpxA* (KanR excised) pBAD18 (AmpR)	Amp+	Figure 4B
BMD1108	MG1655	WT pBAD18-NlpE (AmpR)	Amp+	Figure 4B
BMD1112	MG1655	Δ*cpxA* (KanR excised) pBAD18-NlpE (AmpR)	Amp+	Figure 4B
BMD982	MG1655	Δ*arcB* (KanR excised)		Figure 6
BMD1006	MG1655	Δ*arcA*::KanR	Kan+	Figure 6
BMD1008	MG1655	Δ*arcB* (KanR excised) ΔarcA::KanR	Kan+	Figure 6
BMD1010	MG1655	Δ*rssB*::KanR	Kan+	Figure 6
BMD1012	MG1655	Δ*arcB* (KanR excised) ΔrssB::KanR	Kan+	Figure 6

BMD890	MG1655	PPK-3xFLAG-KanR	Kan+	Figure 7
BMD892	MG1655	Δ*cpxA* (Kan excised) PPK-3xFLAG-KanR	Kan+	Figure 7
BMD882	MG1655	PPX-3xFLAG-KanR	Kan+	Figure 7
BMD884	MG1655	Δ*cpxA* (Kan excised) PPX-3xFLAG-KanR	Kan+	Figure 7
BMD891	MG1655	PPK-3xFLAG-KanR	Kan+	Figure 8
BMD893	MG1655	Δ*cpxA* (Kan excised) PPK-3xFLAG-KanR	Kan+	Figure 8

*Whole-genome sequencing later revealed that this is not a Δ*cpxA* mutant