A Stringent Analysis of Polyphosphate Dynamics in *Escherichia coli*

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ABSTRACT During stress, bacterial cells activate a conserved pathway called the stringent response that promotes survival. Polyphosphates are long chains of inorganic phosphates that modulate this response in diverse bacterial species. In this issue, Michael J. Gray provides an important correction to the model of how polyphosphate accumulation is regulated during the stringent response in *Escherichia coli* (M. J. Gray, J. Bacteriol, 201:e00664-18, 2019, <https://doi.org/10.1128/JB.00664-18>). With other recent publications, this study provides a revised framework for understanding how bacterial polyphosphate dynamics might be exploited in infection control and industrial applications.

KEYWORDS *Escherichia coli* PPK, polyphosphate, stringent response

PolyP: A CONSERVED POLYMER WITH DIVERSE FUNCTIONS

Polyphosphate (polyP) is a simple polymer of three to hundreds of orthophosphate moieties that are joined together by high-energy phosphoanhydride bonds (1, 2). While polyP itself is ubiquitous (1), the pathways regulating its synthesis and turnover appear poorly conserved across the kingdoms of life. However, there are commonalities in the function of polyP in divergent species. In bacteria, the polyphosphate kinase PPK synthesizes polyP from ATP, while the exopolyphosphatase PPX hydrolyzes phosphoanhydride bonds to release free orthophosphate (3–6). In bacteria, polyP dynamics play roles in diverse processes, including cell cycle control (7, 8), translation (9), motility (10–13), and biofilm formation (10, 14, 15). The budding yeast *Saccharomyces cerevisiae* does not have a PPK homolog, and in this model system polyP is instead synthesized by the vacuolar transporter chaperone (VTC) complex (16–18). PolyP is translocated into the vacuolar lumen during synthesis (19) and, remarkably, comprises over 10% of the dry weight of the cell (1). In yeast, links between polyP and phosphate homeostasis and energetics (20–22), metal tolerance (23, 24), cell cycle control (25), and translation (26) have been suggested. Finally, there are no convincing VTC or PPK homologs in mammals, and the identities of enzymes that synthesize and degrade polyP are unknown. PolyP concentrations in most mammalian cells and tissues are considerably lower than those in yeast (<100 μ M versus >200 mM, respectively) (27, 28), although the dense granules of platelets are an exception (29). Here, local polyP concentrations can reach ~100 mM, and the release of polyP from these structures following injury plays important roles in blood coagulation (29–36). PolyP also impacts cell cycle and growth control (37–39), mitochondrial functions (40–42), and neuronal signaling (43–45).

MOLECULAR FUNCTIONS OF polyP

How does polyP modulate such diverse functions across model systems? The answer to this question is unclear, but several studies hint at exciting possibilities. In 2014, work from the Jakob lab provided evidence that polyP functions as a molecular chaperone in bacteria (46). A role in protein folding is likely to be conserved, as polyP also

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modulates aggregation of other proteins, including amyloidogenic proteins associated with human neurological disease (47–49). Intriguingly, polyP can also be added to the N-ε of lysine as a posttranslational modification called polyphosphorylation (26, 50–52). Azevedo et al. showed that polyphosphorylation of yeast proteins Top1 and Nsr1 modulates the topoisomerase activity of Top1 and its ability to bind Nsr1 *in vitro*, as well as the nucleolar localization of both proteins *in vivo* (50). Although it has been studied only in the context of eukaryotes, the robust and nonenzymatic nature of polyphosphorylation suggests that it is likely to occur in any system where polyP is found at high concentrations (50, 53).

THE BACTERIAL PERSPECTIVE: polyP AND THE STRINGENT RESPONSE

Much of the foundational work in the polyP field was spearheaded by the late Arthur Kornberg, who studied polyP metabolism in a variety of bacteria (54). Bacteria remain a premiere system to study polyP biology. One of the critical findings to emerge from Kornberg's work, and that which followed, is a seemingly well-defined role for polyP during the stringent response that promotes survival in response to diverse stresses (55, 56). This response includes a reprogramming of transcription via the stress-responsive RpoS σ factor and the alarmones guanosine-5',3'-tetraphosphate (ppGpp) and guanosine-5',3'-pentaphosphate (pppGpp) (57). Collectively referred to as (p)ppGpp, these molecules are synthesized by RelA or SpoT and serve as allosteric modulators of RNA polymerase (58).

How does polyP fit into the stringent response? In the enterobacterium *Escherichia coli*, polyP levels are low when cells are grown in LB medium but increase dramatically during periods of stress such as starvation (59, 60). PolyP binds the ATP-dependent Lon protease with high affinity and promotes its activity toward select ribosomal proteins to slow translation and cell growth (61, 62). Critically, the impact of polyP on Lon function may extend to other substrates beyond those involved directly in protein translation. For example, recent work in *Pseudomonas aeruginosa* demonstrates that polyP interaction with Lon stimulates its activity toward the XseA subunit of the XseA/B exonuclease complex (63). This triggers a signaling cascade that decreases the proton gradient across the plasma membrane, inhibiting intracellular ATP accumulation (63). Together, these data point to a model where polyP functions as a general activator/adaptor of Lon protease to drive metabolic changes important for survival during stress. For its role in this process, the complex of polyP and Lon has been dubbed the "stringent protease" (64). polyP also promotes transcription of *rpoS* in various species (65–68), indicating that it impacts the stringent response via multiple mechanisms.

How does the stringent response promote the synthesis of polyP in the first place? Although there is limited evidence for regulation of *ppk* transcription by RpoS (69), the focus has been on posttranslational regulation of the PPX exopolyphosphatase. (p)ppGpp synthesized during the stringent response binds to PPX and inhibits its activity (60). With PPX out of play, or so goes the theory, polyP synthesized by PPK accumulates to high levels because it is not degraded (55). Critical support for this model comes from previous studies showing that strains with mutations in *relA* and/or *spoT*, which cannot synthesize (p)ppGpp, have defects in polyP accumulation in response to stress (70, 71).

COURSE CORRECTION

If PPX, unencumbered by (p)ppGpp, is responsible for the degradation of polyP under unstressed conditions, *ppx* mutation might be expected to result in constitutive polyP production. However, *ppx* mutants fail to accumulate polyP in the absence of stress and have only slightly more polyP than wild-type cells after stress induction (59). Thus, at best, the current model is incomplete. A study published by Michael J. Gray in this issue now challenges the role of (p)ppGpp altogether (72). Gray reports the generation of *E. coli relA spoT* double mutants, in two strain backgrounds, that are competent to induce polyP during amino acid starvation to levels mirroring those in wild-type cells (72). These results directly contradict the assertion that (p)ppGpp is

required for polyP accumulation during the stringent response. Gray supports his intriguing observations with additional experiments showing that RNA polymerase mutants that cannot bind (p)ppGpp behave like wild-type cells in terms of polyP accumulation. The same is true of cells that are engineered to overproduce (p)ppGpp (72). The overall conclusion is that (p)ppGpp does not control polyP dynamics, at least during amino acid starvation.

Too often, the reasons for incongruent results are never determined. This can leave a field muddled with a sense of uncertainty. Instead, Gray delivers a sense of clarity by demonstrating that *relA spoT* double mutants accumulate suppressor mutations in RNA polymerase (72). He shows that these suppressor mutations alone are capable of preventing polyP accumulation during amino acid starvation (72). Gray postulates that similar second-site suppressors in *relA spoT* mutants used in earlier work are the root cause of the erroneous conclusion that (p)ppGpp drives polyP accumulation. Of course, these suppressors may have impacted other studies of the stringent response, and relevant experiments should be revisited on a case-by-case basis where required. Gray's findings emphasize the importance of conducting rescue experiments to confirm that mutant phenotypes are indeed due to the loss of the mutated gene.

So if not (p)ppGpp, what does regulate polyP accumulation? Gray finds that treatment of cells with rifampin, which prevents transcription, prevents polyP accumulation during the stringent response. In a candidate-based approach, Gray then identifies the conserved RNA polymerase binding protein DksA and transcriptional elongator GreA as antagonistic regulators. PolyP accumulation depends on the presence of DksA and is inhibited by GreA during amino acid starvation (72). The nature of the interaction between these two factors and their downstream transcriptional target(s) required for polyP regulation are unknown. The *ppk-ppx* operon is unlikely to be a relevant target, because changes in transcription measured in reporter assays did not match with polyP accumulation (72).

OPEN QUESTIONS

The present study is exciting because it points to the existence of an as-yet-unknown pathway(s), modulated by DksA and GreA, that impinges on *ppk*, *ppx*, or both. Large-scale analyses of gene expression in *dksA*, *greA*, and *dksA greA* strains may identify genes whose expression pattern mirrors polyP expression in these mutants. Proteomics and metabolomics may also be useful in this regard, and together such experiments are ideally placed to find novel regulators of PPK and PPX. The most direct regulators could be proteins or small molecules.

PPK has been suggested to function as a dimer for purposes of polyP synthesis (73, 74), and it can also form tetramers that may function in PPK autophosphorylation (74). It would be intriguing to test whether the formation of such higher-order structures is impacted in *dksA* or *greA* mutants. Notably, previous work from the Gray lab identified point mutations in PPK that are well-positioned to disrupt dimerization, although this was not tested experimentally (59). *E. coli* expressing these mutant forms of PPK accumulates high levels of polyP even under unstressed conditions, suggesting a decoupling of polyP dynamics from the stringent response (59). In the current work, an epistasis analysis with one of these mutants suggests that it operates at least partially independently of *dksA* (see Fig. 6 in reference 72), although additional genetic experiments are warranted to further clarify the relationship between *dksA*, *greA*, *ppk*, and *ppx*. Notably, all of the experiments in the present study used amino acid starvation to induce the stringent response. As such, whether (p)ppGpp does indeed play a role in promoting polyP accumulation during other stresses remains to be tested. In the same vein, it will be important to test whether DksA and GreA homologs impact polyP accumulation in other bacterial species, some of which harbor additional PPK enzymes that can synthesize polyP (75). Finally, while this work focuses on what happens to polyP levels when the stringent response is turned on, future work should also include examination of how mutation of *dksA* and *greA* impacts polyP dynamics during the recovery from stress. To separate a role in recovery from a role in the initial accumu-

lation of polyP, a system to rapidly inactivate the DksA and GreA proteins would be useful.

BROADER IMPACT OF THE WORK

The regulation of polyP synthesis in bacterial systems has important therapeutic implications. This is highlighted by recent work suggesting that mesalamine, an FDA-approved drug used to treat ulcerative colitis, inhibits PPK functions in multiple species (76). Mesalamine treatment (i) decreased the occurrence of antibiotic-resistant *E. coli* persister cells epistatically with a *ppk* mutation, (ii) inhibited the ability of *Pseudomonas aeruginosa* to infect *Caenorhabditis elegans*, and (iii) reduced the polyP level of gut microbiota in human subjects (76). This remarkable work shows that there is significant potential for PPK inhibitors in the clinic. Indeed, additional inhibitors are currently being sought by a number of groups (77–79). The importance of understanding polyP metabolism extends beyond clinical applications. PolyP-accumulating microorganisms are being explored as means of removing phosphorous, heavy metals, or other contaminants from contaminated wastewater (80–82). The generation of bacteria that produce elevated levels of polyP or chains with unique properties may enhance these applications. Whether the goal is drugs targeting PPK for infection control or synthetic biology approaches aimed at optimizing bioremediation, a detailed understanding of the pathways regulating polyP dynamics will increase the probability of success. In this way, Gray's contribution has the potential to extend beyond the generation of new knowledge in a specific area of bacteriology to facilitate applications of broad interest.

FINAL THOUGHTS

Altogether, Gray's latest effort represents an important correction to a model of polyP dynamics that has persisted for many years. The study reinforces the need to probe inconsistencies in the literature and to support work that challenges established ideas. Models, after all, are made to be tested.

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