Poly-gamma-glutamic acid secretion protects *Bacillus subtilis* from zinc and copper intoxication

Running title: *Bacillus subtilis* produces poly-gamma-glutamic acid in response to zinc and copper excess

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Keywords: zinc, copper, poly-gamma-glutamic acid, biofilm

Abstract

Zinc and copper are essential micronutrients that serve as cofactors for numerous enzymes. However, when present at elevated concentrations, zinc and copper are highly toxic to bacteria. To combat the effects of zinc and copper excess, bacteria have evolved a wide array of defense mechanisms. Here, we show that the Gram positive soil bacterium, *Bacillus subtilis*, produces the extracellular polymeric substance, poly-gamma-glutamate (γ -PGA) as a protective mechanism in response to zinc and copper excess. Furthermore, we provide evidence that zinc and copper dependent γ -PGA production is independent of the DegS-DegQ two component regulatory system and likely occurs at a post-transcriptional level. These data provide new insight into bacterial metal resistance mechanisms and contribute to our understanding of the regulation of bacterial γ -PGA biosynthesis.

Importance

Zinc and copper are potent antimicrobial compounds. As such, bacteria have evolved a diverse range of tools to prevent metal intoxication. Here, we show that the Gram-positive model organism, *Bacillus subtilis*, produces poly-gamma-glutamic acid (γ -PGA) as a protective mechanism against zinc and copper intoxication and that zinc and copper dependent γ -PGA production occurs by a yet undefined mechanism independent of known γ -PGA regulation pathways.

Introduction

Transition metals ions, such as zinc and copper, are essential for life, yet toxic in excess. Metals have long been appreciated for their antimicrobial properties. Ancient Egyptians used copper salts as early as 2400 BCE as an astringent, food preservative, and disinfectant (1). Since zinc and copper disrupt antibiotic-resistant biofilms, display synergistic activity with other antimicrobials, and kill antibiotic-resistant bacteria, metal-based antimicrobials are currently used in industry, agriculture, and healthcare as metal-impregnated coatings and surfaces and in combination with traditional antibiotics (2). Furthermore, growing evidence suggest that zinc and copper intoxication play a key role in the host-microbe interaction as antibacterial agents (3).

At elevated concentrations, zinc and copper can outcompete other metals, including manganese and iron, for binding to metal containing proteins (4). Bacteria have evolved diverse mechanisms to prevent zinc and copper toxicity. Under zinc and copper excess, the intracellular level of these ions can be maintained by reduced uptake and/or increased efflux through the regulation of expression and activity of membrane associated metal transporters (5). Zinc and copper can also be detoxified by intracellular sequestration by small proteins or low molecular weight thiols, such as bacillithiol and glutathione (6, 7). The specific proteins and cellular processes poisoned by metals can also be repaired by chaperones or antioxidants, or bypassed through the expression of alternative metabolic pathways (8).

During the course of our investigations into the molecular mechanisms underlying bacterial zinc toxicity, we observed that *B. subtilis* colonies became highly mucoid when grown in the presence of zinc. Here, we demonstrate that the mucoid phenotype is due to the production of the secreted polymer, poly-gamma-glutamic acid (γ -PGA), show that γ -PGA

protects *B. subtilis* from zinc and copper intoxication, and provide evidence that zinc and copper induce γ -PGA production independently from known regulatory mechanisms.

Results

Zinc and copper induce *B. subtilis* γ -PGA production

While studying the mechanisms of zinc intoxication, we observed that *B. subtilis* colonies displayed a mucoid phenotype (Figure 1A). *Bacillus subtilis* serves as a model organism for the study of biofilm formation and extracellular polymeric substance (EPS) production (9). There is a growing body of evidence suggesting a link between zinc and copper homeostasis and EPS production. In *B. subtilis*, zinc and copper decrease biofilm hydrophobicity, thereby increasing antibiotic effectiveness (10). Additionally, excess zinc decreases *Streptococcus pyogenes* hyaluronic acid capsule production by inhibiting phosphoglucomutase, a key enzyme in central carbon metabolism (11). Recent studies on *Escherichia coli* virulence suggest that the presence of capsule may exacerbate bile salt mediated zinc starvation (12).

The EPS is composed of polysaccharides, proteins, nucleic acids, and poly-glutamic acid (γ -PGA) (13). γ -PGA is a biopolymer consisting of repeating units of L- or D-glutamic acid or a combination of both, and plays a role in virulence, survival during harsh conditions, and sequestration of toxic metal ions (14). In *B. subtilis*, the γ -PGA biosynthesis machinery is encoded by the *pgsBCAE* operon. In some bacteria, such as *B. subtilis*, γ -PGA is secreted and released from the cell surface, whereas in others like *B. anthracis* and *S. pneumoniae*, PGA remains attached to the cell as a capsule (15). When γ -PGA is made and

secreted by *B. subtilis*, the colonies produce a mucoid slime layer. Therefore, we hypothesized that mucoid material is the result of γ -PGA production.

To determine if the mucoidy observed in the presence of zinc is due to increased γ -PGA production, we compared the colony phenotype of wild-type and a *pgsB* deletion mutant in the presence of zinc (Figure 1B). We observed that in the presence of a sublethal concentration of zinc (250 μ M ZnSO₄) wild-type colonies are mucoid and raised, whereas the *pgsB* mutant colonies are dull and flat. Furthermore, the mucoid phenotype is restored to the *pgsB* mutant when *pgsB* is expressed ectopically from an IPTG-inducible promoter (*P_{hyspank}*).

To determine if the increase in γ -PGA production is specific to zinc or a general response to metals, we tested for colony mucoidy in the presence of a number of physiologically relevant transition metals (iron, manganese, or copper). Previously reported sublethal metal concentrations (2mM FeSO₄, 500 μ M MnCl₂, or 1 mM CuSO₄) were individually added to LB agar (16, 17). As previously reported, exposure to manganese leads to a wrinkled colony phenotype (18). Of the metals tested, only zinc and copper are able to induce colony mucoidy (Figure 1B) As observed with zinc, the colony mucoidy observed in the presence of copper is dependent on the presence of *pgsB*. Additionally, we utilized a spectrophotometric based assay to measure γ -PGA production in liquid media. Exposure to 250 μ M ZnSO₄ or 100 μ M CuSO₄ resulted in 3- or 5- fold increase in γ -PGA secretion (Figure 1C). From these data, we conclude that zinc and copper increase *B. subtilis* γ -PGA production.

γ -PGA protects *B. subtilis* from zinc and copper intoxication

γ -PGA can directly bind to metals, such as zinc, copper, and silver, consistent with a proposed protective role for γ -PGA (19, 20). To determine if γ -PGA production serves as a means of protecting *B. subtilis* from high levels of zinc and copper, we compared the zinc and copper sensitivity of wild-type and a $\Delta pgsB$ mutant by a disk diffusion assay (Figure 2A). The $\Delta pgsB$ mutant is more sensitive to zinc and copper than wild-type. Furthermore, the zinc and copper resistance of the $\Delta pgsB$ mutant is restored when *pgsB* is expressed ectopically from an IPTG-inducible promoter.

We also assessed the contribution of γ -PGA to zinc and copper resistance by monitoring cell growth in LB broth in the presence or absence of zinc or copper (Figures 2B and C). Wild-type growth is impaired in the presence of 250 μ M ZnSO₄ or 100 μ M CuSO₄ as indicated by an extended lag phase, decreased growth rate or decreased final OD₆₂₀. In the absence of metal, growth of the *pgsB* mutant is similar to wild-type. Upon the addition of zinc or copper, the *pgsB* mutant is unable to grow. When *pgsB* is expressed from an IPTG-inducible promoter, growth of the *pgsB* mutant in the presence of zinc or copper increases. Restoration of growth is to near wild type levels in the case of zinc. In the presence of copper, wild-type like growth is restored during exponential phase. However, the *pgsB* complemented strain is unable to achieve the same final OD₆₀₀ as wild-type. Since metals are involved in numerous cellular processes, the partial rescue in the presence of copper may be indicative of differences in the physiological processes inhibited by zinc and copper. From the disk diffusion and growth data, we conclude that γ -PGA protects *B. subtilis* from zinc and copper intoxication on both solid and liquid media.

Zinc and copper dependent γ -PGA production is not controlled at the level of transcription

γ -PGA production can be regulated by altering the expression of the *pgs* operon, which encodes the PGA biosynthetic machinery, or of *pgdS*, which encodes a gamma-DL-glutamyl hydrolase involved in γ -PGA degradation (21, 22). Activation of *pgsB* operon expression involves the DegS-DegU two-component system, the ComP-ComA quorum sensing system, and the SwrA protein. (23). DegS-DegU control the expression of genes involved in many cellular behaviors, including biofilm formation, motility, and competence (24). In response to a variety of stimuli, the cytoplasmic DegS sensor histidine kinase phosphorylates the DegU response regulator (DegU-P), while DegQ enhances DegS-dependent phosphorylation of DegU (23, 25). Once phosphorylated, DegU-P activates *pgs* operon expression by binding upstream of the *pgsB* promoter (26, 27). ComP-ComA and SwrA indirectly activate *pgsB* operon expression by modulating DegU phosphorylation, in the case of SwrA or by regulating DegQ expression, in the case of ComP-ComA (27–29). Since DegS-DegU is downstream of ComA-ComP and SwrA, we decided to focus on DegS-DegU for further study.

To test if DegU is involved in the zinc or copper dependent production of PGA, we monitored the colony morphology of a *degU* mutant on LB agar in the presence of zinc or copper (Figure 3). Similar to wild-type, the *degU* mutant forms a highly mucoid colony in the presence zinc or copper. We infer that DegU is not required for zinc or copper dependent PGA production.

We next explored the possibility *pgs* operon or *pgdS* expression may be regulated in response to zinc or copper excess. To determine if expression of the *pgs* operon or *pgdS* is differentially regulated in response to zinc or copper, we used a β -galactosidase assay to measure *pgs* operon and *pdgS* promoter activity in the presence of increasing concentrations of

zinc or copper (Figure 3B). Even when grown in the presence of zinc (250 μ M) and copper (100 μ M) concentrations known to stimulate γ -PGA production, *pgs* operon and *pgdS* promoter activity did not substantially change. We conclude that zinc and copper dependent γ -PGA production does not occur through changes at the level of *pgs* or *pgdS* gene expression.

Discussion

Our studies are consistent with previous work showing that *B. subtilis* produces γ -PGA in response to excess zinc. We extend these previous studies to demonstrate that γ -PGA production is also induced by copper. We demonstrate that γ -PGA provides *B. subtilis* protection from zinc and copper intoxication to provide a more complete picture of bacterial metal resistance.

Since γ -PGA is a non-toxic, biodegradable compound with utility in medical and commercial applications, microbial biosynthesis of γ -PGA is an extensively studied process (30, 31). Given its anionic charge and affinity for metals, γ -PGA is particular interest as a tool for the bioremediation of toxic metal ions from contaminated water (32). Thus, there is great interest in optimizing growth conditions and understanding the genetic circuitry underlying γ -PGA production. Based on our results, the mechanism of zinc and copper induction of γ -PGA production is independent of the DegS/U two component system known to activate *pgs* operon expression. Furthermore, our data suggest that regulation may be post-transcriptional, since neither γ -PGA biosynthesis (*pgsBCAE*) nor degradation (*pgdS*) gene expression is affected by the presence of excess zinc or copper.

Future investigations will explore the post-transcriptional regulation of γ -PGA biosynthesis. An intriguing candidate is PgsE, a small, 55 amino acid protein encoded last gene in the *pgs* operon. The *B. anthracis* PgsE homolog (CapE) is required for γ -PGA synthesis and is believed to play a structural role (33). Interestingly, when PgsE is overexpressed from an inducible promoter on high copy plasmid in the presence of zinc, γ -PGA production by *B. subtilis* (*chungkookjang*) increases 3-fold compared to the absence of PgsE overexpression (34). However, its role under physiologically relevant conditions is unclear. PgsE (*B. anthracis* CapE) is thought to associate with the PgsBCA (*B. anthracis* CapBCA) biosynthetic machinery in *B. anthracis* and when heterologously expressed in *E. coli* (33, 35). It is tempting to speculate that zinc and copper may be modulating PGA levels by affecting the interaction between PgsE and the PgsBCA protein complex. In total, our continuing work will provide further insight into the molecular mechanism underlying zinc and copper dependent γ -PGA production.

Materials and Methods

Strain and growth conditions

Strains were grown in Luria-Bertani broth (10 g tryptone, 5 g yeast extract, 5 g/l NaCl) or plates containing 1.5% agar at 37°C. When necessary, antibiotics were added to the growth media at the following concentrations: 1 μ g/ml erythromycin plus 25 μ g/ml lincomycin (mls), 100 μ g/ml spectinomycin, 5 μ g/ml kanamycin, 5 μ g/ml chloramphenicol or 100 μ g/ml ampicillin. One mM of isopropyl β -D-thiogalactopyranoside (IPTG) was added to the growth medium when appropriate.

Strain construction

All strains and primers used in this study are listed in Tables 1 and 2, respectively. To facilitate genetic manipulation, all experiments were performed using the highly competent derivative of *Bacillus subtilis* 3610 carrying a *comI* deletion (3610 *comI*) (36). Chromosomal DNA was used for transformation as previously described (37). To induce natural competence, cells were grown in minimal competence media (1.7 g K₂HPO₄, 0.52 g KH₂PO₄, 2 g dextrose, 0.088 g sodium citrate dehydrate, 0.2 g l-glutamic acid monopotassium salt, 1 ml of 1,000× ferric ammonium citrate, and 1 g/l casein hydrolysate and 1% 300 mM MgSO₄).

Colony mucoidy assay

To evaluate the colony mucoidy phenotype, strains were grown to mid-exponential phase (OD₆₀₀~0.4) in LB broth. Five µl of the culture were then spotted on to freshly poured LB plates containing 1.5% agar that had been dried in the laminar flow hood for 10 minutes. The spots were allowed to dry an additional 10 minutes in the laminar flow hood. The plates were then incubated at 37°C for 16-18 hours. Images of the colonies were recorded using an iPhone 12 held in a tripod 36 inches from the agar surface.

PGA quantification

B. subtilis PGA production was measured using a cetyltrimethylammonium bromide (CTAB) assay as previously described (38, 39). Cells were grown in LB broth containing various concentrations of ZnSO₄ or CuSO₄ in LB media overnight at 37°C for 16-18 hours. Cells were then pelleted from 15 ml of culture by centrifugation at 10000 x g for 30 minutes at 4°C and the

supernatant was retained. To precipitate the PGA, thirty ml of ethanol (96%) was added to the supernatant and incubated at 4°C overnight. The precipitated PGA was harvested by centrifugation at 10000 x g for 10 minutes at 4°C. The resulting pellet was resuspended in 1 ml of distilled water. In a 96 well flat bottom microtiter plate, one hundred µl of 0.07 M CTAB in 2% (w/v) NaOH was added to 100 µl of the resuspended PGA and mixed gently by pipetting to avoid bubble formation. The OD₄₀₀ of the resulting solution was measured in a Tecan Sunrise microplate reader. A standard curve using purified PGA (Sigma, G1049) was used to calculate the PGA concentration in the broth.

***pgsB* complementation**

The plasmid pDR111 was used to generate the IPTG-inducible *amyE::P_{hyspank}-pgsB* construct. Briefly, a PCR product containing the *pgsB* coding region was amplified from *B. subtilis* 3610 *comI* chromosomal DNA using primers 1003/1004, digested with Sall and SphI, and cloned into the Sall and SphI sites of the pDR111 plasmid. The plasmid was then transformed in to *B. subtilis* 3610 *comI*. Successful transformation was indicated by spectinomycin resistance and verified by PCR.

In frame markerless gene deletions

Gene deletion mutants were constructed from the BKE strains as previously described (40). BKE strains carrying the gene deletion of interest marked by a kanamycin or erythromycin resistance cassette were acquired from the Bacillus Genetic Stock Center (www.bgsc.org) . Chromosomal DNA was isolated and transformed in to our wild-type 3610 *comI* strain. To

remove the antibiotic resistance cassette, the temperature sensitive pDR244 plasmid encoding the *cre* recombinase was introduced into the transformants by selecting for spectinomycin resistance at 30°C. The strains was subsequently cured of the plasmid by growth at 42°C and screened for sensitivity to spectinomycin (loss of the plasmid) and kanamycin or erythromycin (loss of the antibiotic resistance cassette). Successful gene deletion was verified by PCR using primers 1001/1002.

β-galactosidase assay

A β-galactosidase assay was used to measure *pgs* operon and P_{pdgS} promoter activity, as previously described (41). Cells were grown in LB broth in the presence or absence of ZnSO₄ (62.5, 125, or 250 μM) or CuSO₄ (25, 50, 100 μM) at 37°C. One ml of cells were harvested by centrifugation at early stationary phase (OD₆₀₀~1) and resuspended in an equal volume of Z-buffer (40 mM NaH₂PO₄, 60 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM KCl, and 38 mM 2-mercaptoethanol. Cells were lysed by addition of lysozyme (0.2 mg/ml) and incubation at 30°C for 15 minutes. Each sample was then diluted in Z-buffer to a final volume of 500 μl. The reaction was started with the addition of 100 μl of 4 mg/ml of 2-nitrophenyl β-D-galactopyranoside (ONPG). Once the sample began to turn yellow, the reaction was stopped with 250 μl of 1 M Na₂CO₃. The OD₄₂₀ was measured, and the specific activity of β-galactosidase was calculated using the equation $[OD_{420}/(\text{reaction time} \times OD_{600})] \times \text{dilution factor} \times 1000$.

Growth curves

To measure the effect of zinc and copper on cell growth, strains were grown to mid-exponential phase (OD₆₀₀~0.4) and subsequently diluted 1:100 into fresh LB media in the presence or absence of ZnSO₄ (250 µM) and CuSO₄ (100 µM) in a 96 well flat bottom microtiter plate. The plate was then incubated in a Tecan Sunrise microplate reader at 37°C with continuous shaking (high setting) and the OD₆₂₀ was measured every 15 minutes for 16 hours.

Disk diffusion assay

Disk diffusion assays were modified from (42). Briefly, a 1:100 dilution of an overnight culture was grown in fresh LB medium at 37°C with 250 rpm shaking until mid-exponential phase (OD₆₀₀~0.4). A 100 µl aliquot of cells was then spread on the surface of a LB agar plate (15 ml of 1.5% agar) that had been dried in a laminar flow hood for 10 minutes. The inoculum was allowed to dry for an additional 10 minutes in the laminar flow hood. A sterile 6.5 mm Whatman filter disk was then placed on the surface of the agar and ZnSO₄ (5 µl of a 500 mM solution) or CuSO₄ (5 µl of a 100 mM solution) was added to filter disk and allowed to absorb into the disk for 5 minutes. The plates were incubated at 37°C for 16-18 hrs, after which the diameter of the zone of inhibition was measured.

Acknowledgements

We would like to thank Daniel Kearns (Indiana University) for insightful discussion and generously providing strains.

References

- 281 1. Ranke H. 1933. Medicine and Surgery in Ancient Egypt. Bulletin of the Institute of the
282 History of Medicine 1:237–257.
- 283 2. Schmidt MG, Attaway HH, Fairey SE, Howard J, Mohr D, Craig S. 2019. Self-Disinfecting
284 Copper Beds Sustain Terminal Cleaning and Disinfection Effects throughout Patient Care.
285 Applied and Environmental Microbiology 86:e01886-19.
- 286 3. Djoko KY, Ong CY, Walker MJ, McEwan AG. 2015. The Role of Copper and Zinc Toxicity in
287 Innate Immune Defense against Bacterial Pathogens. J Biol Chem 290:18954–18961.
- 288 4. Waldron KJ, Rutherford JC, Ford D, Robinson NJ. 2009. Metalloproteins and metal sensing.
289 Nature 460:823–830.
- 290 5. Braymer JJ, Giedroc DP. 2014. Recent developments in copper and zinc homeostasis in
291 bacterial pathogens. Current Opinion in Chemical Biology 19:59–66.
- 292 6. Ma Z, Chandrangsu P, Helmann TC, Romsang A, Gaballa A, Helmann JD. 2014. Bacillithiol is
293 a major buffer of the labile zinc pool in Bacillus subtilis. Mol Microbiol 94:756–770.
- 294 7. Stewart LJ, Ong CY, Zhang MM, Brouwer S, McIntyre L, Davies MR, Walker MJ, McEwan
295 AG, Waldron KJ, Djoko KY. 2020. Role of Glutathione in Buffering Excess Intracellular
296 Copper in Streptococcus pyogenes. mBio 11:e02804-20.
- 297 8. Chandrangsu P, Rensing C, Helmann JD. 2017. Metal homeostasis and resistance in
298 bacteria. Nat Rev Microbiol 15:338–350.

- 299 9. Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R. 2013. Sticking together: building a
300 biofilm the *Bacillus subtilis* way. *Nat Rev Microbiol* 11:157–168.
- 301 10. Falcón García C, Kretschmer M, Lozano-Andrade CN, Schönleitner M, Dragoș A, Kovács ÁT,
302 Lieleg O. 2020. Metal ions weaken the hydrophobicity and antibiotic resistance of *Bacillus*
303 *subtilis* NCIB 3610 biofilms. *npj Biofilms Microbiomes* 6:1.
- 304 11. Ong CY, Walker MJ, McEwan AG. 2015. Zinc disrupts central carbon metabolism and
305 capsule biosynthesis in *Streptococcus pyogenes*. *Sci Rep* 5:10799.
- 306 12. Olson MA, Grimsrud A, Richards AC, Mulvey MA, Wilson E, Erickson DL. 2021. Bile salts
307 regulate zinc uptake and capsule synthesis in a mastitis-associated extraintestinal
308 pathogenic *Escherichia coli* strain. *Infect Immun* IAI0035721.
- 309 13. Marvasi M, Visscher PT, Casillas Martinez L. 2010. Exopolymeric substances (EPS) from
310 *Bacillus subtilis* : polymers and genes encoding their synthesis. *FEMS Microbiology Letters*
311 313:1–9.
- 312 14. Ogunleye A, Bhat A, Irorere VU, Hill D, Williams C, Radecka I. 2015. Poly-γ-glutamic acid:
313 production, properties and applications. *Microbiology* 161:1–17.
- 314 15. Ezzell JW, Welkos SA. 1999. The capsule of *Bacillus anthracis*, a review. *J Appl Microbiol*
315 87:250.

- 316 16. Guan G, Pinochet-Barros A, Gaballa A, Patel SJ, Argüello JM, Helmann JD. 2015. PfeT, a
317 P1B4-type ATPase, effluxes ferrous iron and protects *Bacillus subtilis* against iron
318 intoxication. *Mol Microbiol* 98:787–803.
- 319 17. Huang X, Shin J-H, Pinochet-Barros A, Su TT, Helmann JD. 2017. *Bacillus subtilis* MntR
320 coordinates the transcriptional regulation of manganese uptake and efflux systems. *Mol*
321 *Microbiol* 103:253–268.
- 322 18. Shemesh M, Chai Y. 2013. A Combination of Glycerol and Manganese Promotes Biofilm
323 Formation in *Bacillus subtilis* via Histidine Kinase KinD Signaling. *J Bacteriol* 195:2747–
324 2754.
- 325 19. Eymard-Vernain E, Coute Y, Adrait A, Rabilloud T, Sarret G, Lelong C. 2018. The poly-
326 gamma-glutamate of *Bacillus subtilis* interacts specifically with silver nanoparticles. *PLoS*
327 *ONE* 13:e0197501.
- 328 20. McLean RJC, Beauchemin D, Clapham L, Beveridge TJ. 1990. Metal-Binding Characteristics
329 of the Gamma-Glutamyl Capsular Polymer of *Bacillus licheniformis* ATCC 9945. *Appl*
330 *Environ Microbiol* 56:3671–3677.
- 331 21. Suzuki T, Tahara Y. 2003. Characterization of the *Bacillus subtilis* ywtD Gene, Whose
332 Product Is Involved in γ -Polyglutamic Acid Degradation. *J Bacteriol* 185:2379–2382.
- 333 22. Urushibata Y, Tokuyama S, Tahara Y. 2002. Characterization of the *Bacillus subtilis* ywsC
334 Gene, Involved in γ -Polyglutamic Acid Production. *J Bacteriol* 184:337–343.

23. Stanley NR, Lazazzera BA. 2005. Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly- γ -dl-glutamic acid production and biofilm formation. *Molecular Microbiology* 57:1143–1158.
24. Verhamme DT, Kiley TB, Stanley-Wall NR. 2007. DegU co-ordinates multicellular behaviour exhibited by *Bacillus subtilis*. *Molecular Microbiology* 65:554–568.
25. Dahl MK, Msadek T, Kunst F, Rapoport G. 1992. The phosphorylation state of the DegU response regulator acts as a molecular switch allowing either degradative enzyme synthesis or expression of genetic competence in *Bacillus subtilis*. *J Biol Chem* 267:14509–14514.
26. Ohsawa T, Tsukahara K, Ogura M. 2009. *Bacillus subtilis* Response Regulator DegU Is a Direct Activator of *pgsB* Transcription Involved in γ -Poly-glutamic Acid Synthesis. *Bioscience, Biotechnology, and Biochemistry* 73:2096–2102.
27. Osera C, Amati G, Calvio C, Galizzi A. 2009. SwrAA activates poly- γ -glutamate synthesis in addition to swarming in *Bacillus subtilis*. *Microbiology* 155:2282–2287.
28. Msadek T, Kunst F, Klier A, Rapoport G. 1991. DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. *J Bacteriol* 173:2366–2377.
29. Ogura M, Tsukahara K. 2012. SwrA regulates assembly of *Bacillus subtilis* DegU via its interaction with N-terminal domain of DegU. *The Journal of Biochemistry* 151:643–655.

- 354 30. Hsueh Y-H, Huang K-Y, Kunene SC, Lee T-Y. 2017. Poly- γ -glutamic Acid Synthesis, Gene
355 Regulation, Phylogenetic Relationships, and Role in Fermentation. 12. International
356 Journal of Molecular Sciences 18:2644.
- 357 31. Moradali MF, Rehm BHA. 2020. Bacterial biopolymers: from pathogenesis to advanced
358 materials. Nat Rev Microbiol 18:195–210.
- 359 32. Mark SS, Crusberg TC, DaCunha CM, Iorio AAD. 2006. A Heavy Metal Biotrap for
360 Wastewater Remediation Using Poly- γ -Glutamic Acid. Biotechnology Progress 22:523–531.
- 361 33. Candela T, Mock M, Fouet A. 2005. CapE, a 47-Amino-Acid Peptide, Is Necessary for
362 Bacillus anthracis Polyglutamate Capsule Synthesis. J Bacteriol 187:7765–7772.
- 363 34. Yamashiro D, Yoshioka M, Ashiuchi M. 2011. Bacillus subtilis pgsE (Formerly ywtC)
364 stimulates poly- γ -glutamate production in the presence of zinc. Biotechnol Bioeng
365 108:226–230.
- 366 35. Motta Nascimento B, Nair NU. 2020. Characterization of a membrane enzymatic complex
367 for heterologous production of poly- γ -glutamate in E. coli. Metab Eng Commun
368 11:e00144.
- 369 36. Konkol MA, Blair KM, Kearns DB. 2013. Plasmid-Encoded ComI Inhibits Competence in the
370 Ancestral 3610 Strain of Bacillus subtilis. J Bacteriol 195:4085–4093.
- 371 37. Harwood CR, Cutting SM. 1990. Molecular biological methods for Bacillus. Wiley,
372 Chichester; New York.

38. Li M, Zhang Z, Li S, Tian Z, Ma X. 2021. Study on the mechanism of production of γ -PGA and nattokinase in *Bacillus subtilis* natto based on RNA-seq analysis. *Microb Cell Fact* 20:83.
39. Halmschlag B, Putri SP, Fukusaki E, Blank LM. 2020. Identification of Key Metabolites in Poly- γ -Glutamic Acid Production by Tuning γ -PGA Synthetase Expression. *Front Bioeng Biotechnol* 8:38.
40. Koo B-M, Kritikos G, Farelli JD, Todor H, Tong K, Kimsey H, Wapinski I, Galardini M, Cabal A, Peters JM, Hachmann A-B, Rudner DZ, Allen KN, Typas A, Gross CA. 2017. Construction and Analysis of Two Genome-scale Deletion Libraries for *Bacillus subtilis*. *Cell Syst* 4:291-305.e7.
41. Chan JM, Guttenplan SB, Kearns DB. 2014. Defects in the Flagellar Motor Increase Synthesis of Poly- γ -Glutamate in *Bacillus subtilis*. *J Bacteriol* 196:740–753.
42. Chandrangu P, Helmann JD. 2016. Intracellular Zn(II) Intoxication Leads to Dysregulation of the PerR Regulon Resulting in Heme Toxicity in *Bacillus subtilis*. *PLoS Genet* 12:e1006515.

Strains used in this study

Strain	Description	Source
PC1	3610 <i>comI</i> (wild-type)	(36)(36)(36)
BKE35900	<i>trpC2</i> Δ <i>pgsB::erm</i>	(40)
PC2	PC1 Δ <i>pgsB</i>	This work

PC3	PC1 Δ <i>pgsB amyE::P_{hypspank}-pgsB spec</i>	This work
BKK35490	<i>trpC2 ΔdegU::kan</i>	(40)
PC7	PC1 Δ <i>degU</i>	This work
DS9309	3610 <i>amyE::P_{pgdS}-lacZ cat</i>	(41)
DS9079	3610 <i>pgsEΩlacZ cat</i>	(41)
PC10	PC1 <i>amyE::P_{pgdS}-lacZ cat</i>	This work
PC11	PC2 <i>pgsEΩlacZ cat</i>	This work

Plasmids used in this study

Plasmid	Description	Source
pDR244	<i>cre ori_{Ts} amp spec</i>	(40)
pDR111	<i>bla amyE' P_{hyperspank} spec lacI 'amyE</i>	Gift from D. Kearns Lab
pPC1	pDR111 <i>bla amyE' P_{hyperspank} -pgsB spec lacI 'amyE</i>	This work

Primer table

Primer number	Description	Sequence
1001	pgsB-check-F	TAGGGAAGATTATGTTACATAATGC
1002	pgsB-check-R	TAAACTGAGTAGTACACCTA
1003	pgsB-Sall-pDR111-F	ACTGGTCGACATGTGGTTACTCATTATAGC
1004	pgsB-SphI-pDR111-R	ACTGGCATGCCTAGCTTACGAGCTGCTTTACC

396 **Figures**

397

398 Figure 1: Zinc and copper induce PGA production. (A) Colony morphology of WT *B. subtilis* 3610
399 *com1* in the presence of 250 μ M ZnSO₄, 1 mM CuSO₄, 2 mM FeSO₄, 500 μ M MnCl₂. (B) Colony
400 morphology of WT, $\Delta pgsB$, and $\Delta pgsB$ P_{hyspank}-pgsB grown on LB agar in the presence of 250 μ M
401 ZnSO₄ or 100 μ M CuSO₄. IPTG (1 mM) was also added to $\Delta pgsB$ P_{hyspank}-pgsB to induce *pgsB*
402 expression. (C) Concentration of γ -PGA produced by cells grown in the presence of 250 μ M
403 ZnSO₄ or 100 μ M CuSO₄. The γ -PGA concentration is expressed as mg of γ -PGA per gram of cell
404 wet weight. Data shown are the mean and standard deviation from three independent
405 experiments. *P* values were calculated by an ANOVA. **, *P* $\leq$ 0.01; ***, *P* $\leq$ 0.001

406

407 Figure 2: PGA protects *B. subtilis* from zinc and copper intoxication. (A) Disk diffusion
408 susceptibility test performed on WT, $\Delta pgsB$, and $\Delta pgsB P_{hyspank} pgsB$ grown on LB agar. Five μ l
409 of 500 μ M ZnSO₄ or 5 μ l of 100 μ M CuSO₄ was added to a filter disk. The zone of inhibition was
410 determined by measuring the diameter of the zone of clearing. Data shown are the mean and
411 standard deviation from three different experiments. *P* values were calculated by an ANOVA.
412 n.s., nonsignificant; **, *P* ≤ 0.01. (B and C) Growth curves of WT, $\Delta pgsB$, and $\Delta pgsB P_{hyspank}$
413 $pgsB$ in the presence and absence of either (B) 250 μ M ZnSO₄ or (C) 100 μ M CuSO₄. IPTG (1
414 mM) was also added to $\Delta pgsB P_{hyspank} pgsB$ to induce *pgsB* expression. Data shown are the
415 mean and standard deviation from three independent experiments.

Figure 3: Zinc and copper dependent PGA production is not controlled at the level of transcription. (A) Colony morphology of WT and $\Delta degU$ grown in LB agar in the presence of 250 μM ZnSO_4 or 1 mM CuSO_4 . (B) *pgs* operon (*pgsEΩlacZ*) and P_{pgdS} promoter activity in cells grown in LB media amended with varying concentrations of ZnSO_4 or CuSO_4 . Data shown are the mean and standard deviation from three independent experiments. No significant difference was detected between samples by ANOVA.