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Identification of the Role of swrA in Copper Induced Poly-gamma-glutamic Acid Production in Bacillus subtilis

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Identification of the Role of *swrA* in Copper Induced Poly-γ-glutamic Acid Production in *Bacillus subtilis*

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by

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Abstract

Each year over 2.8 million Americans are infected by an antibiotic resistant strain of bacteria with over 35, 000 dying as a result (CDC). The lack of development of new antibiotics has renewed interest in the antimicrobial properties of copper. Gram positive bacterium such as *Bacillus subtilis* produce poly-gamma-glutamic acid (γ-PGA) to sequester excess copper ions. *swrA* has shown to be essential for proper activation of the *pgs* operon by phosphorylated DegU via an unknown mechanism. This study seeks to determine the role of *swrA* in copper induced γ-PGA production and if *swrA* and *ppsB* are involved in copper resistance. Copper Morphology assays were conducted with *ΔswrA*, GJ014, GJ015, and 3610 *B. subtilis. swrA* mutants did not produce γ-PGA in the presence of copper. Kirby Bauer assays were conducted with *ΔswrA*, *ΔppsB*, *ΔswrAΔppsB* mutants and 3610 *B. subtilis* to assess copper resistan

ce There were no significant differences in the average zone of inhibition of all four strains tested. The results of this study indicate that *swrA* is essential for copper induced γ-PGA production. This study also points to a regulatory pleotropic role of *swrA* of the *pgs*, *fla/che*, and *srfA* operon via the *deg* system to allow for continued copper resistance. Further understanding the role of γ-PGA production will assist in developing future antimicrobials using copper.

Background

The Post Antibiotic Era

In 1928, Sir Alexander Fleming found *Penicillium notatum* growing on his equipment. The antimicrobial properties of this fungus would lead to the development of the first modern antibiotic, Penicillin (**Figure 1**). Antibiotics are defined as any compound that can inhibit the growth of another organism . Penicillin opened a new chapter of medicine by providing a new way to prevent and fight bacterial infections. Before the introduction of modern antibiotics in

1928, mortality rates for common infections such as pneumonia were as high as 40 percent and even higher for staph infections at around 80 percent(1). Wound infections were generally treated with amputations with around 70 percent of amputations in World War I being the result of wound infections (2). Being able to control infections, such as wound infections, allowed for advances in medicine such as surgery, organ transplants, dialysis, and cancer treatments (3). Since the introduction of antibiotics, the average lifespan in the United States has increased from 56.4. years to 80 years which has been attributed to infection control and the mentioned medical advancements.

Within a decade of Penicillin's discovery, clinicians identified Penicillin resistant strains of bacteria (4). In response, researchers developed stronger antibiotics to fight this Penicillin resistant such as Methicillin which target bacterial cell walls and ; however, resistant strains to these new antibiotics were found also a decade later (**Figure 1**). With each introduction of new antibiotics, resistant strains followed soon after. In 1968, the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) in the United States; the rise of MRSA from 1968-1980 alarmed clinicians as methicillin was a part of the more powerful class of tetracyclines (1, 5). Clinicians in the 1990s officially defined antibiotic resistance as a phenotypic or genotypic property of bacteria that allows it to inactivate, block, or exclude the killing or inhibitory effects of antibiotics (6). The relatively quick evolution of bacteria is owed to its ability to pass along genes to other bacteria for resistance through horizontal gene transfer (the ability of bacteria to pass along its genes to other species of bacteria)(7). Bacteria to quickly develop defenses against antibiotics has led to a silent epidemic of antibiotic resistance strains of bacteria. In 2019, nearly 80 years after the introduction of modern antibiotics, the Center for the Disease

Control declared the 21st century as the 'Post Antibiotic Era' in response to the continual increase in resistant strains of bacteria.

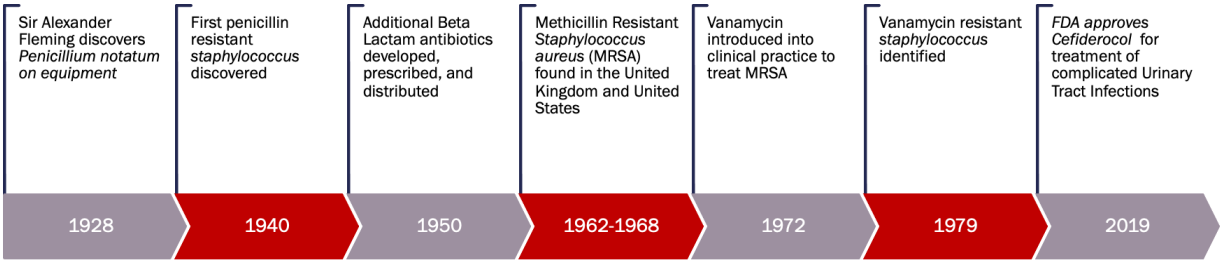

Figure 1. Timeline of the development of modern antibiotics and acquired resistance

The CDC in a 2019 report declared the 21st century as the 'Post Antibiotic Era"(3). The CDC and World Health Organization warned that this era would be a time where common infections and minor injuries could result in death (3, 8). This post-apocalyptic warning was becoming a reality; the report stated that over 2.8 million Americans were infected with an antibiotic resistant strain of bacteria each year with over 35,000 dying as a result (3). At the same time, the CDC reported an increase in sepsis – an extreme immune response to an infection that can result in organ and tissue damage (9, 10) The CDC pointed to this as an example of what a future without antibiotics may look like. Treatments for sepsis involve high doses of intravenous antibiotics and surgery to remove damaged and dead tissue (9, 10). With over 1.7 million Americans developing sepsis each year and over 270,000 dying from the condition, the lives of many Americans are threatened by the inability to treat this deadly condition(9). Sepsis reveals one consequence of antibiotic resistance and how reliant modern medicine is on effective antibiotics. Antibiotic resistance threatens the very foundation of modern medicine; modern medicine depends on the ability to prevent and counteract bacterial infections. In the case of surgery, antibiotics are needed to reduce the likelihood of complications of postoperative

infections. However, it must be noted that antibiotic resistance did not form overnight, and that the CDC declaration did not arrive without cause. The quick progression of this new era can be pinpointed to four key factors

A combination of factors are key contributors to this silent epidemic. The potential causes of the current epidemic all point to an excessive and inappropriate use of antibiotics as the catalyst for the rise of resistant bacteria (4). One factor is associated with the high usage of antibiotics in livestock. Nearly 80 percent of antibiotics produced in the United are given to livestock as a growth supplement (4). Molecular detection has shown that antibiotic use can make its way to humans through the food supply; bacteria with resistance are able to outcompete "susceptible" strains of bacteria (4). These bacteria are transferred to humans through meat products and can multiply and pass resistance through horizontal gene transfer (HGT) (4). Another factor is the overuse and inappropriate prescribing of antibiotics. Around 30 to 50 percent of prescribed antibiotics are incorrect (4). Inappropriate prescribing gives preexisting bacteria ample opportunities to increase in virulence and resistance through random mutations and HGT (4). While misuse plays a big factor, overuse of antibiotics also a play a large role. Countries outside of the US can have little to no regulation against antibiotic use; some antibiotics can even be bought over the counter (OTC) (4). The internet has also supplied another way to obtain antibiotics in countries that do regulate its use (4). Overuse provides bacteria with the same opportunities for bacteria to develop resistance and pass it to other bacteria (4, 11). At the same time, overuse also provides an intense selective pressure which allows bacteria via natural selection to quickly adapt and multiply (11). The final and largest contributor is the lack of development of new antibiotics in the past two decades. Unlike the response seen in the 50s and 60s, the continued discovery of antibiotic resistant bacteria has not

spurred the development of new antibiotics (**Figure 1**)(1, 4, 5). In the past decade, 15 of the 18 major pharmaceutical companies have abandoned antibiotic development completely (1, 4). Economists point to the lack of financial incentive towards developing antibiotic therapies; antibiotics are curative, used for a relative short period of time, and are sold for $1000-$3000 compared to chemotherapy which is priced at $10,000 per treatment (1, 4). The slow development of new antibiotic therapies also provides pathogenic bacteria with the opportunity to develop resistance, especially against stronger classes of drugs like Vanamycin. The misuse and overuse provide the perfect storm for an epidemic of resistant strains; slow drug development serves as an additional catalyst. Without new antibiotics, and procedures using modern medical practices depend on infection control and prevention. Antibiotic resistance this threatened to destroy the very foundation of medicine. To combat antibiotic overuse, misuse, and slow development, researchers have investigated how early human civilizations prevented and treated bacterial infections. They that early humans took advantage of the antimicrobial properties of metals such as copper.

Antimicrobial Properties of Copper

The earliest use of copper can be traced to human civilizations dating back to 5 and 6 BC (5). The first recorded use of copper in medicine appeared in the Smith Papyrus – an Egyptian medical text written around 2600 and 2400 BC (7). Egyptian physicians cited copper as a way to disinfect chest wounds, treat scalds, relieve itching, and purify drinking water (12). On the Greek island of Cyprus, copper was found in abundance; the Hippocratic Collection (coauthored by Greek physician Hippocrates), recommended copper to dress wounds and treat leg ulcers (8). In the 19th and 20th centuries, copper served as a popular antimicrobial to treat common infections like cholera, syphilis, and tuberculosis until the introduction of commercial antibiotics

in 1940 (7). One key study in 1983 found brass doorknobs (alloy of copper and zinc) were more effective in preventing the spread of microbes than plastic or commonly used stainless Steele in hospitals (13).

Copper is a fascinating trace element that is essential to most living organisms but toxic in excess due to its high reductive potential. Copper has a redox potential that ranges from +200 to +800 mv allowing it to serve as an electron donor, acceptor, and carrier (5). For instance, the 13 protein cytochrome C oxidase (CCO) contains three copper ions that are essential to its function; cytochrome C serves as a key electron acceptor in the electron transport chain in aerobic organisms (ETC) (14). These same However, these same reductive properties of copper can damage the cellular membranes of pathogenic organisms such as bacteria. Excess copper can participate in a Fenton-type reaction to generate reactive hydroxyl radicals (7).

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{+}$$

Schematic 1. Equation for a fenton-type reaction involving copper

Hydrogen peroxide can pass through the cell membrane and when combined with a reducing agent such as copper, can produce radicals that damage genetic material, cause oxidation of lipids in the membrane, and inactivate enzymes associated with the cell membrane of bacteria (15). There is evidence that copper plays an important part of the innate immune system. During phagocytosis, some cells will accumulate Cu(I) to kill pathogens (16). Copper also can harm the host's cell's too; however, the concentrations of copper needed to inhibit or destroy pathogenic organisms differs from the cells of the host. Copper overall serves as a great bactericidal because its high redox potential and ability to serve as an electron carrier or acceptor.

Bacterial Defenses Against Metals

Just as bacteria has developed defenses against antibiotics, they have done the same with metals. Gram-negative bacteria sequester excess copper into the periplasm and bind it to metallochaperones (17). Gram-positive bacteria (and some gram-negative) use metallochaperones to buffer Cu(I) to areas of lower concentrations in the cytoplasm of the cell (14). Bacteria can also form biofilms to protect themselves against metal intoxication. Biofilms are a cluster of bacteria that are attached to a surface in a matrix. Biofilms allow bacteria to adapt to their environment by altering their metabolism, gene expression, and protein production (18). In extreme environmental conditions, biofilms reduce the minimum cellular conditions needed for the bacteria to survive. To protect themselves from antimicrobials such as copper, biofilms can contain neutralizing agents or barriers to counteract metal toxicity. One such compound produced in bacterial biofilms to fight metal toxicity is poly-γ-glutamic acid.

Poly-γ-glutamic acid

Poly-γ-glutamic acid (γ-PGA) is a biodegradable polymer made up of repeating units of L-glutamic and D-glutamic acids connected by γ-amide linkages (**Figure 2**)(19). γ-PGA, discovered in 1937 in *Bacillus subtilis* comes in two forms, the α and γ, which depends on the location of the carboxyl group; the γ form of the compound is typically found in bacterial species. This γ form prevents the degradation of the compound by proteases in the cell that cleave α-linkages (19). γ-PGA function is highly variable and dependent on if it is released or bound by peptidoglycan to the bacterium. γ-PGA that is released can protect the bacterium from extreme conditions, while attached γ-PGA can help the bacterium evade phage attacks or

recognition by the immune system (19). Soil bacteria such as *B. subtilis* can release γ-PGA into the environment to sequester high concentrations of toxic metal ions (19).

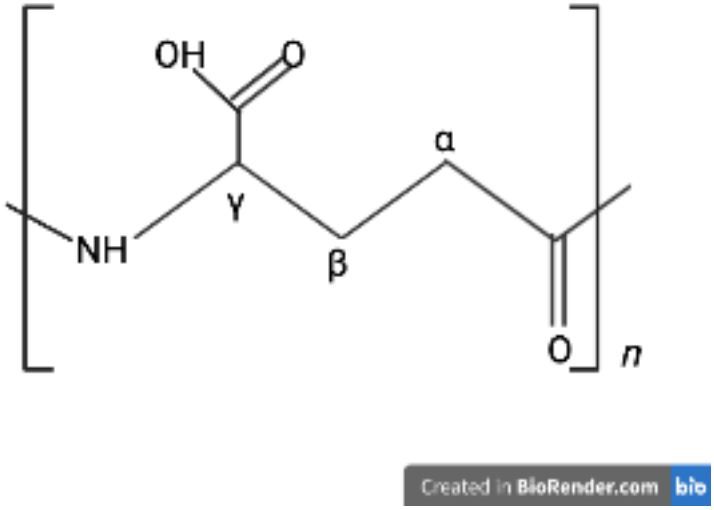

Figure 2. Chemical structure of a polymer of γ-PGA. Created using Biorender.com

γ-PGA synthesis

To develop copper as an antimicrobial and potential antibiotic, the mechanisms of γ-PGA production must be known. Understanding these mechanisms will assist in targeting an important defense measure taken by gram positive bacteria; targeting this mechanism can improve copper's antimicrobial activities. γ-PGA production begins with the generation of L-glutamic acid. L-glutamic acid can come from two sources, either through the conversion of carbon using acetyl-CoA and α-Ketoglutaric acid from the TCA or exogenously and converted to L-glutamine(19). γ-PGA contains a mixture of D and L enantiomers of glutamic acid, using glutamate racemase, bacteria such as *Bacillus subtilis* will convert L-glutamic acid to D-glutamic acid (19)L and D glutamic acids are next involved in a ATP-dependent polymerization reaction. The phosphoryl

group of ATP is transferred to a terminal group of the carboxyl group via hydrolysis (19). An amino group on the glutamic acid performs a nucleophilic attack on the phosphorylated carboxyl group forming an amide linkage (19). Chains of γ-PGA are formed with the help of the PgsBCA synthase complex. The genes *pgs* operon are involved in the polymerization of released γ-PGA and are the key production machinery of γ-PGA in *Bacillus*.

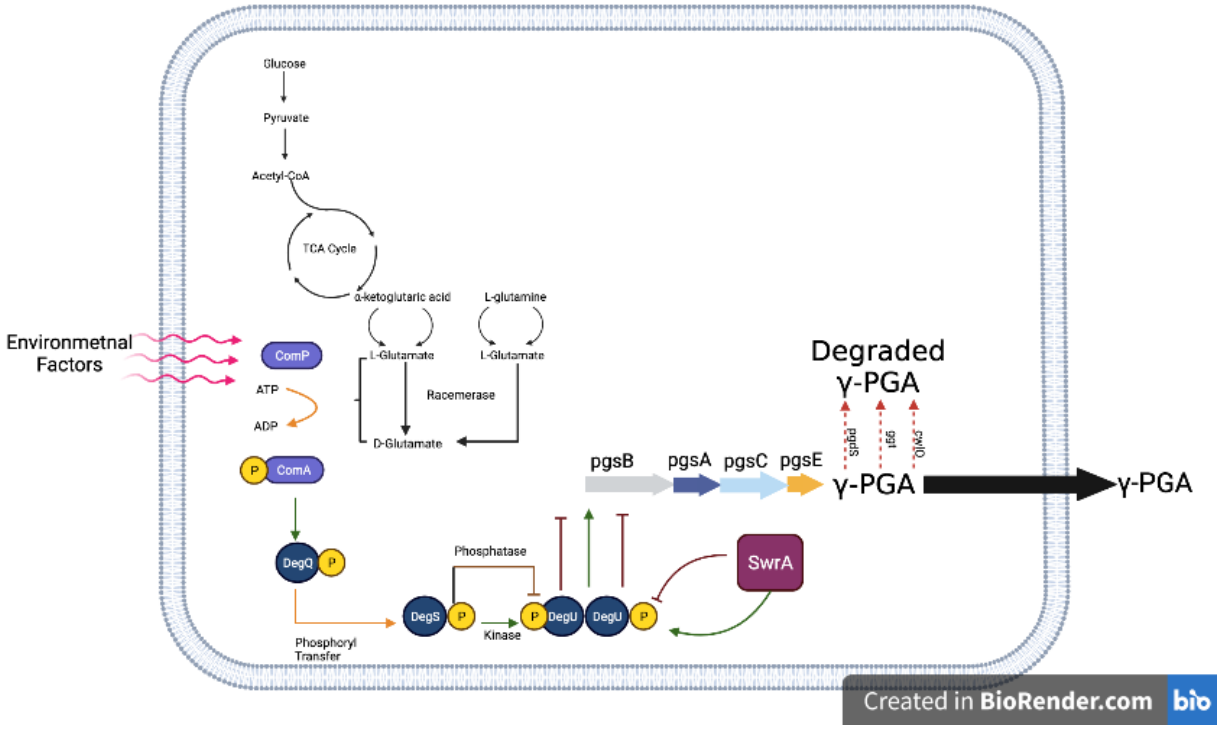

Figure 3. Overview of γ-PGA biosynthesis. Figure generated using BioRender

pgsbBCAE operon regulates γ-PGA

Regulation is another component of γ-PGA production that is also important in observing copper's bacterial fighting abilities by understanding the genes involved in turning on the machinery responsible for γ-PGA synthesis (**Figure 3**). The production of released γ-PGA is regulated by four identified genes – *pgsB, pgsC, pgsA,* and *pgsE* (20). These genes make up the

pgs operon which is responsible for polymerizing γ-PGA at the active site of the γ-PGA synthase (21). PgsA and PgsE mediate the transport of γ-PGA while PgsB and PgsA catalyze the polymerization of γ-PGA via ATPase activity (20). The genes *pgdS*, *cwlO*, and *ggt* are responsible for γ-PGA degradation by encoding components of glutamate dehydrogenase (20).

The *pgs* operon is regulated by a two component DegS-DegU system and ComP-ComA quorum sensing system (18). In *Bacillus*, high cell density will trigger the phosphorylation of Deg and Com proteins to drive the transcription of *pgsBCA* (**Figure 3**). Environmental signals will first promote phosphorylated ComP to phosphorylate ComA (18). ComA-P activates *degQ* transcription (a key regulator) (22). DegQ-P encourages the phosphoryl transfer of the DegS protein kinase to DegU (23). DegS also has phosphatase activity and can deactivate DegU via dephosphorylation (21). DegU-P controls the expression of *pgsBCA* by binding near the promotor; however, cooperation between DegU-P and SwrA is necessary to full activate transcription of the *pgsBCA* operon (24). The direct relationship between DegU-P and SwrA is unknown but previous work by Stanley and Lazazzera has determined the two proteins are essential for γ-PGA production (25). The regulatory machinery for γ-PGA synthesis and degradation are believed to be involved in the ability for *Bacillus* bacteria to use γ-PGA as a defensive measure against copper intoxication.

γ-PGA production in response to metal toxicity

γ-PGA is hypothesized to protect *B.subtilis* from metal intoxication by sequestering metal ions (18). Previous work done by Deol et al. demonstrated that *B.subtilis* exposed to excess levels of copper and zinc produced a mucoid phenotype which was later identified as γ-PGA

(26). Deol et al. also found that γ-PGA protects *B. subtilis* from zinc and copper intoxication. *B. subtilis* mutants with *ΔpgsB* (the removal of the *pgsB* gene involved in activating the *pgs* operon) did not produce γ-PGA and showed decreased growth when compared to the wild type (26). However, the results of this study differed from previous literature, as the *ΔdegU* mutant still produced γ-PGA in the presence of zinc and copper (26). Additionally, when *B. subtilis* was grown at metal concentrations determined to induce γ-PGA production, increased *pgs* and *pgdS* promotor activity was not found (26). The authors suggested a potential alternative pathway with *pgsE* – the last gene involved in the *pgs* operon (26). Additional research conducted in the Chandrangsu Lab by Grace Johnson (unpublished) however pointed to *swrA* – a gene essential for γ-PGA production – as a key regulator of γ-PGA production in response to metal intoxication. Johnson evolved wild-type *Bacillus subtilis* (NCIB3610) by exposing the it to excess concentrations of Cu^{2+} until resistance appeared. Two resistant strains – GJ014 and GJ015 - were found to not produce γ-PGA in the presence of copper and contained dull, flat colony morphology. Genome sequencing found that these two resistant strains carried mutations in the genes *swrA* and *ppsB*; the mutation found was identified later to encode for a premature stop codon (Johnson, unpublished). The work by Grace Johnson indicated that *swrA* and *ppsB* may have an essential role in regulating copper induced γ-PGA production.

swrA

The *swrA* gene encodes for a 117 amino acid long protein that cooperatively works with phosphorylated DegU (DegU-P) to drive *pgsBCA* expression. *swrA* is involved in both the *pgsBCA* operon and the *fla/che* operon which regulates *B. subtilis* swarming motility (24). SwrA supports DegU-P by stimulating the *fla/che* promotor to enable swarming and elevating the

number of flagella present (27). Two studies indicated SwrA is essential for γ-PGA production in *B. subtilis*. Osera et al. found that SwrA and DegU-P work in a cooperative manner (22). DegU-P alone can activate the pgs transcription at a minimal level without *swrA* expression; however, overexpression of *swrA* in a strain with a mutated *degU* did not activate the *pgs* operon (24). At the same time, Oshawa et al. found that high levels of DegU-P could fully activate the *pgs* operon in the absence of *swrA* and the transcription factor *degQ* minimally but also concluded that *swrA* stimulated the DegU-P dependent expression of *pgsB* for the proper production of γ-PGA (22). Ermoli et al. indicated that SwrA is a key modulator of DegSU on the *pgsB* promotor (19). Wild type $DegS^{Hy}$ with mutated *swrA* did not produce γ-PGA; mutated *degS* (which encodes for a Kinase that phosphorylates DegU) with mutated *swrA* also did not produce γ-PGA (21). The mutated $degS^{Hy}$ (a Hy mutation was used to cause excess accumulation of DegU-P) with a functional *swrA* resulted in the hyperproduction of γ-PGA (19). Ermoli et al. concluded that SwrA is a regulator of the *pgsB* along with DegU; *swrA* is needed for sufficient production of γ-PGA (19). While the literature has shown that *swrA* is needed for γ-PGA production, it has not determined if *swrA* is essential for copper induced γ-PGA production or the relationship between *swrA* and *ppsB* in γ-PGA production.

swrA regulates γ-PGA expression in response to copper toxicity

This study aims to determine if *swrA* has a role in regulating γ-PGA production in the presence of excess copper. We will determine if *swrA* or *ppsB* cooperatively or independently regulate γ-PGA production. Understanding *swrA's* role in γ-PGA production in response to metal toxicity will allow us to further study the anti-microbial properties of copper and the defenses bacteria develop against it.

Additionally, this study will also investigate how *ppsB* is involved in γ-PGA production. *ppsB* has not been previously cited to be involved γ-PGA production or copper resistance in *Bacillus* bacteria. It is known *ppsB* is one of five genes that encode for plipstatin synthetase. Plipstatin is one of the three metabolites (in addition to γ-PGA) produced by *B. subtilis* and is potentially involved in the anti-fungal properties of the bacteria. Surfactin is the third metabolite produced by B. subtilis that also has antifungal properties. The only association that *ppsB* shares with *swrA* is they are both regulated by deg system through an unknown mechanism (28). This project seeks to understand the potential relationship between *swrA* and *ppsB* in copper induced γ-PGA production and resistance. Understanding the roles of these two genes will further clarify the genes and proteins necessary for copper induced γ-PGA production and how it is regulated

We used the model organism, *Bacillus subtilis* to observe γ-PGA production. *B. subtilis* is a gram-positive soil bacteria that is utilized in industry for its excellent secretion ability(29). *B. subtilis* is a great model organism because it has a fermentation cycle of four hours, genetic stability, no strong codon expression, and a vast database of well-known and developed genetic tools and knockouts (19, 30) The two main strains of *B. subtilis* used are NCIB3610 (undomesticated Bacillus) and NCIB168 (domesticated Bacillus). The laboratory strain, NCIB168, has lost the ability to produce γ-PGA; however, NCIB3610, a derivative of the Marburg line, is a lab strain that can produce γ-PGA (19).

NCIB3610 *B. subtilis* was transformed with a *ΔswrA* from NCIB168 from the Bacillus Genomic Stock Center (BSGC). The transformed NCIB3610 with*ΔswrA* was exposed to increasing levels of copper to determine if swrA is needed γ-PGA production in response to copper toxicity. Colony morphology assays were performed to assess the γ-PGA production and

colony appearance. NCIB3610 *ΔswrA* was compared to two evolved strains of *B. subtilis,* GJ014 and GJ015, that were shown to have mutations in *swrA* and *ppsB*. GJ014 and GJ015 colonies were observed to have irregular edges, undulate margins and a flat, large appearance. We hypothesized that *swrA* is involved in γ-PGA production in *B. subtilis* in response to copper intoxication. We predict that the NCIB3610 strain with the *swrA* knockout will not produce γ-PGA in the presence of copper. The colonies will appear irregular in shape, with undulate margins and flat in appearance. The lack of γ-PGA production in NCIB3610 *ΔswrA* points to *swrA* playing an important role in regulating γ-PGA secretion in response to copper.

We performed Kirby-Bauer assays to assess the role of *swrA* and *ppsB* in copper resistance in *B. subtilis.* Kirby-Bauer assays assess how resistant a bacterium is to an antibiotic, in this experiment, the assay was used to assess copper resistance. We generated a double mutant of *swrA* and *ppsB* by transforming the NCIB3610*ΔswrA* with NCIB3610*ΔppsB*. NCIB3610, NCIB3610*ΔswrA,* NCIB3610*ΔppsB*, and NCIB*ΔswrAΔppsB* were incubated in 0.75% Luria Broth (LB) agar and poured onto a solid 1.5% LB agar plate. A disk was be saturated with 1M copper (II) sulfate and allowed to diffuse overnight. We assessed resistance by measuring the zone of inhibition of each strain. The zone of inhibition occurs around the saturated disk where the most copper is present and where the most bacterial die off should occur. Bacterial strains that are more resistant have smaller zones of inhibition. We hypothesized that strains with the *swrA* knockout will be less resistant to copper intoxication because *swrA* has a potential regulatory role in γ-PGA production and copper resistance. From the disk diffusion assays, we can determine if *ppsB* and *swrA* work together or separately in helping *B. subtilis* resist copper intoxication. We predict that NCIB3610*ΔswrA* will have a larger zone of inhibition than the

wild type if *swrA* is involved in copper resistance. The same can be said of NCIB3610*ΔppsB*. We will use a double mutant to identify if *swrA* and *ppsB* are involved in a potential pathway involved in γ-PGA production. We predict that is swrA and ppsB work together, the zone of inhibition will be larger than the other three strains tested.

Overall, this study will identify the if *swrA* is involved copper induced γ-PGA production in *B. subtilis*. At the same time, the results of this study will determine if *swrA* or *ppsB* is responsible for the copper resistance seen in the evolution studies by Grace Johnson. Identifying the genes involved in copper resistance in *B. subtilis* can provide further insight into how bacteria protect themselves from metals. Antibiotic resistance is a serious threat to our healthcare system and modern medical practices. Identifying the genes involved in bacterial defenses against metals, such as γ-PGA, will allow for treatments involving metals to be developed. These treatments could target the machinery of these bacteria and allow for treatments in the futures using metals combat the future to combat antibiotic resistant strains of bacteria.

Methods

Generation of Evolved Strain 14 and Evolved Strain 15 (GJ014 and GJ015). The evolved strains were obtained from research conducted by Grace Johnson. The methods outlined below are from her research conducted this summer. To identify potential genes involved in copper resistance and γ-PGA production, wild type of *B. subtilis* was exposed to increasing concentrations of Cu^{2+} to generate resistant strains. Colonies 3610 *B. subtilis* were streaked onto solid 1.5% LB agar plates and incubated overnight at 37°C (**Table 1**). Colonies were selected from the solid LB agar plates and inoculated in LB media containing Cu^{2+} at concentrations ranging from 0mM to 2mM. These cultures were incubated overnight at 37°C with shaking

(200rpm) or until a OD_{600}~0.6 was obtained. 3610 was grown again in 1mM Cu^{2+} LB medium and incubated at 37°C. This same culture was removed and placed into another 1mM Cu^{2+}LB media again with the same conditions to place additional stress on the bacteria. A 1:100 dilution of the overnight culture was placed into a 1.5mM Cu^{2+} LB media and incubated overnight at 37°C with shaking (200rpm). This culture was plated onto a solid 1.5% LB agar plate, incubated overnight at 37°C, and selected for colonies based on their morphologies. Evolved strains 14 and 15 (GJ014 and GJ015) were shown to form flat, large, and matte colonies. These colonies did not produce γ-PGA. DNA sequencing revealed these strains contained mutations in *swrA* and *ppsB*.

Strain	Description	Phenotype
3610	NCIB3610 Wild type isolate	γ–PGA production Circular, raised, moderate sized colonies
Δ*swrA*	NCIB3610 *trpC2* ΔswrA::erm	Erythromycin resistance Irregular, flat, moderate sized colonies
Δ*ppsB*	NCIB3610 *trpC2* ΔppsB::kan	Kanamycin resistance Irregular, flat, dull, moderate sized colonies
GJ014	NCIB3610 Premature Stop Codon	No γ-PGA production Circular, flat, large sized colonies
GJ015	NCIB3610 Premature Stop Codon	No γ–PGA production Irregular, flat, dull, large sized colonies
ΔswrAΔppsB	NCIB3610 *trpC2* ΔswrA::erm ΔppsB::kan	Kanamycin Resistance Erythromycin Resistance

Table 1. *B. subtilis* strain features and associated phenotypes

Transformation of NCIB3610 *B. subtilis* with *ΔswrA*. To assess the importance of *ΔswrA* importance in γ-PGA production in the presence of copper NCIB3610 was transformed with *ΔswrA* with an erythromycin marker from NCIB168*ΔswrA*::erythromycin from the BSGC

(31). NCIB168 B. *subtilis* does not produce γ-PGA. To mitigate this problem, *ΔswrA* was transformed into the NCIB3610 *B. subtilis* strain (undomesticated) (**Table 1**). The transformation method outlined in Kunst et al. using a single crossover event was used (34).

NCIB3610 and NCIB168*ΔswrA*::erm were streaked and incubated at 37°C overnight on solid 1.5% LB agar and LB agar with erythromycin. MC media was developed using 1M potassium phosphate buffer, 1M Trisodium citrate, 1M $MgSO_4$, 50% glucose, 10% Casein hydrolysate, 50% potassium glutamate, 22mg/mL ferrous ammonium citrate, and 2 mg/mL tryptophan (31). The MC media was used for liquid cultures the next day as a starvation agent. The following morning, the recipient strain (NCIB3610) and donor strain (NCIB168*ΔswrA*) were inoculated in MC media. Both strains were incubated at 37°C with shaking at 200 rpm overnight.

A 1:100 dilution of the recipient strain was created and incubated at 37°C with shaking (200 rpm) until an OD_{600} ~ 0.6-0.8 was reached. The donor strain was filter sterilized using a syringe filter. The filtrate contained DNA from the lysed cells. Donor filtrate was mixed with the recipient strain and incubated at 37°C with shaking (200 rpm) for two hours. The donor filtrate-recipient media was transferred into an Eppendorf tube and centrifuged at 5000 rpm for 5 minutes to pellet the cells. The supernatant was removed with 100 µL of supernatant remaining to resuspend the pellet. The pellet was plated and smeared onto a solid 1.5% LB agar plate with erythromycin to assess success of the transformation. The transformed NCIB3610 (NCIB3610*ΔswrA*) was incubated overnight at 37°C (**Table 1).**

Morphology Assays with Copper (II)Sulfate. Copper morphology assays were conducted to assess if *ΔswrA* contributes to γ-PGA production when exposed to excess levels Cu^{2+}. NCIB3610, GJ014, and GJ015 were streaked onto separate 1.5% LB agar plates while NCIB3610*ΔswrA* was streaked on 1.5% LB agar with erythromycin. All the plate were incubated

overnight at 37°C. A colony from each plate was inoculated into four separate tubes with 1.5% LB media and incubated overnight at 37°C with shaking (200 rpm) until an OD_{600} ~ 0.4-0.6 was reached.

Solid 1.5% LB agar plates were prepared with 0mM, 1mM, 2mM, and 3mM of $CuSO_4$. A 1:100 dilution of the four overnight cultures was prepared with 1.5% LB liquid media and again incubated at 37°C with shaking (200rpm) for 3-5 hours or until an OD_{600} ~ 0.6-0.8 was reached. The LB agar and copper plates were divided with four quadrants and labeled. A 5 µL dot of each diluted culture was micro pipetted onto the appropriate quadrant on all four concentrations of $CuSO_4$. The four plates of LB agar and copper sulfate were incubated overnight at 37°C and analyzed for growth.

Transformation of NCIB3610*ΔswrA* B. subtilis with *ΔppsB*. A double mutant of the transformed NCIB3610*ΔswrA B. subtilis* (recipient) was generated to determine if swrA or ppsB are responsible for copper resistance and γ-PGA production. NCIB3610*ΔppsB::kan* served as the donor in this transformation. The MC media and methods outlined in Kunst et al. were used in this transformation (citation needed).

The NCIB3610*ΔswrA* and NCIB3610*ΔppsB B. subtilis* strains were prepared using the transformation written above. A 1:100 dilution of the recipient strain was performed with MC media and incubated at 37°C with shaking (200 rpm) until an OD_{600}~0.6-0.8 was obtained. The donor strain was filter sterilized with a syringe filter and mixed with the diluted recipient media. This mixture was transferred to an Eppendorf tub and centrifuged at 5000 rpm for 5 minutes. Part of the supernatant was removed with some remaining to resuspend the pellet. The pellet was plated on a solid 1.5% LB agar with kanamycin to determine if the Δ*ppsB* had been successfully transformed into the NCIB3610*ΔswrA*.

Kirby-Bauer Assay. Diffusion discs assays were performed to assess the role of *swrA* and *ppsB* in copper resistance (**Figure 4**). NCIB3610, NCIB3610*ΔswrA*, NCIB3610*ΔppsB*, and NCIB3610*ΔswrAΔppsB* were streaked and incubated overnight on solid 1.5% LB agar plates and their corresponding antibiotic. The four strains were inoculated in 10 mL of LB and incubated overnight at 37°C with shaking (200 rpm) or until an $OD_{600} > 0.6$ was met. The overnight cultures were diluted (1:100) with 5 mL of 0.75% LB agar and placed on a heat block at 60°C to prevent the agar from resolidifying.

Four 1.5% LB agar plates were prepared and brought to room temperature. The overnight cultures were poured evenly across the surface of the 1.5% LB agar plate and allowed to solidify. Once solidified, sterilized filter discs were placed in the center of the plate. A 5µL drop of 1M copper sulfate was micropipetted onto the disk. The plates were incubated overnight at 37°C. The zone of inhibition of each strain was measured the following morning.

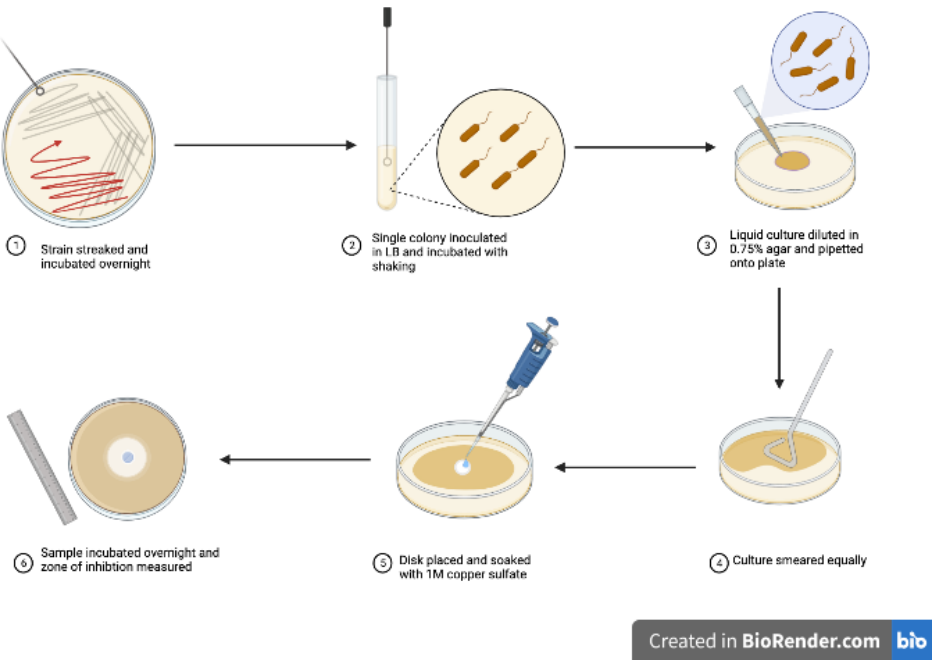

Figure 4. Kirby-Bauer assays were conducted to determine if *swrA* and *ppsB* contribute to copper resistance in *B. subtilis*. This figure was created using Biorender.com

Statistics. A One-Way ANOVA was used to analyze the average zone of inhibition from the disc diffusion assay. Due to the small sample size, normality was assessed in the residuals of the dataset overall than within each mutant group. A Shapiro Wilk Test of normality was conducted and showed the residuals of the data set were normally distributed (W=0.86, p=0.02). A Levene's test demonstrated that the zone of inhibition data did possess homogenous variances (df=3, F=1.42, p=0.29). The requirements of a parametric One-Way ANOVA were met.

Results

B. subtilis strains with swrA mutations are shown to not produce γ-PGA when exposed to varying concentrations of copper.

We performed copper morphology assays to determine if swrA has a role in γ-PGA production in response to copper intoxication. We transformed a undomesticated lab strain of *B. subtilis* (NCIB3610) with a s*wrA* knockout (*ΔswrA*) and plated on varying concentrations of copper sulfate. The two evolved strains, GJ014 and GJ015, were used as a reference as these strains contained mutations in *ppsB* and *swrA* along with NCIB3610 which is known to produce γ-PGA in response to metal toxicity. The four strains were all plated on 1.5% solid LB agar with 0mM, 1mM, 2mM, and 3mM of copper sulfate, incubated overnight, and assessed for γ-PGA production. *B. subtilis* strains containing mutated *swrA* did not produce γ-PGA at all concentrations of copper sulfate (**Figure 5**). At 1mM and 2mM, the production of γ-PGA is seen with NCIB3610; the colony contains a shiny appearance and is round (**Figure 5**). At 0mM the most growth is seen in all strains (**Figure 5**). GJ014, GJ015, *ΔswrA* formed a matte colony with irregular edges at 0mM and 1mM. The *ΔswrA* colony formed at 2mM resembled NCIB3610 with round edges and small when compared to GJ014 and GJ015; however, no γ-PGA production is

present (**Figure 5).** At 3mM, no growth occurred in GJ015 and NCIB3610 while minimal growth is seen *in ΔswrA* and GJ014. The copper morphology assays may demonstrate that *swrA* has a role in γ-PGA production in response to excess copper.

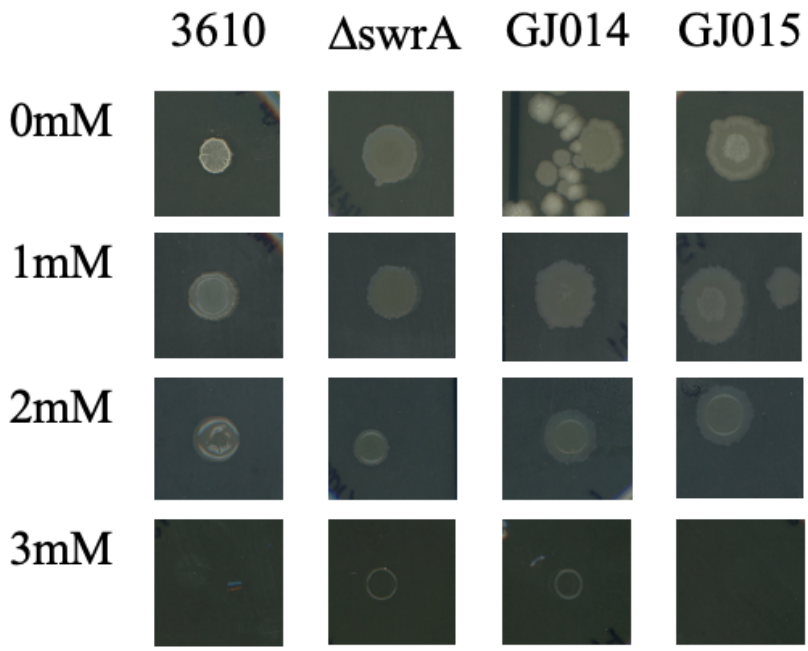

Figure 5. Colony morphology assays on 1.5% LB agar plates with 0mM, 1mM, 2mM, 3mM of copper sulfate. Refer to table 1 in methods for strain descriptions

B. subtilis strains with ΔswrA and ΔppsB knockouts showed no difference in resistance to excess copper when compared to the wild type and the double mutant

Kirby-Bauer assays were conducted to test determine if *swrA* or *ppsB* contribute to copper resistance in B. subtilis. A double mutant was generated by transforming NCIB3610ΔswrA with NCIB3610*ΔppsB*. Sequencing results revealed that GJ014 and GJ015 contained mutations in both *ppsB* and *swrA*. The strains 3610, *ΔswrA*, *ΔppsB,* and *ΔswrAΔppsB* were inoculated into liquid cultures and incubated overnight at 37.1°C with shaking. The liquid

cultures were diluted with 0.75% LB agar and placed in a heat block at 60°C. The 0.75% LB agar solutions were poured onto 1.5% LB agar plates. A disk was placed in the center and soaked with 1M copper (II) sulfate. All strains were incubated for 24 hours at 37.1°C with the copper-soaked disk diffusing. The diameter of bacterial die off around the disc – the zone of inhibition - was measured with a ruler.

Copper diffusion was present in all four plates. 3610 had additional colonies form on top of the agar and γ-PGA production on the outer boundary of the zone of inhibition (**Figure 6**). No γ-PGA production was viewed on the other strains (**Figure 7**). Bacterial die off in *ΔppsB* mainly concentrated to the center of the plate compared to the other strains where die off appeared as a gradient among the halo (**Figure 7**).

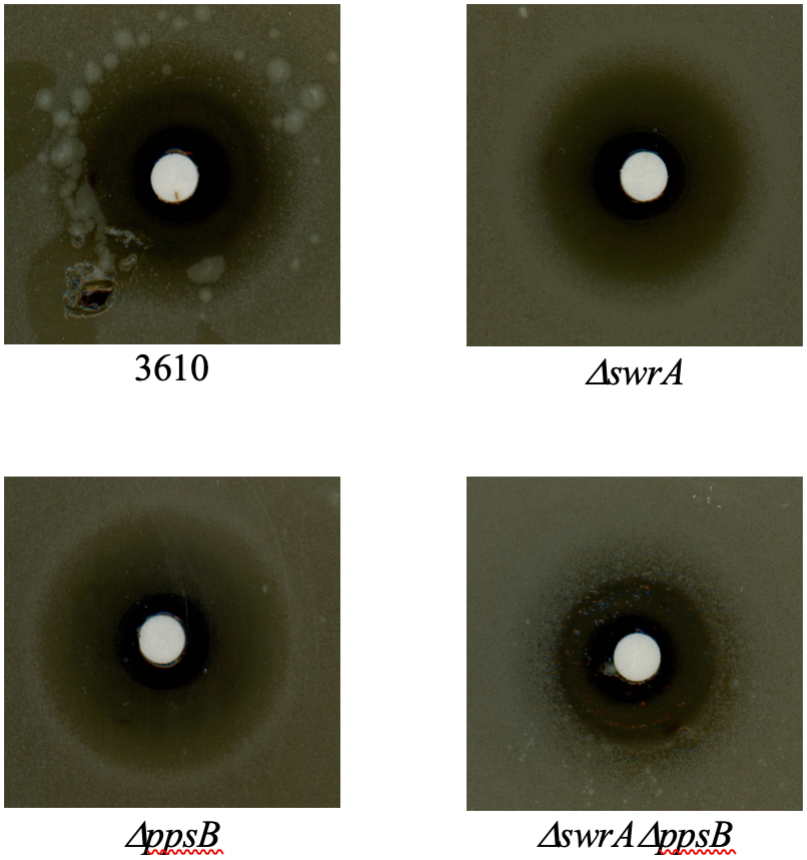

Figure 7. Kirby-Bauer resistance assays on 1.5% LB agar and diluted cultures in 0.75% LB agar poured on top. The disk at the center contains 1M of copper (II) sulfate. Refer to Table 1 in methods for strain descriptions

A One-Way ANOVA was conducted to compare the average zone of inhibition in *Δswr*A, *ΔppsB*, *ΔswrAΔppsB,* and 3610. There were no significant differences in the average zone of inhibition between all four strains of *B. subtilis* (**Figure 8**)

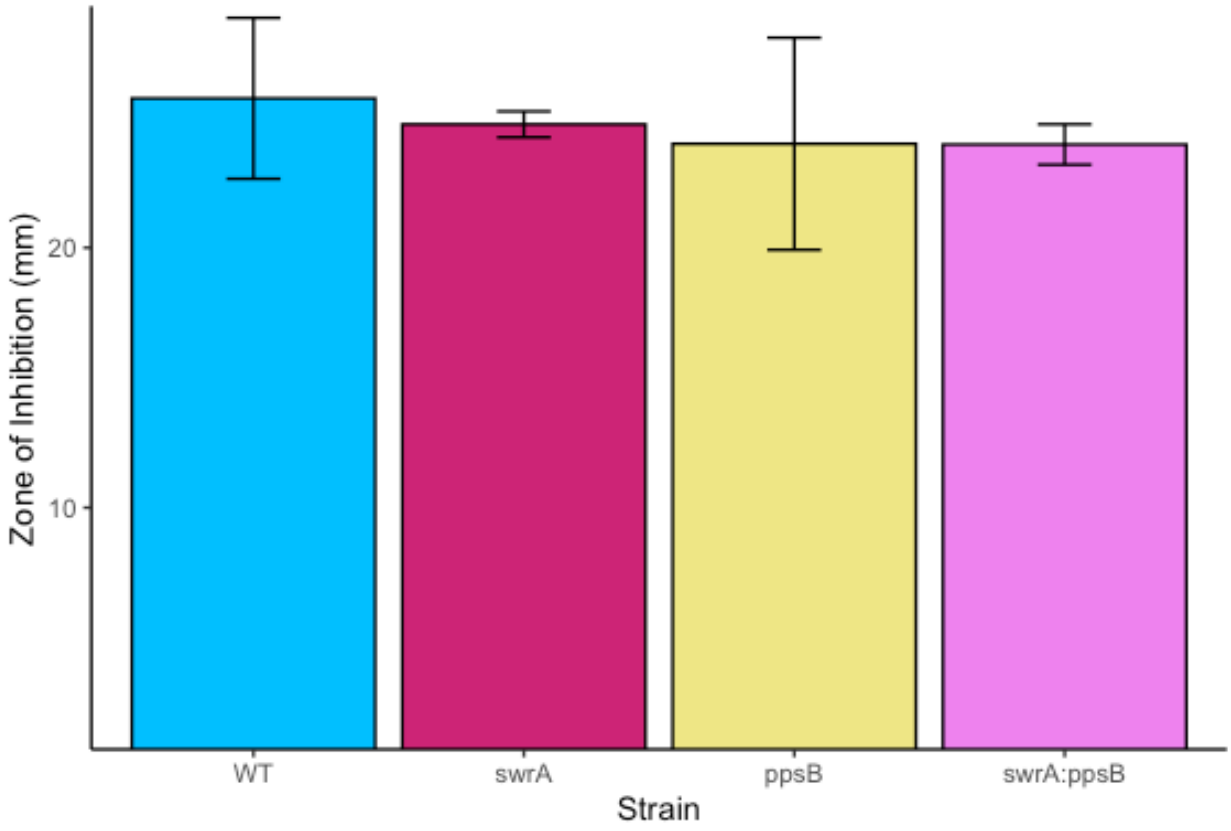

Figure 8. Bar graph of the average zone of inhibition of 3610, *ΔswrA*, *ΔppsB*, and *ΔswrAΔppsB* (mm) with standard deviation (F=0.412, df=3, p=0.747).

Discussion

swrA is involved in copper induced γ-PGA production

B. subtilis strains with mutated *swrA* did not produce γ-PGA when exposed to 0mM, 1mM, 2mM, and 3mM of copper (II) sulfate (**Figure 5**). The wild type displayed a mucoid phenotype which has shown to be γ–PGA when exposed to copper (26). The lack of γ-PGA production in these mutated strands follows previous literature that found that *swrA* is essential for effective γ-PGA production. SwrA has been shown to work cooperatively with the transcription factor DegU in activating the *pgs* operon (24). The mechanism of how SwrA cooperatively works with DegU-P to induce the *pgs* operon is unknown. Excess phosphorylated

DegU can overcome the loss of SwrA but does not restore γ-PGA production abilities in B. subtilis (24). SwrA may strengthen the binding phosphorylated DegU to *pgsB* to activate transcription *pgs* operon. Additionally, SwrA may assist in binding by orienting DegU to properly bind with *pgsB* (24). Additional studies are needed to determine the exact interaction of SwrA with DegU.

swrA and ppsB are not involved in copper resistance when compared to the wild type B. subtilis

γ-PGA has been found to defend soil bacteria such as *B. subtilis* from metal intoxication by sequestering metal ions (32). The free carboxyl groups in γ-PGA form complexes with free metals to absorb metal ions from the soil in B. subtilis (33). Because we identified *swrA* involved in copper induced γ-PGA production, we conducted Kirby-Bauer assays to assess if it also had a role in copper resistance. We sought to understand if *swrA* or *ppsB* were responsible for any observed resistance to copper. *swrA* and *ppsB* were both identified in GJ014 and GJ015 *B. subtilis* strains (**Table 1**). These strains did not produce γ–PGA when exposed to copper. The Kirby-Bauer assays indicated that *swrA* and *ppsB* may not be involved in copper resistance in *B. subtilis*.

We first investigated the role of growth medium on *B. subtilis* growth. *B. subtilis* can effectively grow on solid agar and liquid media (34). Solid and liquid mediums require *B. subtilis* to engage in different lifestyles. As shown in figure 5, *B. subtilis* will develop robust biofilms to protect against copper intoxication. Biofilm production occurs in both media types; however, how the biofilms form differs. In solid agar plates, biofilms grow and thicken away from the place of inoculation due to an osmotic pressure gradient from the agar below(34). In liquid media, biofilms form as a pellicle that initially grow in a thin layer on interface (34)

Biofilms developed on agar are unable to penetrate the substrate below the interface; however, in liquid medias, B. subtilis can swim to the media underneath (34).

Kirby Bauer assays are conducted using solid plates with a disc diffusing highly concentrated copper (II) sulfate (**Figure 7**). The lifestyle requirements for *B. subtilis* to survive on solid media may not be conducive for resisting copper intoxication. A Minimum Inhibitory Concentration (MIC) assay could better assess copper resistance in *B. subtilis*. MIC assays are conducted similarly to Kirby-Bauer assays but done in a liquid solution; the assays determine the minimum concentration of antibiotic needed to reduce bacterial growth (35) An MIC assay would rule out if the biofilms formed in liquid media are more effective in resisting copper intoxication. Additionally, we can rule out if the type of assay contributed to the results seen in this study.

We also investigated other methods *B. subtilis* can protect itself against metal intoxication. SwrA is a master regulator of two operons, the *fla/che* operon for movement and *pgs* operon for γ-PGA production. SwrA regulates both operons via the Deg system (**Figure 3**). Because we did not see significant differences in the average zone of inhibitions in *swrA*, *ppsB*, and *ΔswrAΔppsB*, we took a closer look into the Deg system. DegQ serves as a pleiotropic regulator by promoting the phosphorylation of DegS and DegU. Previous literature has shown that *degQ* and *degU* expression may influence *srfA* expression (36). Miras et al. found that reduced expression of *degQ* and *degU* led to the derepression of the *srfA* operon prompting surfactin production. Surfactin is a cyclic lipopeptide produced by *B. subtilis* (37). It is a member of a class of molecules called biosurfactants – amphillic compounds made of hydrophilic and hydrophobic components (**Figure 9**) (37). Biosurfactants like surfactin can reduce surface tension to allow for swimming motility (38) Additionally surfactin has shown to be effective in

bioremediation of soils contaminated with copper sequestering excess copper similar like γ-PGA (39).

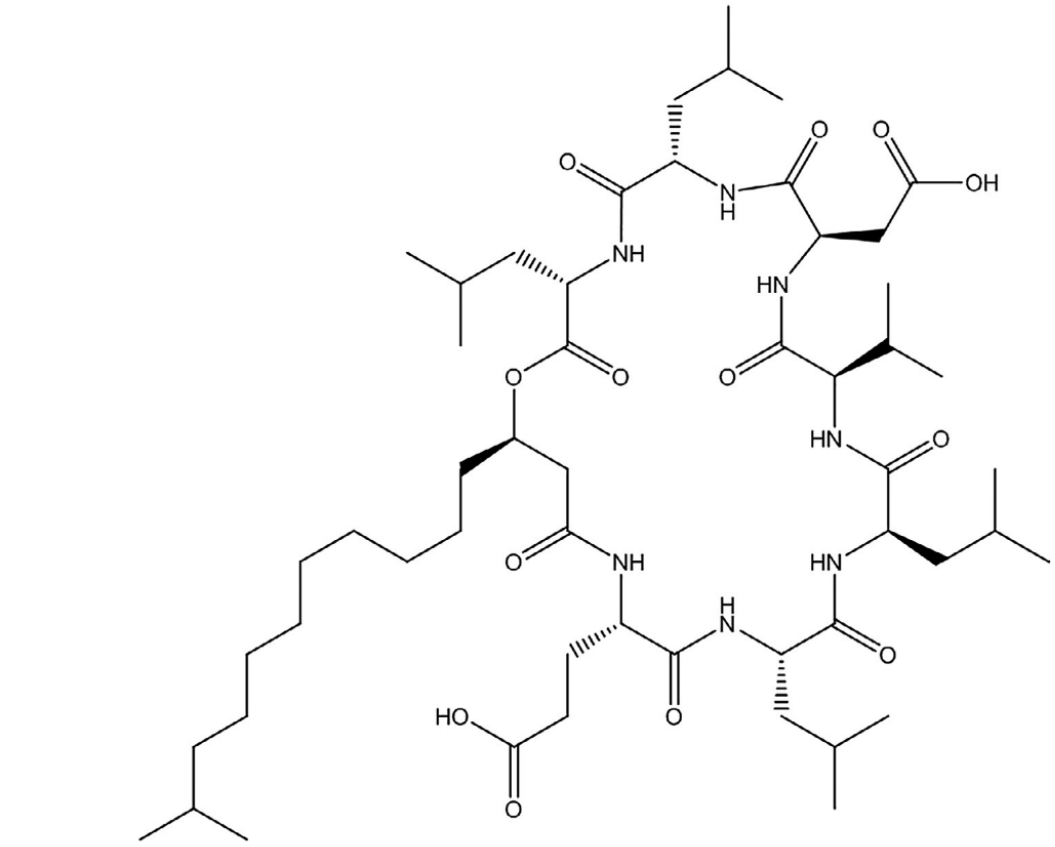

Figure 9. Chemical structure of surfactin from B. subtilis. Adapted from Henkel and Hausmann 2019

Our results may indicate that the reduced expression of the *deg* system may have led to the derepression of the *srfA* operon leading to the production of surfactin (**Figure 9**). The loss of *swrA* showed no effect on *B. subtilis* ability to resist copper intoxication; however, no γ-PGA was produced (**Figure 4**). The loss of γ-PGA production in the *ΔswrA* mutant indicates that *swrA*

plays a role in *pgs* activation. *swrA* works with the Deg system to regulate the *fla/che* operon for motility and γ-PGA production. It was previously shown that even with phosphorylated DegU, *swrA* expression was still needed to effectively produce γ-PGA. The interaction between SwrA and DegQSU is unknown. Our results may indicate that *swrA* is a pleiotropic regulator of the *pgs* and *srfA* operon through the *deg* system. The loss of *swrA* expression could lead to the reduced expression of *degQSU* through some unknown mechanism (**Figure 10**). Reduced expression of the DegQSU system causes repression of the *pgs* operon and activation of the *srfA* operon through an unknown mechanism. Activation of *srfA* operon would induce surfactin production to maintain copper homeostasis in the event of excess copper exposure.

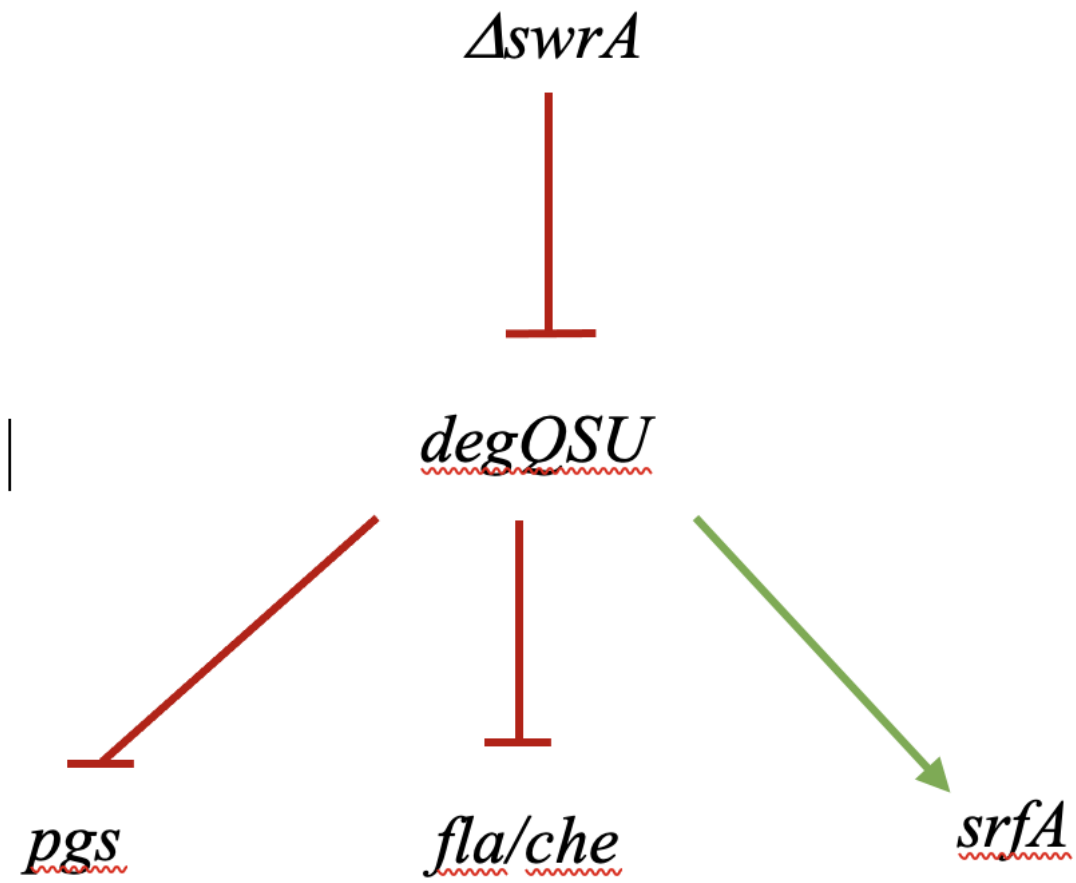

Figure 10. Schematic of the pleiotropic regulatory role of *swrA* in copper resistance

Surfactin, like γ-PGA, could help B. subtilis resist excess levels of copper. Future experiments could expose *ΔswrAΔdegU* and *ΔswrAΔdegQ* double mutants to excess levels of copper. MIC or Kirby Bauer assays could be conducted to assess resistance to copper. Decreased resistance could indicate that *swrA* pleiotropically regulates *pgs* or *srfA* through the *deg* system. With this information, we can further understand the defenses bacteria such as *B. subtilis* use to resist metal intoxication.

Conclusion

The continued rise of antibiotic resistant superbugs brought a renewed interest in the antimicrobial properties of metals such as copper. This study demonstrated that *swrA* is involved in copper induced ©-PGA production and could potentially serve as a pleiotropic regulator of ©-PGA and surfactin production. Future experiments could test *swrA*, *degQ* and *degU* knockouts against excess levels of copper. These experiments could determine if *swrA* is a master regulator of the *pgs* and *srfA* operons which both produce compounds that allow *B. subtilis* to maintain metal homeostasis by sequestering excess copper. Understanding *swrA's* role as a potential pleiotropic regulator can help us better understand the defenses bacteria employ to resist metal intoxication. Copper has shown to be an excellent antimicrobial due to its high reductive potential. Treatments in the future can utilize the antimicrobial properties of copper while targeting the defense mechanisms bacteria have developed against metal intoxication. The continued rise of antibiotic resistant bacteria has defined the 21st century as the "Post Antibiotic Era. " With an estimated 35,000 Americans dying from antibiotic resistant bacterial functions each year and the lack of development of new antibiotics, further inquiry into copper and bacterial defenses against it are essential in protecting and maintaining the very foundation of modern medicine.

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