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Mutations of *Bacillus subtilis* to Increase Production of Riboflavin (Vitamin B2) Intermediate Guanosine Triphosphate (GTP)

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Abstract

B. subtilis is a Gram-positive bacterium that produces a wide range of naturally occurring products. A few of these products are antibiotics, such as sublancin or surfactin, and vitamins, such as riboflavin (vitamin B2). Riboflavin is a natural vitamin that is an important part of the function of the electron transport chain (ETC) within cells. Through this project, the production of the key precursor to riboflavin, guanosine triphosphate (GTP), was increased by isolating purine analog-resistant mutants of *B. subtilis* that redirect the carbon flow to guanine and away from adenine. To do this, a library of randomly mutated *B. subtilis* was prepared using EMS (ethyl methyl sulfonate) mutagenesis, and mutants were then screened for azaguanine (Az) resistance, a toxic analog of guanine. Increasing guanine levels should correlate to an increase in GTP levels. Azaguanine acts as a competitive inhibitor of biosynthetic enzymes that convert guanine to GTP; the *B. subtilis* strain used in the EMS mutagenesis contains a preexisting *purB* mutation that enhances the recovery of azaguanine-resistant mutations in the GMP-IMP-AMP junction of the purine pathway. Twelve azaguanine-resistant mutants (Az^r) were recovered and characterized for azaguanine resistance using a maximum inhibitory concentration (MIC) assay, and guanine levels were measured using a reverse halo (growth halo) plate assay and High-Performance Liquid Chromatography (HPLC). Of these 12 mutants, one mutant, Az8, was determined to have the highest-produced guanine; the next best was a spontaneous mutant, Az2. We speculated that the mutation in Az8 favors the production of guanine and guanosine over the production of hypoxanthine or inosine. In the future, the Az8 strain will be used to further increase guanine/GTP production in a second mutagenesis and selection round, using the guanosine toxic analog decoyinine.

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Introduction

Riboflavin, also known as vitamin B2, is an essential vitamin in the electron transport chain (ETC) in animals and humans. It is capable of controlling both electron acceptors and electron donors within the mitochondria of the cell during the production of adenosine triphosphate (ATP). However, riboflavin is only a precursor for flavin derivatives, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN), which are responsible for many different processes within the body. Despite being an important cofactor, riboflavin must be converted into either FAD or FMN by the bifunctional enzyme RibC to be used in cellular processes. FMN, which is the phosphorylated product of riboflavin, is responsible for the oxidation reaction associated with NADH during the electron transport chain. The FMN molecule, which is adenylated from FAD, is an important cofactor in maintaining the homeostasis of functional hemoglobin. Some of the functions FAD is associated with are ATP production, DNA repair, chromatin remodeling, protein folding, and many other functions within all animals including humans (Pinto and Zempleni 2016).

Riboflavin is not produced by animals, so it must be acquired from the diet and has become a staple part of many human food and feed additives of livestock. Because it was first isolated from lactose-containing materials foods, riboflavin was originally named lactoflavin (Kutsal and Özbas 1989). Lactoflavine encompasses multiple different types of flavin derived from D-ribose. Some of those types are ovoflavine, isolated in eggs, hepatoflavine, isolated from the liver, lactoflavin, isolated from milk, and verdoflavine, isolated from plants. Riboflavin is a yellow, water-soluble vitamin that is relatively stable (in cooking) up to 120°C for up to six hours. It is stable in the air and under many oxidizing agents but degrades rapidly in alkaline

(basic) conditions. Another important feature of riboflavin, especially when isolating riboflavin overproducing microbial strains, is that it fluoresces under ultraviolet light.

The concentration of riboflavin within different food varies depending on how the food is produced. Some human food containing high concentrations of riboflavin are pork liver at a concentration of 2900-4400 μg/g and cheese at a concentration of 330-565 μg/g. After being digested within the human body, it is spread throughout the body in very small concentrations (Konishi and Shiro et al., 1968). It is found within the kidney at the greatest concentration of 20.0 μg/g and the liver at a concentration of 16.6 μg/g. When specifically looking at humans, many symptoms are present as a result of riboflavin deficiency (Pinto and Zempleni, 2016). The most apparent is skin abnormalities resulting from hepatic mitochondria fatty acid oxidation (NIH, 2023). Riboflavin active forms have also been known to act upon the free radicals within the body (Pinto and Zempleni, 2016). To counteract these deficiencies, many food companies add riboflavin to baby food and cereals, and bread products. In addition, riboflavin can be obtained through single or multivitamin supplements. In animals, riboflavin deficiency can result in stunted growth and deformed limbs. Consequently, when given the appropriate amount (in feed), riboflavin not only counteracts these deficiencies but functions as a growth promoter (Perkins et al., 1999)

The first process, developed in 1935, utilized chemical synthesis (Kutsal and Özbas, 1989). In the process, D-ribose is used as the starting product, which is typically produced by fermentation. Other chemical synthesis uses non-fermentative compounds O-xylene and alloxan as starting materials. A few other methods have been developed since which use similar starting materials with slightly different steps. Despite the ability to chemically synthesize riboflavin, there are many drawbacks to these methods. The largest is the low yield of synthesis. Next is the

use of solvents and chemical intermediates that are toxic, which not only need to be removed from the final product but also need to be disposed of safely. Because of the low green chemistry and low yield, chemical synthesis is not commercially used.

In place of chemical processes, commercial riboflavin fermentation processes have been developed using bacteria, yeast, and fungi (Averianova et al., 2020). The two best production strains are *Bacillus subtilis* (bacteria) by DSM and *Ashbya gossypii* (fungus) by BASF. In both of these microorganisms, the wild-type cells contain the necessary cellular machinery to produce riboflavin, but at trace levels. However, by using a combination of classical strain improvement and recombinant genetic engineering techniques, these strains have been altered to contain a combination of host mutations, to increase precursor pools, and/or engineered riboflavin biosynthetic genes (*rib*), to increase the yield of riboflavin (Liu et al., 2020; Averianova et al., 2020). However, there are differences between the two production strains. Although *Ashbya gossypii* contains all of the required genes for riboflavin production, they are located on different chromosomes, whereas for *B. subtilis*, all the required riboflavin biosynthetic genes are located at a single site of the chromosome (called the *rib* operon). The clustering of the *rib* genes allows for faster genetic engineering as opposed to engineering genes from multiple loci. Moreover, the growth of *B. subtilis* is much faster than *A. gossypii*, resulting in higher productivity rates, an important consideration for developing any commercial production strain (Averianova et al., 2020, Perkins et al., 1999). For these reasons, *B. subtilis* is used as the standard in the commercial production of riboflavin. Before *B. subtilis'* riboflavin fermentation can be discussed, a better understanding of the riboflavin biosynthetic pathway is required.

B. subtilis and Biosynthesis of Riboflavin

B. subtilis is a versatile bacterium that is capable of producing many different products and reactions in an industrial production and academic research setting. A few of the many different products are listed below in **Table 1**. The *B. subtilis* is a model Gram-positive bacterium, with over 70 years of research history. It contains a thick cell wall that is composed of peptidoglycan which allows the bacteria to maintain its function and shape under stress. The rod-shaped bacterium is capable of forming heat-resistant, solvent-resistant endospores through the process of sporulation. The process of sporulation is brought on by periods of low to no nutrients causing stress to the cell. It is naturally competent, allowing foreign deoxyribose nucleic acid (DNA) to be taken up into the cells and integrated into the genome. As such, researchers can insert, delete or even change nucleotides in the *B. subtilis* chromosome with relative ease. This is an important feature when engineering biosynthetic pathways of *Bacillus subtilis* for the production of a commercial enzyme or small molecule, especially for the manipulation of genes associated with the riboflavin biosynthetic pathway (see below). Any of these changes could drastically alter the viability and function of the cell. Another important characteristic is that the genomic sequence of *B. subtilis* has been determined and with this knowledge comes an understanding of synthetic biologic, genetic engineering, and omics tools which can be used to further improve commercial production strains. These tools can be used to optimize gene expression of biosynthetic genes, identify and quantify gene transcript level and protein levels, and provide a roadway of carbon flow from glucose to the final product, e.g. riboflavin. In addition, there are several different categories of "libraries" to enhance gene expression and manipulation. These include promoter libraries, ribosome binding sites libraries, knockout libraries (perfect excision of all coding regions of *Bacillus*), etc.

Product Class	Example
Chemicals	1. Isobutanol 2. 2-methyl-1-propanol 3. glucosamine 4. Heparosan 5. Amorphadiene 6. *scyllo*-inositol 7. Acetoin
Proteins	1. Human growth protein 2. Interferon 3. Proinsulin 4. Tissue plasminogen activator 5. Insulin-like growth factor (IGF-1)
Food	1. Riboflavin 2. Purine nucleosides 3. Poly-γ-glutamic acid 4. D-ribose 5. L-malate 6. Acetoin
Enzymes	1. α-Amylase 2. β-Amylase 3. β-Glucosidase 4. Glucose isomerase 5. Alkaline phosphate 6. Lipases 7. Penicillin acylase
Antibiotics	1. Subtilin 2. Pneumolysin 3. Tetanus toxin fragment C (TTFC) 4. Omps and Hsp60 5. Adjuvant

Table 1. Commercial products of *B. subtilis*. Adapted from Zeigler and Perkins, 2008.

In addition to genetic tools, there is a wealth of fermentation knowledge of *B. subtilis* at the commercial scale (Arbige et al., 1996). For example, *B. subtilis* can be fermented at a larger scale, over 100 m^3 (100,000 liters) in batch or fed-batch mode. Sugar sources can vary from

processed starch from corn (e.g. DE95) to sucrose (sugarcane or sugar beets). Nitrogen sources can vary but the preferred is ammonium or ammonia gas. For riboflavin production by DSM, multiple 120,000-liter fermenters containers are used (Konishi and Shiro et al., 1968). Riboflavin can be recovered at titers over 10 g/L in about 48-72 hours of growth. This process requires a constant supply of O_2 for the reaction to occur Perkins et al., 1999.

Considering government regulatory approval, products made by *B. subtilis* are classified "As Generally Regarded as Safe" (GRAS) by the United States (Perkins and Pero, 1993). This means that any products that are produced by *B. subtilis* are safe for human and animal consumption. Additionally, within the European Union, *B. subtilis* is classified as a qualified presumption of safety (QPS) microorganism. QPS means that the cell is presumed to be safe for human consumption (e.g. probiotic) or product thereof. This classification differs by assessing the organism instead of the products but together, the products and cells of *B. subtilis* are regarded as safe.

Riboflavin Biosynthetic Pathway and Precursor Pools

The two precursors to riboflavin are ribulose 5-phosphate (Ru5P) and GTP (**Figure 1**), the former is derived from the pentose pathway and the latter from the purine pathway. Both are needed to be produced at high levels in the cell in order to increase riboflavin production (Perkins et al., 1999).

So when assessing GTP levels, the synthesis and interconversion of purines within this pathway is done through a combination of different enzymatic reactions (**Figure 1**). Enzymes being the biological catalysts that when present within a reaction can increase the reaction rates. Kinases are also needed to phosphorylate purines as a way to store energy for enzymatic

reactions and other cellular functions (Pedro et al., 2019). Kinases are a specific type of enzyme that catalyzes the addition of phosphate groups onto a molecule. Kinase activity is seen with the addition of multiple phosphate groups on adenosine and guanosine. This reaction can occur in one, two, or three sequential steps. For example, guanosine is first converted to guanine monophosphate (GMP), then guanine diphosphate (GDP), and then guanine triphosphate (GTP). In addition to storing energy, GTP has another function, that is the precursor to riboflavin biosynthesis entering at the RibG reaction (**Figure 1**). Other than directly measuring GTP levels, measuring the levels of various purines in the pathway (e.g. guanine, inosine, etc.) can be used as an indirect way to determine that activity of the purine pathway to GTP formation (Perkins et al., 1999)

The other riboflavin precursor is ribulose-5-phosphate (Ru5P), which is derived from pentose pathway, is used in the RibB reaction (Xiu et al., 2015). Interesting, it can also be converted to ribose-5-phosphate (R5P) which enters the purine pathway (via PRPP) and eventually to form GTP. It is assumed that the cellular level of Ru5P is sufficient for increasing riboflavin production (Perkins et al., 1999 Averianova et al., 2020).

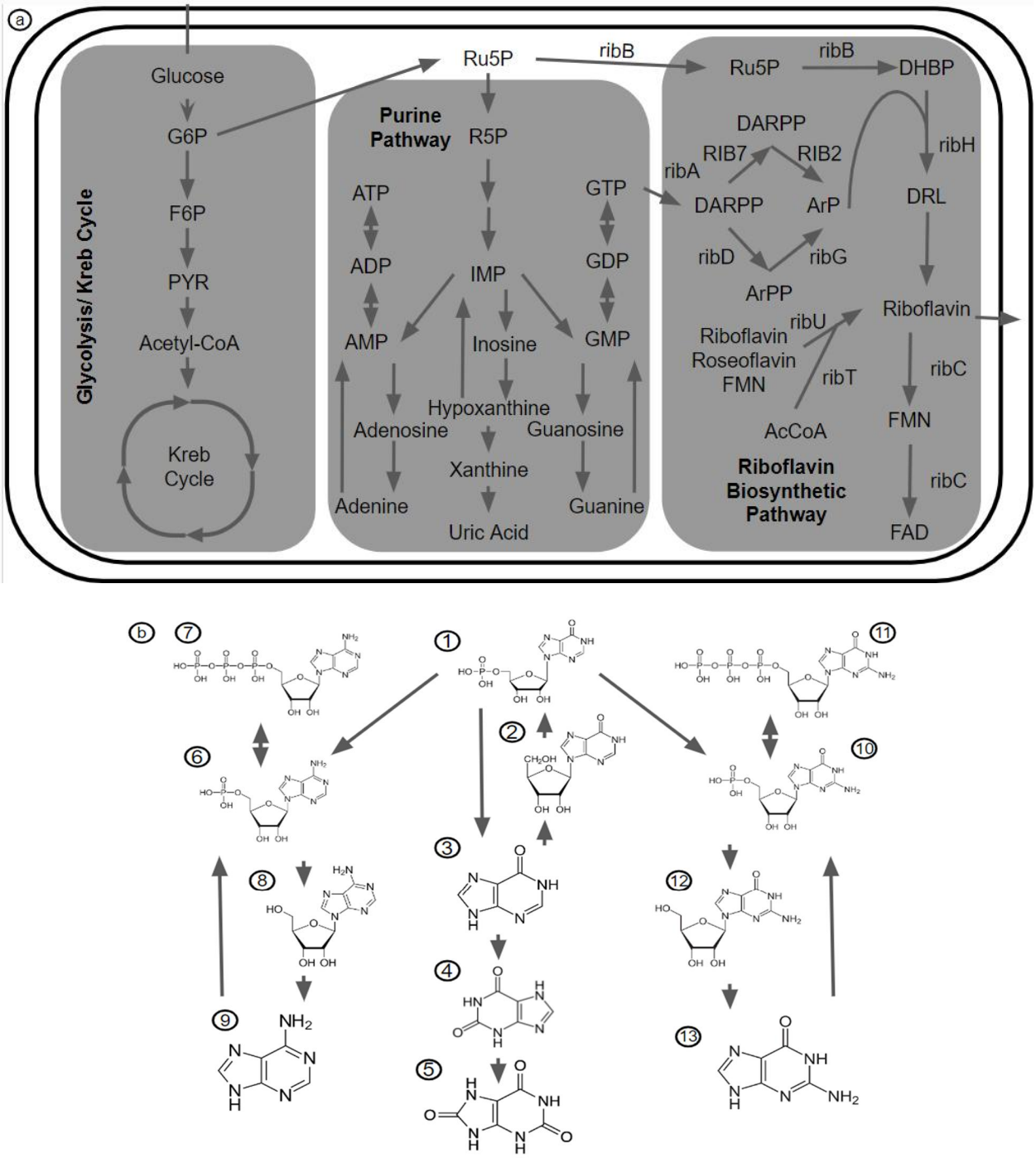


Figure 1. Overview of Riboflavin Biosynthesis. (a) The three major pathways present in *B. subtilis* are glycolysis, the purine pathway, and the riboflavin biosynthetic pathway; adapted from Averianova et al., 2020 and Xu et al., 2015. (b) Structures of major purines within the purine pathway (1: IMP/2: Inosine/ 3: Xanthine/ 4: Hypoxanthine/ 5: Uric Acid/ 6: AMP/ 7: ATP/ 8: Adenosine/ 9: Adenine/ 10: GMP/ 11: GTP/ 12: Guanosine/ 13: Guanine.

Rib Operon

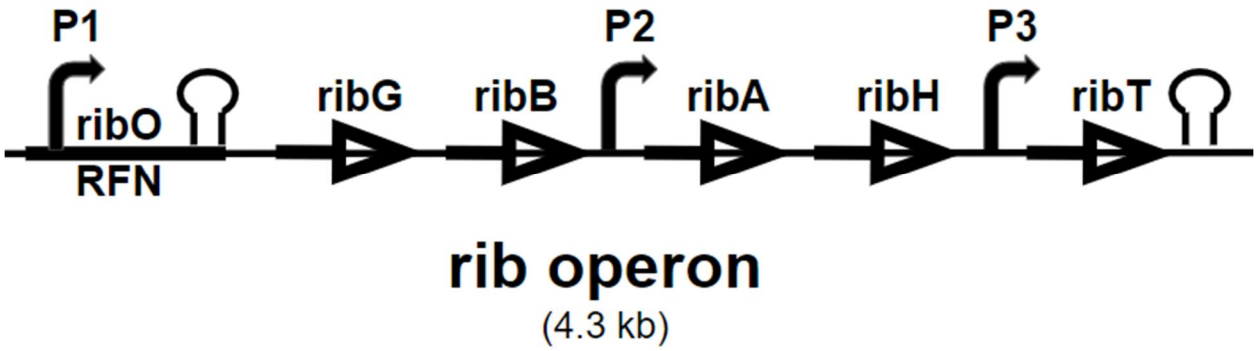


Figure 2. The general structure of *rib* operon. The *rib* operon is 4.3 kb (4,300 bp) in length. These genes have been recently renamed to account for those encoding bifunctional enzyme activities: *ribDG* (*ribG*); *ribE* (*ribB*); *ribAB* (*ribA*); *ribH* (*ribH*), and *ribT* (*ribT*). The protein encoded by the rib gene varying in length: *ribT* (122 aa), *ribH* (154 aa), *ribAB* (398 aa), *ribE* (215 aa), *ribDG* (361 aa). The operator region, *ribO*, contains the riboswitch site that in conjunction with FMN regulates the transcription of the *rib* operon. aa: amino acids; kb: kilobase; bp: base pairs. Adapted from Perkins et al., 1999, Averianova et al 2020.

The synthesis of riboflavin in *Bacillus subtilis* can be enhanced by increasing the expression of the *rib* biosynthetic genes. For example, introducing a second copy of the *ribA* and *ribG* genes (inserted at the secondary chromosomal locus *sacB*) increases riboflavin production by 25% (Humbelin et al. 1999). These genes encode riboflavin synthase and GTP cyclohydrolase, respectively. However, within this pathway, there were multiple rate-limiting steps in the production of riboflavin. The most important limiting steps are the synthesis of ribulose-5-phosphate or guanosine thiophosphate (GTP). Both of these biochemical compounds are irreplaceable precursors for the different pathways that make up the riboflavin production pathway.

The riboflavin biosynthetic genes are organized within the *B. subtilis* genome as a single operon, that is conserved through most Gram-positive bacteria. Within the *rib* operon, ribGBAHT, there are a total of five major genes (Perkins and Pero 1993) (It is important to note that these genes have been recently renamed to account for those encoding bifunctional enzyme

activities: *ribDG* (*ribG*); *ribE* (*ribB*); *ribAB* (*ribA*); *ribH* (*ribH*), and *ribT* (*ribT*) – for clarity, especially with respect to older literature, the original gene names will be used). The *rib* operon is vital for the production of riboflavin as it catalyzes many of the steps involved in the riboflavin biosynthetic pathway. The first encoded region on the operon is *ribO*, which acts as a regulatory site for the operons. The structural genes within the operon are *ribG*, *ribB*, *ribA*, and *ribH*, the gene order of which does not follow the order of enzyme reactions (Perkins, 1999). Starting at the first structural gene, *ribG* encodes a bifunctional enzyme that is involved in pyrimidine deaminase and reductase activities, the second and third steps in the riboflavin biosynthetic pathway This gene is responsible for the conversion of 2,5-diamino-6-(ribosylamino)-4(3h)-pyrimidinone 5'-phosphate into 5-amino-6-(ribosylamino)-2,4(1h,3h)-pyrimidinedione 5'-phosphate. The next structural gene, *ribB*, encodes riboflavin synthase which converts 6,7-dimethyl-8-ribityllumazine to riboflavin, the last step in the riboflavin pathway. This protein forms a multiunit capsid structure needed for its reaction (Rotter et al, 2022). The third structural gene, *ribA* encodes a bifunctional enzyme that controls the entry of the precursors GTP and Ru5P into the pathway. One activity of RibA is GTP cyclohydrolase II activity which catalyzes the formation of 2,5-diamino-6-(ribosylamino)-4 (3H)-pyrimidinone-5′-phosphate from GTP. The second enzymatic activity in RibA synthesizes a four-carbon unit (3,4-dihydroxy-2-butanone 4-phosphate) from ribulose-5-phosphate and formate that is utilized in the later lumazine synthase reaction. The fourth gene, *ribH*, encodes for lumazine synthase, an enzyme that forms a cycle at the end of the biosynthetic pathway where it produces both 5-amino-6-tributylamine-2,4 (1H,3H)-pyrimidineione, which is recycled by this synthase and produces a condensed L-3,4-dihydroxy-2-2 butanone 4-phosphate. The last gene in the operon encodes an

acetyl transferase-like enzyme. Rotter et al. recently showed that RibT is involved in disassembly of the RibB capsid structure (Rotter et al., 2022).

RibO, as mentioned before, is an important regulatory site used to control the transcription of the *rib* operon. The *ribO* region is approximately 300 bp in length and is located between the start vegetative promoter (P_1) and the first structural gene, *ribG*. Within this sequence lays the riboswitch regulatory signal which when transcribed generates two different overlapping secondary RNA structures associated with a *rho*-independent transcription termination site (structure in **Figure 2**). If the level of FMN is high in the cell, FMN will bind to the nascent mRNA leader in such a way one of the secondary structures is formed that allows the establishment of the *rho*-independent terminator, and transcription is prematurely aborted. This is observed by the appearance of a 300 bp RNA transcript in Northern blots (Perkins et al., 1999). Alternatively, when the level of FMN is low, a different mRNA secondary structure is formed preventing the formation of the terminator, and thus allowing read-through transcription of the *rib* structural genes by RNA polymerase. This is observed by the appearance of a full-length 4.3 kb RNA transcript in Northern blots (Perkins et al., 1999). Thus, the riboswitch functions as a negative feedback loop where a high concentration of FMN causes FMN to bind to the *ribO* RNA leader and form the terminator structure while at low concentration, the FMN is not present to form the terminator structure and transcription continues. Finally, since FMN (and FAD) is synthesized by RibC, a bifunctional kinase and adenylation enzyme, mutations in *ribC* and *ribO* result in the deregulation of *rib* operon transcription. The *rib* operon also contains two additional internal promoters, P_2 and P_3; P_2 (between *ribB* and *ribA* structural genes) appears to be regulated by riboflavin levels, but the mechanism is not known, whereas P_3 (upstream of *ribT*) is constitutive (Perkins et al., 1999).

Decoyinine and Azaguanine

To increase the levels of the riboflavin precursor, GTP, toxic purine analogs are often used to isolate resistant mutants that display higher purine production (Perkins et al., 1999).; the higher level of purine pools in the cell overcomes the negative effects of the toxic analog. How this occurs is dependent on the nature of the interaction between the toxic analog and enzymatic reaction. There are multiple different types of inhibitor interactions such as non-competitive, uncompetitive, and competitive inhibition. The first type of inhibition is non-competitive inhibition which is where the inhibitor does not fight for the active site. In this type of inhibition, the inhibitor binds to a different active site than the substrate, changing the confirmation of the enzyme. This change in conformation prevents the binding of the substrate, completely stopping the formation of the enzyme-substrate complex (ES complex). However, upon the removal of the inhibitor, the conformation returns to normal, and the ES complex can form once again. The next type of inhibition is uncompetitive inhibition where the inhibitor binds to the active site of the enzyme. In this irreversible inhibition, the inhibitor binds to the active site of the enzyme, preventing the substrate from bonding and stopping the reaction. The final type of inhibition is competitive inhibition where the inhibitor binds to the active site of the enzyme. While this function is similar to uncompetitive inhibition, competitive inhibition can be reversed if there is an excess of the substrate. This excess is capable of removing the inhibitor and forming the ES complex. Toxic purine analogs act as competitive inhibitors for specific prunes. This means that if the cell produces purines in excess, it displayed toxic purine analog resistance. Important toxic purine analogs are azaguanine (Az), an analog for guanine, and decoyinine (Dc), an analog for guanosine. Structures of toxic analogs can be seen in **Figure 3**, along with their equivalent purine or vitamin. The use of a toxic purine analog is a common practice when selecting for an

increase or decrease in production due to the specificity of the resistance. Resistance will only be observed if the chosen compound is produced in excess within the cell.

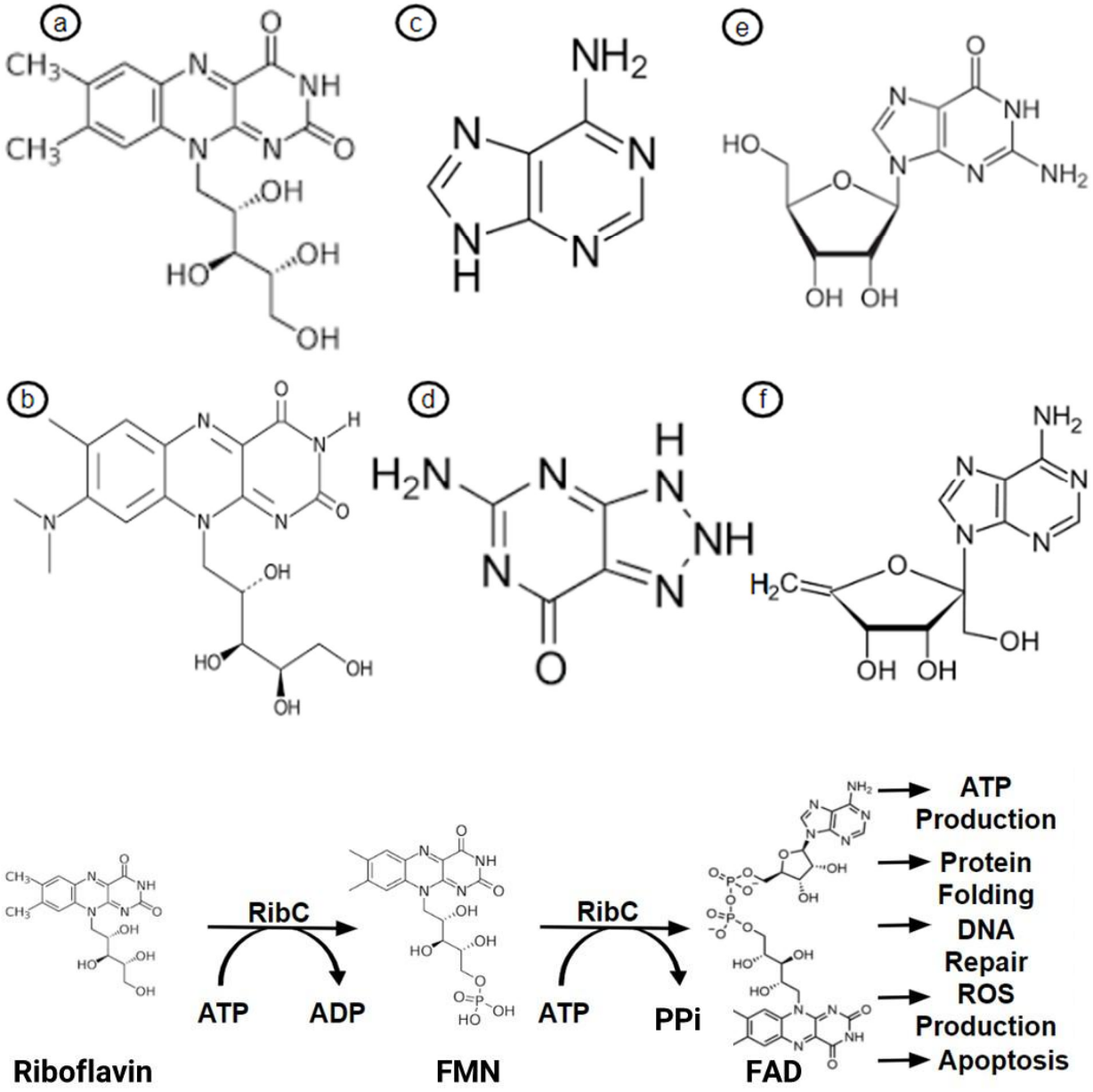


Figure 3. Structures of Purines/ Vitamins and Their Analogs. (a) riboflavin. (b) roseoflavin. (c) guanine. (d) azaguanine. (e) guanosine. (f) decoyinine. (g) biochemical synthesis of FMN and FAD from riboflavin. The conversion of riboflavin to FMN and FMN to FAD is catalyzed by the bifunctional RibC protein having both kinase and adenylation activities, respectively. FAD is used for multiple applications: ATP synthesis, DNA repair, ROS (reactive oxygen species) production, etc. Reaction scheme adapted from Giancaspero et al. 2015.

The competitive inhibitor characteristic of toxic purine analogs has been used to isolate strains that produce several different purines. For example, the Az-resistant strain AJ-1993 was isolated and was observed to have increased guanosine production with only the addition of adenine (Konishi and Shiro, 1968). So in the case of toxic purine analogs, the amount of purine produced by a cell can be assumed to have increased. Variation in the specific purine production and yields occurs if other toxic metabolites involved in purine biosynthesis are used, such as methionine sulfoxide (MS). Methionine sulfoxide is a glutamate/glutamine antagonist that can affect the nitrogen donation for adenine and guanine (Matsui et al., 1977). Here, they use MS to isolate a methionine sulfoxide-resistant mutant of *B. subtilis* (named 14119) that produced 9.6 grams of guanosine per liter (Matsui et al., 1977), a greater amount of guanosine than was generated by the azaguanine-resistant mutant isolated by Konishi and Shiro (Konishi and Shiro, 1968). Moreover, methionine sulfate (MS) was also used to isolate a mutant with increased inosine 5'-monophosphate (IMP) compared to the parent strain (Matsui et al., 1977).

Purine analog-resistant mutants can be recovered by just growing bacterial cells in media containing the toxic analog. The number of such mutants that result from this treatment is very low, arising through spontaneous mutation of the chromosome, usually at a frequency of 10^{-5} to 10^{-8} (Watford and Warrington, 2023). These mutants are referred to as spontaneous mutants. To enhance the appearance of these purine-resistance mutants, chemical mutagens that modify DNA, are often used. This is done by first treating bacterial cells with the chemical mutagen to generate a library of cells with random mutations circumventing the chromosome. These mutant libraries are then screened for those cells resistant to toxic purine analogs. Typically, the appearance of chemically induced mutants increases approximately 100-fold or more above the

spontaneous mutagenesis rate (reference). In addition, the variety of mutational changes in the DNA is often increased over spontaneous mutagenesis

Two typical chemical mutagens are methyl methane sulfonate (MMS) and ethyl methane sulfonate. Both mutagens generated GC to AT transitions. In chemical reactions on DNA, methylation by MMS is five to ten times more effective than ethylation by EMS. However, in mutagenesis, MMS is less effective than EMS (Rhaese and Boetk, 1973). This is due to differences in the repair mechanisms within the cell. This is true for *B. subtilis*: EMS is the preferred mutagen since the *B. subtilis* repair mechanism is not as effective as those for MMS; mutation(s) in the recombination or polymerase genes is needed to increase the frequency of MMS mutagenesis (Prakash and Strauss, 1970). Neither of these mutagens are purine analogs but are compounds that are used to increase the frequency of Az and Dc-resistant mutants.

Mutations within a genetic sequence can take on many forms. The first is a deletion when one or more base pairs are removed from the genetic sequence. A frameshift mutation alters the reading frame to one that produces a protein with a different amino acid sequence. Reading frame refers to the three neighboring nucleic acids which code for individual amino acids. Another mutation is an insertion where a nucleotide is added to the sequence. Such insertions can shift the reading frame. A final mutation that could happen is a base pair substitution, where a base pair is switched out without affecting the reading frame. The result of this comes from the change of code and a possible switch in coded amino acids. The most drastic of the changes can come from changes in a stop or start codon, where the translation machinery is no longer able to bind and start translating a sequence if a start codon is altered. However, if a stop codon is altered, the translation would continue so there are a lot of noncoding sequences that are translated. In terms of the overall effect on the production of a metabolite in a bacterial cell,

some mutations will enhance production, some reduce it, while the majority will have no effect: ultimately the phenotype of the strain that is obtained will represent the total of all these mutagenic effects.

Current Research

The first step to overproduce riboflavin in *B. subtilis* is to increase the production of guanosine triphosphate, one of the riboflavin's precursors. (We assume that there are sufficient intracellular amounts of the other precursor, ribulose-5-phosphate). To increase GTP production in *B. subtilis,* cells are randomly mutated with a chemical mutagen and then cells are selected that have resistance to azaguanine and then decoyinine. It has been previously shown that increasing GTP production in a strain containing both Az and Dc-resistance mutations, results in higher production of riboflavin when a third mutation, conferring roseoflavin resistance, is added to the strain; resistance to this riboflavin toxic analog increases (by deregulation) the expression of the riboflavin biosynthetic genes due to a mutation in either the *ribC* gene or *ribO* operator region (Perkins et al., 1999).

However, since GTP is only one of many products of the purine pathway, the focus of the mutagenesis will be on the GMP-IMP-AMP branch of the purine pathway, so that the carbon flow is directed towards GMP rather than AMP. To do this, a *B. subtilis* strain containing a *purB* mutation will be used to generate an EMS mutant library from which azaguanine mutants can be selected for and recovered. The *purB* mutations inactivate the adenyloscuccinate lyase (ADLS) enzyme which blocks the purine pathway at two points, SAICAR-to-AICAR and S-AMP-to-AMP reactions; cells containing a *purB* mutation can grow if adenine is present in the growth medium. Thusly, guanine and GMP (and GTP) synthesis will be dependent on exogenously

added adenine by reversible enzymatic reactions from AMP-to-IMP-to GMP (see **Figure 1**); Az-resistance mutations (and subsequent Dc-resistant mutations) located at the IMP junction should enhance this carbon flow resulting in increased production of guanine, GMP, GDP, and GTP (perhaps as well as other purines around this junction). In later strain construction steps, this *purB* mutation will be eventually replaced with the wild-type gene to allow enhanced carbon flow from glucose down through the purine pathway to GTP.

Since resistance to azaguanine and decoyinine can also occur by mutations that prevent their uptake into the cell (Thakar and Kalle, 1987), several methods will be used to confirm purine overproduction in these mutants. One method used in this study is the maximum inhibitory concentration (MIC) assay that determines the mutant's level of resistance to the toxic analogs. We assume that the higher the resistance to azaguanine, the better chance that the mutant is a purine overproducer than deficient in analog uptake. The setup of this assay, like the minimum inhibitory concentration assay, uses a 24-well MTP format. A second assay is the reverse halo assay to detect the extracellular production of guanine. This assay is analogous to the diffusion halo assay, except the growth of the sensitive indicator (in this case a guanine-requiring bacterium) is monitored on agar plates. The third method used in this study is high-performance liquid chromatography (HPLC) to identify and quantify the levels of extracellular production of individual (non-phosphorylated) purines. In all, eight different purines could be identified by our HPLC method, however, identification of key purines guanine and guanosine was difficult due to interference with purines, hypoxanthine, and xanthine. Production of guanine and guanosine should be correlated to the amount of GTP produced by the mutants (Perkins et al., 1999), however, this is just an assumption. However, by monitoring guanine and guanosine levels, we should have a good idea if the cellular pathway to guanine and guanosine is

hyperactive in our mutants, and that there is a corresponding increase in the production of GMP, GDP, and GTP.

Now our studies were not done in a vacuum. A similar (if not identical) experimental approach was conducted by Perkins et al to increase riboflavin production in *B. subtilis* (Perkins et al., 1999). During their experiments, however, the assays used in the identification of the best guanine and GTP overproducing azaguanine or decoyinine-resistant mutant are not known (Perkins, 2023, personal communication). That is why we developed and used three to analyze our mutants throughout this study. Nevertheless, Perkins et al. were able to isolate a strain of *B. subtilis*, called RB50, that contained a combination of purine analog resistant mutations (azaguanine and decoyinine) and a riboflavin-analog resistant mutation (roseoflavin) that produced up to 1 g/liter riboflavin in 10-liter fermentation. This strain was subsequently used to introduce engineered *rib* biosynthetic genes to substantially increase riboflavin production to over 15 g/liter. Despite this achievement, the exact number and location of host mutations that led to high riboflavin production were not fully determined. Only three mutations were definitively identified: *guaA* (GMP synthase), *spo0A* (major sporulation regulatory gene), and *ribC* (FAD kinase/FMN adenylate). That combination of these three mutations into a strain with a clean genetic background did not yield riboflavin production at levels generated by RB50, indicating that a combination of additional host mutations introduced into RB50 by multiple rounds of mutagenesis and selection is needed for full riboflavin overproduction. It is the hope of this project that by repeating the mutagenesis strain lineage, we can obtain an RB50-like strain, which we can subsequently sequence and compare to the RB50 genome sequence to identify common mutations.

To this end, the first step of generating this new RB50-like strain is to isolate azaguanine-resistant mutants that demonstratively overproduce guanine and/or other purines. Consequently, we report here the isolation of 12 azaguanine-resistant mutants after one round of EMS mutagenesis of a *B. subtilis* strain containing *purB*::*kan* and *spo0A*::*erm* knockout mutations. One of these mutants, Az8, was determined (by HPLC) to produce 2-fold more purines than the parental strain. The next best mutant was Az2, which produces a combination of guanine and hypoxanthine, but at lower levels. Az8 will be used in subsequent studies to generate a double azaguanine- and decoyinine-resistant mutant that should produce even higher levels of purines.

Materials and Methods

Media and Bacterial Strains

Bacillus subtilis JP5 (*purB*::*kan spo0A*::*erm*) was used for the isolation of increased purine-producing mutant strains. The *purB* and *spo0A* mutations are "perfect" deletions in which the sequences from the start codon to the stop codon were removed and replaced by a kanamycin resistance gene (*kan*) or an erythromycin resistance gene (*erm*). Strains containing the individual mutations were obtained from the *Bacillus* Genetic Stock Center (The Ohio State University, Columbus, Ohio) and the mutations were combined into a single strain using DNA transformation techniques (data not shown).

Brain Heart Infusion agar (BHI) was used for general growth and tittering of *B. subtilis* strains. In most cases, it was prepared with kanamycin (10 μg/mL) or a combination of erythromycin (1 μg/mL) and lincomycin (25 μg/mL) to confirm the presence of the *kan* and *erm* antibiotic markers, respectively. Spizizen salt (SS) minimal medium agar containing adenine (SS

Ade) was used for the selection of purine analog resistant mutants; SS contained the following components: 1x T-Base (0.2% ammonium sulfate, 1.4% dipotassium phosphate, 0.6% monopotassium phosphate, 0.1% tri-sodium citrate [$3H_2O$]), 0.02% magnesium sulfate [$7H_2O$], 0.5% glucose, 1x Fe•citrate solution (0.1% tri-sodium citrate [$3H_2O$] and 0.0135% $FeCl_3$ [$6H_2O$]), 0.04% glutamate, 1.5% agar, and 0.002% adenine (20 μg/mL final concentration). This medium was prepared in the following manner (per liter): 750 mL distilled water and 15 g agar were first mixed and autoclaved. After cooling to 50°C in a water bath the following sterile components were added and plates poured (30 mL per plate): 200 mL of 5x T-Base, 10 mL of 1.2% magnesium sulfate, 15 mL of 40% glucose, 1 mL of 350x Fe•citrate, 1 mL of 40% glutamate, 10 mL of 0.2% adenine. This minimal media contains the minimum components possible for the growth of *B. subtilis*; it is supplemented with adenine to satisfy the *purB* auxotrophy. SS Ade broth (no agar) was also used to grow candidate mutants to test for purine production. In some experiments, the concentration of adenine was increased either 5- or 10-fold. azaguanine (Selleckchem) was used at a final concentration of 500 μg/mL to select for azaguanine-resistant mutants; azaguanine stock solutions were prepared at a concentration of 100 mg/mL in water titrated with NaOH to get the azaguanine in solution. Adenine, azaguanine, Fe•citrate, and glutamate stock solutions were filtered sterilized using a 0.45-micron Millipore filter prior to use.

EMS Mutagenesis

An overnight culture of JP5 strain (*purB*::*kan spoa0A*::*erm*) was grown in SS minimal media with adenine (20 μg/mL). Cultures were centrifuged (4000 rpm, 10 min) to separate the supernatant from the cells and the cells were resuspended in 0.2 M [KPO4] buffer (pH 7.0). Resuspended samples (2.0 mL) were added to 3, 30 mL sterile tubes containing 10% EMS. After 30, 60, and 90 minutes of incubation at 37°C with slow shaking, mutagenesis was terminated by the addition of a 10-fold excess (18 mL) of 0.16 M sodium thiosulfate ($Na_2S_2O_3$). The non-mutagenized control was treated the same as the 30 min samples but with H_2O being added instead of EMS. Also, a duplicate of the 60-minute EMS treatment was made. Cells were centrifuged (4000 rpm, 10 minutes) and washed two times in 20 mL SS minimal media containing 20 μg/mL adenine. Finally, washed cells were resuspended in 2 mL SS minimum containing 20 μg/mL adenine and 20% glycerol, and stored at -80°C in 0.2 mL aliquots. Frozen cells from each EMS treatment were used to determine the cell titer kill curve. To do this, a frozen aliquot was thawed at room temperature and used to prepare the following dilution series 10^{-2},10^{-3},10^{-4},10^{-5},10^{-6}, and 10^{-7} in SS minimal media. Samples (0.1 mL) from each dilution were plated on BHI (Blood Heart Infusion) agar media containing 10 μg/mL kanamycin. After 18 hours of incubation at 37□, the cell count was determined. The frequency of non-adenine auxotrophic mutants was determined

Selection of Azaguanine-Resistant (Az^r) Mutants

Cells from the non- and EMS-treated mutant libraries were used to isolate Az-resistant mutants. To do this, 0.1 mL samples were plated onto SS minimal agar containing 20 μg/mL adenine with and without the addition of 500 μg/mL azaguanine in soft agar overlays (calculated for the entire 30 mL agar plate). Plates were incubated at 37°C for several days until the appearance of single colonies. To confirm azaguanine resistance, colonies were tested for two rounds of growth on SS Ade plates with 500 ug/mL azaguanine. To do this, colonies from the primary azaguanine selection plates were transferred via sterile toothpick sequentially onto three agar plates, SS Ade (1), SS Ade with 500 μg/mL azaguanine (2), and SS Ade (3). After incubation at 37°C, the growth of putative Az-resistant mutants was compared to the parental strain, JP5. Az-resistant candidates that grew better than JP5 were then streaked for single colonies on SS Ade plates containing azaguanine. Single colonies were given a strain name (e.g. Az1), the presence of kanamycin and erythromycin resistance confirmed, and stored at -80°C.

Maximum Inhibitory Concentration (MIC) Assay

Two-fold dilutions of azaguanine (starting at 2000 μg/mL) were prepared in 24 well MTP wells containing 1 mL SS minimal media with 10 μg/mL adenine. The serial dilution was performed by adding 20 μL of a 100 mg/mL azaguanine stock solution to 980 μL SS minimal medium with 10 μg/mL adenine in the first well and then transferring 500 μL to the second well containing 500 μL of the same medium; this mixing and transferring continued until the azaguanine concentrate in the well reached 125 μg/mL. Afterward, two microliters of an overnight culture of each Az^r mutant were added to each well, and growth turbidity was scored after 24 hr. incubation at 37C with slow shaking.

Reverse Halo Assay

Individual wells of 24 well MTP plates containing 2 mL of SS minimal agar with 20 μg/mL adenine were first overlaid with 1 mL of SS minimal soft agar containing 20 μg/mL adenine and 2 μL of an overnight LB culture of the guanine-dependent *B subtilis* mutant RM1 (*guaB*::*kan*). Sodium tetrazolium, a red growth indicator, was also included in the overlay (0.05 μg/mL final concentration) to help visualize the growth of the indicator bacteria. After hardening of the soft agar overlay, duplicate wells were inoculated with each mutant (Az-1 to Az-7A). This was done by toothpick transfer of a small number of bacteria from single colonies of the mutants previously grown for 24 hr. at 37°C on SS minimal agar plates containing 20 μg/mL plate. As a positive control, 10 μL of 0.2%, 0.1%, and 0.05% authentic guanine was added to the first three wells.

High Performance Liquid Chromatography (HPLC) Analysis

Frozen cells (2 mL) were plated on SS minimal media with 20 μg/mL adenine as a large patch. After incubation at 37°C for 24 hours, the cell patch was stripped off the plate using 2-3 mL SS minimal media with 20 μg/mL adenine, and the resuspended cells were used to inoculate shake flasks containing 30 mL of SS minimal 20, 50, or 100 μg/mL adenine as indicated. The cultures were incubated at 37°C with shaking (200 rpm) or 72 hours. Cells were removed from each culture by centrifugation (4000 rpm, 10 minutes) and the supernatant was filtered using a 0.45-micron filter to remove any additional bacteria. Filter sterilized supernatants (1 mL) were transferred to HPLC vials, and purine compounds were separated using a C-14 column with linear acetonitrile and KPO_4 (0.1 mM; pH 7) 10:90 gradients over a 30-minute run time at a flow rate of 0.5 mL/min. This method was adapted from Hou and Ding, 2010. Unphosphorylated

purines were detected at 260 nm by comparing peak retention times with authentic purine standards: guanine (0.005 μg/mL), guanosine (0.005 μg/mL), adenine (0.005 μg/mL), adenosine (0.005 μg/mL), xanthine (0.005 μg/mL), inosine (0.005 μg/mL), and hypoxanthine (0.005 μg/mL). Spiking 10 μL of individual authentic purines (0.2% stock solution) into 1 mL of filter-sterilized supernatant was used to confirm the peak.

Strategy to Analyze Azaguanine-Resistant (Az^r) Mutants

We assume that azaguanine resistance in our mutants is due to increased production of guanine/GTP which overcomes the negative growth effects of azaguanine. However, there are other types of resistance that can occur. One type of resistance comes from the strengthening of the cell wall to prevent the intake of azaguanine or other toxic purine analogs. Another type of resistance arises if the cellar intake remaining unaffected but the cell pumps out the toxic purine analog faster than the analog can take effect.

To confirm the overproduction of guanine, a combination of the three tests was performed on the Az^r mutants: maximum inhibitory concentration (MIC), reverse halo assay, and HPLC. However, not every test was performed on each mutant strain. As shown in **Table 2**, only mutants Az1 through Az7A had all three tests performed but Az-8 through Az-11 were only analyzed for purine content by HPLC. These two groupings of mutants arose from different rounds of isolation. After the first round of isolation, the method for HPLC was developed, which allowed for a more precise measurement of the purines produced by the cell. Due to this reason, the second round of isolated mutants were not analyzed by the MIC and reverse halo assays.

	Assay		
Mutant	MIC	Reverse Halo	HPLC
Az1	✓	✓	✓
Az2	✓	✓	✓
Az3	✓	✓	✓
Az4	✓	✓	✓
Az5	✓	✓	✓
Az6	✓	✓	✓
Az7	✓	✓	✓
Az7A	✓	✓	✓
Az8	×	×	✓
Az9	×	×	✓
Az10	×	×	✓
Az11	×	×	✓

Table 2. Test performed on 12 isolated strains of JP5 mutants. Comparison of the tests performed on the 12 different Az-resistant mutants.

Results

Mutagenesis

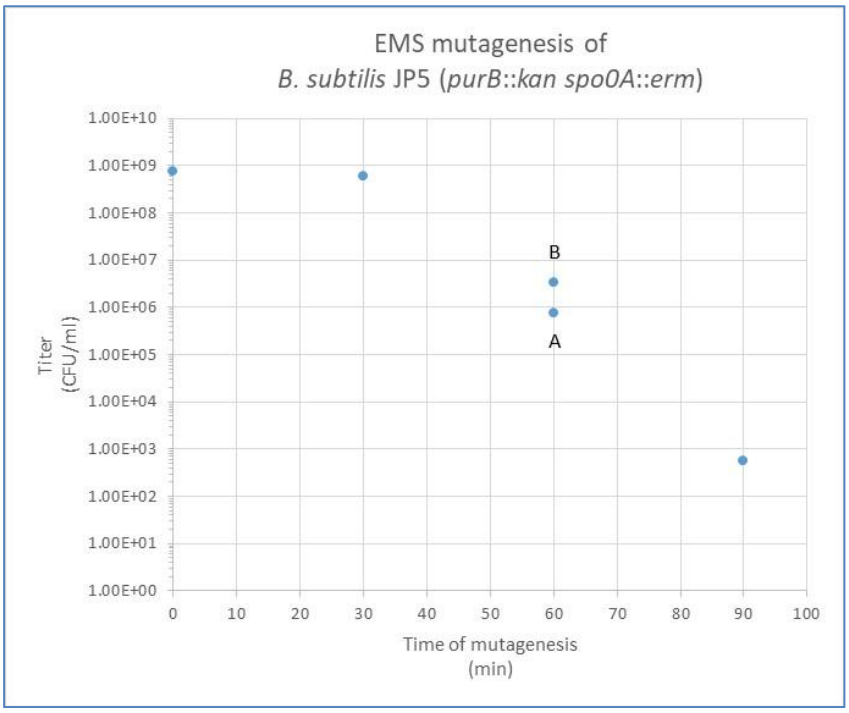


Figure 4. EMS mutagenesis of *B. subtilis* (*purB*::*kan spo0A*::*erm*). Kill curve of *B. subtilis* JP5 using EMS mutagenesis. A and B represent duplicate samples.

To prepare mutant libraries of JP5, cells from an overnight culture were exposed to EMS for 30, 60, and 90 minutes (see **Materials and Methods**). Cell titers of each treatment were then determined to assess the decrease in cell titer (i.e. kill curve), a common approach to assess mutagenesis treatment. As shown in **Figure 4**, samples exposed to EMS for 30 minutes had a similar cell titer compared to no EMS treatment (0 minutes). The 60 minutes treated samples (A and B) had a significant decrease in cell titer than the 30-minute sample, reaching about 3-logs. The 90-minute treatment had the highest reduction of cell titer at 6-logs. Typically, optimum mutagenesis, in terms of mutagenesis (about 300-500 mutations per genome) and a sufficiently

high cell titer for screening or selecting for a desired trait, occurs at a 2-3 log reduction in cell titer. A greater reduction in cell titer (>2-3 logs) usually indicates too few cells in the mutant library, containing too many mutations per genome, also known as genetic load, to maintain cell vitality. Finally, a too-low reduction in cell titer (< 2-3 logs) results in a mutant library with a sufficient number of cells, but with too few mutations per genome to warrant recovery of the desired trait. Using these criteria, we concluded that the 60-minute EMS treatment was optimal for the generation of a mutant library. However, to ensure we obtained a sufficient number of azaguanine-resistant mutants, we tested cells from all EMS treatments.

Isolation of Azaguanine-Resistant (Az^r) Mutants

EMS Treatment	**Mutants Obtained**
No treatment	Az1, Az2, Az3, Az4, Az5, Az6
60 min (B) EMS treatment	Az7, Az7A, Az10, Az11
30 min + 60 min (A) EMS treatment	Az8, Az9

Table 3. Mutants obtained from different EMS treatments. Screening of the mutant library for Az-resistant mutants. Mutants were isolated from mutant libraries isolated by the following EMS mutagenesis: no treatment, 30+60-minute EMS treatment. and 90-minute EMS treatment.

Four different EMS mutagenized libraries of JP5 were used in two rounds of selection for mutants that displayed greater Az resistance compared to the original strain *B. subtilis* JP5. Those libraries were 30 minutes, 60-minutes (A and B duplicates), and 90-minutes EMS treatments; control cells not treated with EMS treatment were also used to isolate spontaneous azaguanine-resistant mutants. Approximately 0.1 mL from the thawed frozen library stocks were applied onto SS Ade agar plates. In the absence of azaguanine, the number of colony-forming units (CFUs) varied. The no EMS treated sample and 30-minute EMS sample treated produced a

lawn of cells; the 60-minute EMS samples (A and B) both generated >300 CFUs (e.g. To-Many-To-Count - TMTC); the 90-minute EMS sample only produced <50 CFU's per plate. Based on the determined cell titers, these results were expected. In the presence of 500 ug/mL azaguanine, only a few colonies appeared compared to the plates without the toxic analog, indicating that the toxic analog killed most of the cells and those cells resistant to azaguanine were able to grow to form colonies. These putative Az^r mutants were further tested to confirm azaguanine resistance by a series of growth experiments on SS Ade plates containing azaguanine as described in **Material and Methods**.

In all, 12 Az-resistant mutants were isolated from two rounds of selection using SS Ade agar plates containing 500 ug/mL azaguanine. In the first round, Az^r mutants were only recovered from the no EMS treated library, Az1 through Az6, and from the 60B-minute EMS library, Az7 and Az7A (these two arose from the same isolated colony but upon further analysis we noted they segregate into two colony morphologies). In the second round, mutants Az8 and Az9 were recovered when cells from the 30 and 60A-minute EMS treatments were plated together, and Az10 and Az11 were recovered from the 60B-minute EMS library. These results are summarized in **Table 3**.

Characterization of Azaguanine-Resistant (Az^r) mutants

A series of three tests were developed to further characterize the Az^r mutants isolated in the EMS-induced mutant library to determine the best mutant in terms of increased purine production. The first assay, the maximum inhibitory concentration assay, was developed to determine the relative resistance of isolated mutants to azaguanine. We assume that a mutant that has greater guanine production should display greater azaguanine resistance. The second assay,

the reverse halo assay, looks at the relative amount of guanine being excreted from the Az^r mutant. In this assay, the growth of guanine-dependent indicator cells around an Az^r mutant colony (e.g. growth halo) is dependent on the amount of excreted guanine, so that the greater amount of purines being produced will cause a greater ring of indicator cell growth surrounding the mutant colony. The final assay, high-performance liquid chromatography, identifies and quantifies individual purines being excreted from the cell. Based on retention times developed for authentic purine standards, purines excreted from the cell can be identified and the relative amount determined.

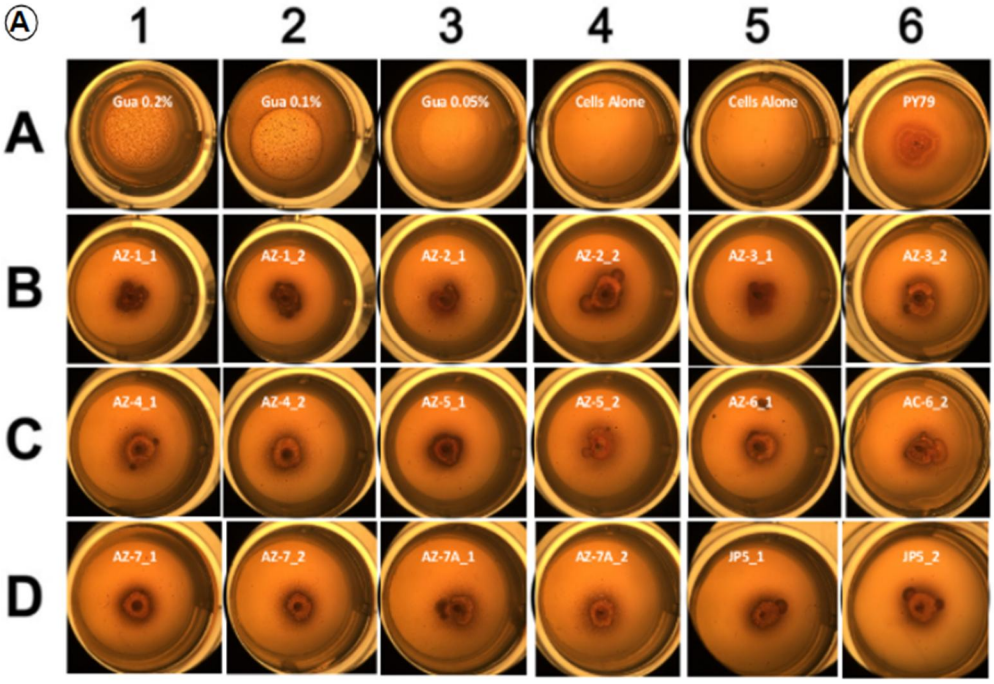
1. Maximum Inhibitory Concentration (MIC) Assay

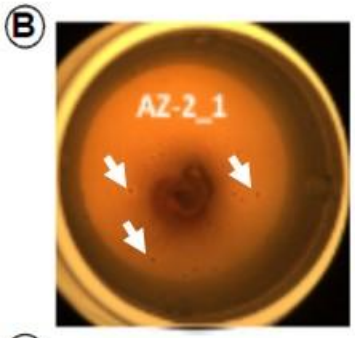
Strain	Az Resistance
JP5	< 125 μg/mL
Az-1	500 μg/mL
Az-2	1000 μg/mL
Az-3	500 μg/mL
Az-4	250 μg/mL
Az-5	500 μg/mL
Az-6	500 μg/mL
Az-7	500 μg/mL
Az-7A	250 μg/mL
Az-8	NT
Az-9	NT
Az-10	NT
Az-11	NT

Table 4. Azaguanine resistance of mutant strains from a maximum inhibitory concentration (MIC) assay. *NT= not tested; Resistance to azaguanine according to a MIC assay at azaguanine concentrations of 2000 μg/mL, 1000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, and 0 μg/mL.

Azaguanine resistance was observed in all of the mutant strains to be greater than the parental strain (**Table 4**). The parent strain of JP5 displayed a resistance that was less than 125 μg/mL. Az2 was displayed to have the greatest resistance at 1000 μg/mL. Az1, Az3, Az5, Az6, and Az7 displayed a slightly lower resistance at 500 μg/mL. Az4 and Az7A displayed the lowest resistance of the mutant strains at 250 μg/mL.

2. Reverse Halo Assay





D

Strain	Indicator Growth Halo*
JP5	+
JP5 AZ-1	++
JP5 AZ-2	+++
JP5 AZ-3	++±
JP5 AZ-4	+++
JP5 AZ-5	++±
JP5 AZ-6	++
JP5 AZ-7	++
JP5 AZ-7A	+±
Cells alone	—
PY79	++++
Gua 0.2%	++++++
Gua 0.1%	+++++
Gua 0.05%	++++

* RM1 (guaB::kan)

Figure 5. Reverse halo assay of azaguanine mutants. RM1 is a guanine-dependent strain of *B. subtilis*. Mutants cells were first transferred onto the center of the well overlaid with SS Ade soft agar containing RM1. After several days of incubation at 37°C, the growth of the RM1 indicator surrounding the Az^r mutant colony was measured using

red tetrazolium. Indicator cells grew outwards from the Az^r mutant colony center, forming a darker ring of growth depending on the amount of guanine excreted from the Az^r mutant colony. (A) A 24-well plate of reverse halo assay. (B) Well B3 contains Az2-1, the white arrows represent RM1 colonies. (C) Well D5 contains JP5-1. (D) Comparison of indicator relative growth and cell density under a microscope. Growth key of RL1 by Az^r mutants: +, growth only around Az^r colony edge, ++, growth around Az^r colony edge with the appearance of individual RL1 colonies near Az^r colony edge, +++, growth around Az^r colony edge with the growth of individual RL1 colonies midway from Az^r colony edge and edge of MTP well, ++++, growth around Az^r colony edge with growth of individual RL1 colonies near edge of MTP well. Growth key of RL1 by authentic guanine and controls: —, no growth; ++++, ~150; +++++, ~300 CFU; ++++++, >500 CFU.

Two-fold dilutions of guanine concentrations of 0.2%, 0.1%, and 0.05% were used as the positive control (**Figure 5, wells A1-A3**). The greatest growth was observed in the 0.2% concentration of guanine and decreased in a dose-dependent manner; growth of the indicator was observed as the appearance of tiny red colonies. No growth of RM1 (negative control) was observed in the absence of guanine (**Figure 5, wells A4-A5**). A wild-type strain of *B. subtilis*, PY79 (**Figure 5, well A-6**), was observed to generate a growth pattern of the indicator similar to that of the 0.05% guanine control.

The growth of the indicator surrounding the Az^r mutant colonies was not as clear-cut. Az2 and Az4 were observed to have the greatest growth of the RM1 indicator (**Figure 5, wells B3-B4 and C1-C2**) compared to the JP5 parent (**Figure 5, wells D-5 and D-6**). This growth was strongest immediately surrounding the two Az^r colonies as judged by the intense red strain of tetrazolium growth dye, but the growth of the indicator was reduced to single colonies toward the edges of the well. Az3 and Az5 were next in terms of promoting the growth of the indicator (**Figure 5, wells B5-B6**): the growth was again strongest immediately surrounding the Az^r colonies, as intense as Az2 and Az4, but the number of singles colonies towards the edge of the well were less than Az2 and Az4. Our interpretation of these results is that Az2/Az4 are producing more exogenous guanine than Az3/Az5 resulting in more individual colonies of the indicator as the guanine diffuses to the edge of the well, irrespective of the intensity of the

indicator growth immediately surrounding the Az^r colonies. Using this interpretation, the next best mutants were Az1 (**wells B1-B2**), Az6 (**wells C5-C6**), Az7 (**wells D1-D2**), and Az7A (**wells D3-D4**) in decreasing order. It is important to note that all the Az mutants produced a stronger RM1 growth signal than the parental strain JP5 (**Figure 5, wells D5-D6**).

3. HPLC Analysis

Acetonitrile/KPO4 Mobile Phase		
Purine	**Avg Peak Time**	**Range**
Adenine	15.99	±0.180
Adenosine	23.73	±0.156
Guanine	10.23	±0.155
Guanosine	7.19	±0.056
Hypoxanthine	9.6	±0.277
Inosine	15.72	±0.382
Xanthine	17.19	±0.237

Table 5. Average retention times of purines. Average purine retention time on a C-14 column using linear acetonitrile and 0.1mM KPO_4 buffer. Average retention time used for identifying peaks on HPLC.

HPLC was used to quantify the level of purines produced from Az^r mutants. Control experiments were first conducted to determine the retention times of authentic purines. These retention times were then used as a reference to identify the purine secreted from the Az^r mutants in cultures.

To do these control experiments, the average peaks for each purine were recorded and observed if there were changes when other purines were present; results are shown in **Table 5.** For adenine, adenosine, and xanthine, we observed sharp peaks with distinct RT's clearly separable from the other purines. However, for the other purines we observed purines with similar or identical retention times. Individually, hypoxanthine and guanine had retention times

of 9.6 min, and 10.23, respectively. However, when hypoxanthine and guanine were mixed together, the difference in retention times was reduced making interpretation of the data difficult. An example of this phenomenon is shown in **Figure 1S**. A similar situation arose with mixtures of authentic inosine and adenine (data not shown). Results with guanosine were more complex (**Figure 2S**). The guanosine retention time was around 7.2 min, however, when was mixed with xanthine we observed that it also, in part, co-migrated with xanthine at 17 min. (i.e. a peak at 7.2 min and a larger peak at 17 min than xanthine alone). To circumvent these issues, spiking of authentic purines (hypoxanthine, guanine, guanosine, and inosine all at final concentration of 50 μg/mL) into the purified culture supernatant prior to HPLC analysis was performed to confirm the purine identity of the peaks.

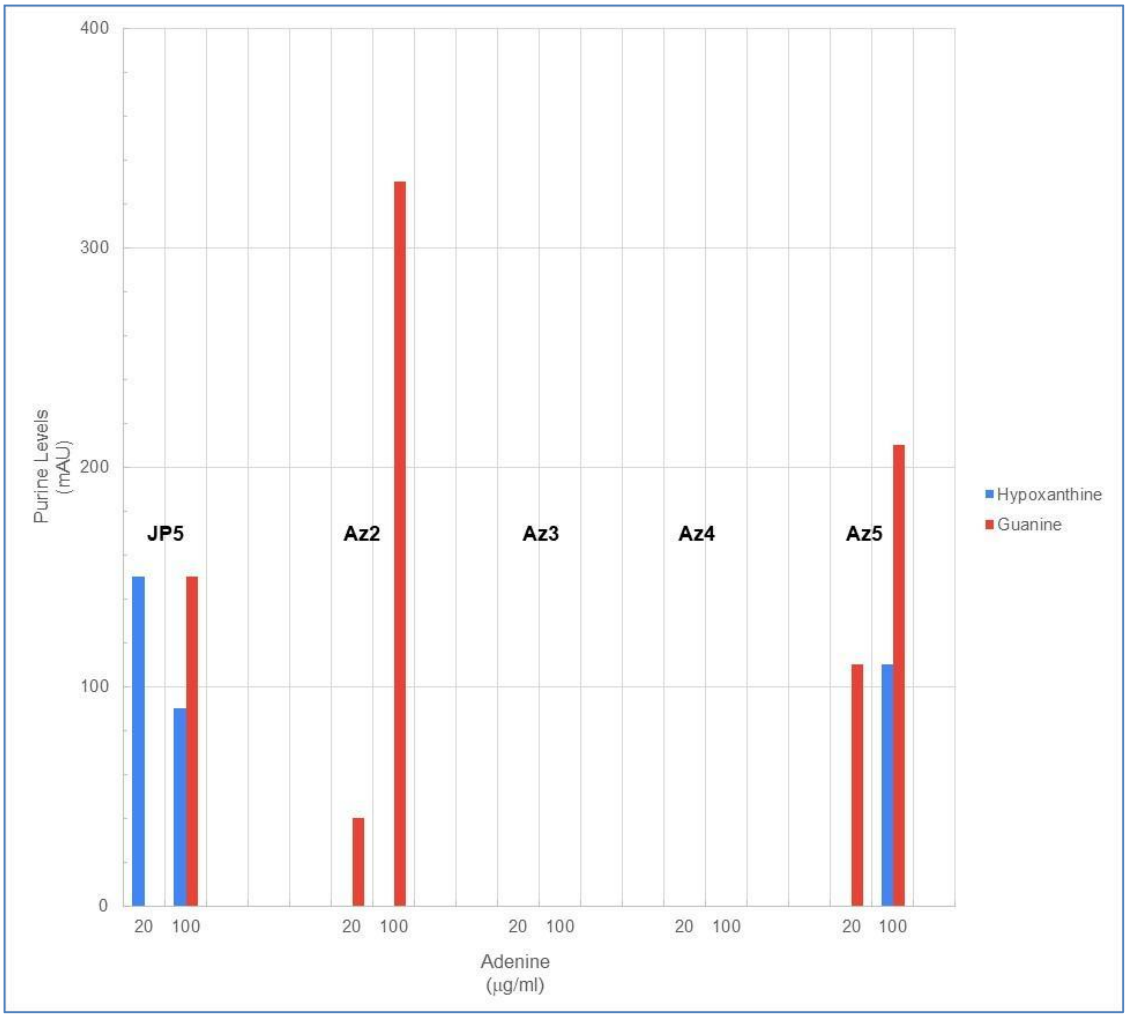


Figure 6. Purine production from Az2 through Az5 in minimal media cultures containing 20 or 100 µg/mL adenine. Samples of JP5, Az2, Az3, Az4, and Az5 were grown for 72 hours, samples were filter sterilized and analyzed for purine content using HPLC; significant peaks (peaks >100 mAU area) were identified by comparison against the authentic purine standards. Purines identified were hypoxanthine and guanine.

To analyze purine production from the Az^r mutants, three sets of HPLC tests were conducted. The first set included Az2, Az3, Az4, and Az5; the second set Az8 thru Az11, and the third set, the best mutant from the first two sets.

In the first set, mutants Az2 thru Az5 along with the JP5 parent were grown for 72 hours in SS medium containing two different levels of adenine 20 and 100 µg/mL adenine since it was unclear which would be best to produce extracellular purine production µg/mL). As shown in

Figure 6, Az2 produced the most guanine (but no hypoxanthine) compared to the JP5 parent and the other Az^r mutants; this level increased as the concentration of adenine increased. Az-5 produced an increasing mixture of guanine and hypoxanthine, whereas the other two mutants produced did not produce guanine or hypoxanthine at these adenine concentrations. Spiking experiments confirmed the detection of guanine and hypoxanthine; an example of guanine spiking of the Az2 sample is shown in **Figure 1S**. We concluded among these mutants that Az-2 was the best purine producer.

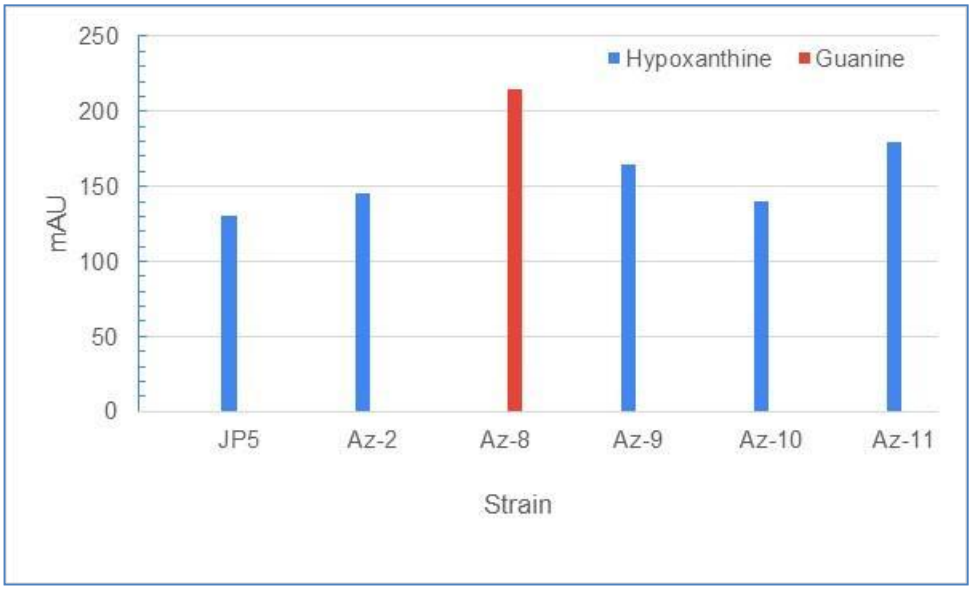


Figure 7. Purine production from Az8 through Az11 in minimal media cultures containing 200 ug/mL adenine. Samples of JP5, Az2, Az8, Az9, Az10, and Az11 were grown for 72 hours, samples were filter sterilized and analyzed for purine content using HPLC; significant peaks (peaks >100 mAU area) were identified by comparison against the authentic purine standards. Purines were identified, hypoxanthine and guanine. Average of duplicate cultures.

In the second set of HPLC experiments, mutants Az8 thru Az11 along with the Az2 and the JP5 parent were tested in duplicate shake flask cultures. Az8 thru Az9 were not previously tested using the MIC assay and reverse halo, going straight to testing by HPLC, since HPLC seemed superior in assessing purine production. To do this, the strains were grown for 72 hours

in an SS medium containing 200 μg/mL adenine; this higher concentration was used to further enhance purine identification. As shown in **Figure 7**, Az-8 clearly was the only mutant making guanine, with the other mutants and the JP5 parent producing just hypoxanthine. We noted, however, that the purine profiles of JP5 and Az2 were different from the previous experiment (**Figure 6**), it was apparent that the purine production profiles of JP5 and Az2 were different from the prior experiment. A series of control spiking experiments were initiated to understand this change in the purine pattern. What we observed was faster RT times of authentic purine compounds and broadening of the peaks (data not shown). We concluded that the C-14 column was degrading and it was subsequently replaced. Nevertheless, we concluded that Az8 was the best purine-producing mutant.

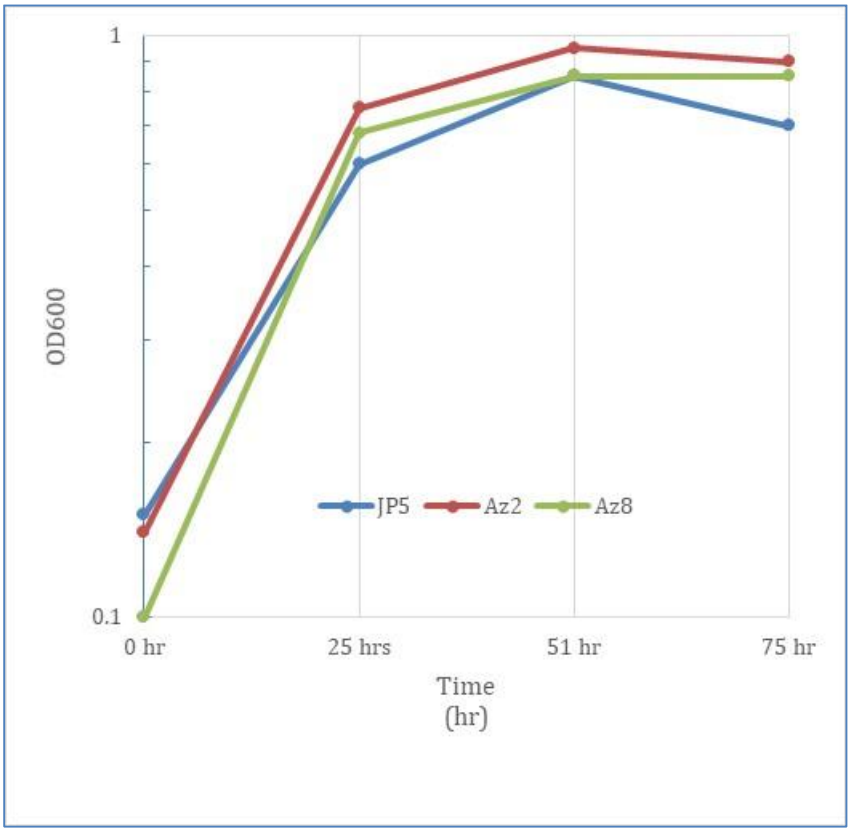


Figure 8. Optical density for 72 hours of growth in SS minimal media containing 50 µg/mL adenine. Optical density (600 nm) of cell cultures of JP5, Az2, and Az8 were taken at 0, 25, 51, and 75-hour time intervals.

In the final set of HPLC tests, only the best mutants from the two prior experiments, Az-2 and Az-8 were tested for extracellular purine production by HPLC. Unlike the previous experiments, samples from each mutant were obtained from a three-day time course experiment (25, 51, and 75 hours) and the growth of the cells was monitored by measuring the optical density at OD600. By dividing the mAU of the purine peak by the OD, this allowed us to normalize the level of purine production among the three strains. Because of the large number of cultures, only one concentration of adenine addition was used (100 µg/mL). Cultures of the parental strain, JP5, served as the control.

As shown in **Figure 8**, the growth pattern of the strains was very similar: log phase growth ended after 25 hours and the stationary phase extended through to 75 hours; no lag was

observed at the beginning of the incubation. Some lysis of the JP5 cells was noted by 75 hr. growth as judged by a slight decrease in the cell density.

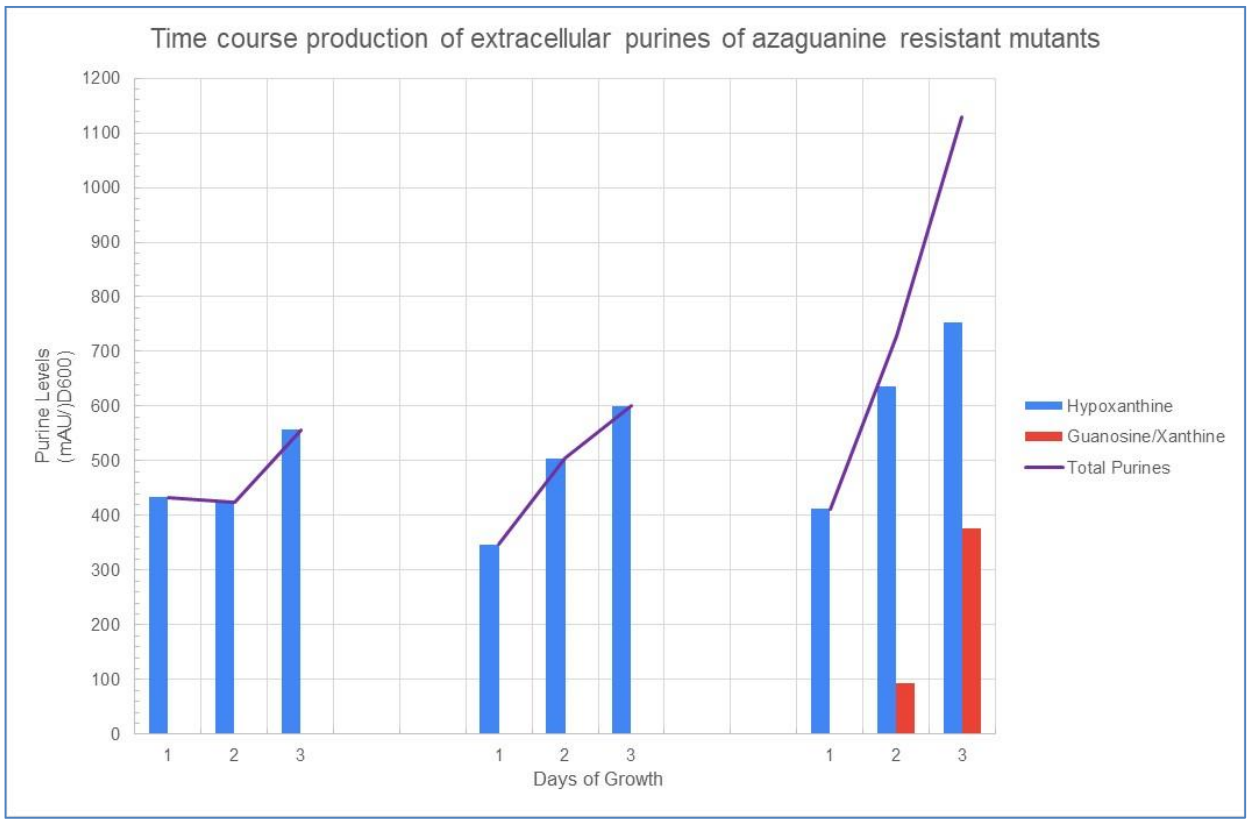


Figure 9. Time course of purine production from Az-2 and Az-8 in SS minimal media with 100 μg/mL adenine. Samples of JP5, Az2, and Az8 were taken at 25-hour, 51-hour, and 75-hour time intervals. Samples were analyzed for purine content using HPLC; significant peaks (peaks >100 mAU area) were identified by comparison against the authentic purine standards, and the mAU areas divided by the OD600 measurement (**Figure 8**). Purines identified: hypoxanthine (blue bars), guanosine/xanthine (red bars), and total sum (purple lines).

HPLC analysis of purine production (per OD) showed a steady increase in purine production of Az2 and Az8 (**Figure 9**). Only the purine hypoxanthine could be clearly identified in this experiment since the new C-14 column was used; the guanosine and xanthine peaks co-migrated so it was not possible to distinguish between the two in this experiment. Az2 produced slightly more hypoxanthine than JP5 and production was linear; no guanosine/xanthine was detected. However, Az8 produced higher levels of hypoxanthine and guanosine/xanthine,

especially after 48-hour growth. In terms of total purine production (the sum of hypoxanthine and guanosine/xanthine), Az8 produced the highest level of total purines compared to either JP5 or Az2, about 2-fold by 75 hr. growth. We noted a small increase in purine production by the parental JP5contol at the 75 hr. time point. We attributed that to a slight increase in cell lysis at this time point (**Figure 8**); otherwise, the level of hypoxanthine was constant. Based on these results, we judged Az8 to be the best purine production strain.

It is important to emphasize again that guanine was not included in the total purine production measurement. This was because the RT of guanine is very close to that of hypoxanthine, and the observed peaks from the strain supernatants were closer to the RT of hypoxanthine than guanine. Moreover, spiking studies by adding authentic guanine or hypoxanthine to the culture samples prior to HPLC were not able to completely resolve the differences between the two peaks. Likewise, we were not able to distinguish whether these cultures produced xanthine or guanosine, since again their RT peaks were identical.

Discussion

Based on our experiments, we were able to generate EMS-mutant libraries of a *B. subtilis* strain from which we were able to isolate azaguanine-resistant mutants, the best of which, Az8, produced about two-fold more purines than the parental strain. The next best mutant was Az2. We assume but did not confirm that the production of GTP by these mutants was increased because our HPLC assay could only detect non-phosphorylated purines. Both spontaneous and EMS-induced Az^r mutants were isolated in our experiments. For the best mutants, Az8 represents the former, whereas Az2 the latter. The isolation of Az8 represents the first step of the

ultimate goal of constructing an RB50-like strain (Perkins et al., 1999). The original RB50 strain described by Perkins et al. had both Az and Dc resistance along with RoF resistance that deregulates the expression of the riboflavin biosynthetic genes, mutations that altogether increased riboflavin production. While the production of an RB50-like is expected to have a phenotype characteristically similar to the original, there is a high chance that the new RB50-like strain will be genotypically different except for those mutations in common that affect riboflavin and purine production.

Mutagenesis and Isolation of Azaguanine-Resistant (Az^r) Mutants

The mutagenesis, as discussed before was intended to shift the carbon flow of the purine pathway to favor the GMP-IMP-AMP branch. This could be accomplished by introducing one or more mutations that decrease the activity of the enzymes responsible for the formation of inosine and/or AMP from IMP. We assume these mutations would not completely stop enzyme activity, since such mutants would lack inosine and hypoxanthine and not survive under our screening protocol. Consequently, to direct mutagenesis in this region of the purine pathway we utilized a *purB* mutation in the parental strain JP5. This mutation blocks the reformation of adenine (and other purine intermediates, so only by the addition of this purine, cells can grow under minimal growth conditions. More importantly, the addition of just adenine controls the formation of guanine and thus allows us to more easily identify mutations in the GMP-IMP-AMP junction that reroutes the carbon flow to guanine.

However, there were multiple issues that arose during the process of EMS mutagenesis. The first issue was there was only one treatment period that fell within the optimal killing range. This would be the 60-minute treatment time which had about a 3-log reduction in cell titer. As

noted before, the 30-minute treatment period had very little difference in killing compared to the untreated sample and the 90-minute treatment period had a higher level of killing. The killing within the 90-minute treatment period was about 6-log which was outside of the optimal range, meaning that there were too many mutations within the cell which caused many of the cells to be non-viable. Those that survive would have too many mutations affecting cell growth. Additionally, with the reduction to robustness this great, there were concerns about there not being enough cells within the library to recover Az^r mutants. Based on these factors, the 60-minute treatment was predicted to have enough cells to isolate Az^r mutants along with multiple potential mutants present in the sample.

Reassessing our mutagenesis scheme, it may have been better to either reduce the concentration of EMS and/or recover more samples (for library preparation) over a given time period to obtain a large pool of mutated cells. Looking back at the 6-log killing at the 90-minute mutagenesis time period, either the EMS concentration or exposure time would need to be decreased to get optimum production killing. More treatment periods within the optimal killing give the possibility of more resistant mutants which can be compared to find the best purine-producing mutant. However, as was shown in the 30-minute treatment period, there can be very few changes in killing within a short exposure time period. As such, decreasing the time periods so that treatment up to 90 minutes at the initial concentration of EMS was not included, was not viable. This left decreasing concentration of EMS added into the sample, which will be done for future mutagenesis experiments (see **Supplemental Information**).

Another issue that arose is that the utilization of a kill curve was not the best measure of desirable mutation formation. The kill curve looks solely at the number of cells that are present after EMS treatment, which when compared over a time course, can show you that loss of cell

viability has occurred, but not the number or location of mutations in individual cells, especially those mutations that affect biosynthetic pathways such as the purine pathway. which was the focus of this project. If mutants only from the 60-minute EMS treatment were isolated, and not the other treatment many other mutants would be missed. Where each of the 12 mutants comes from, in both rounds of isolation, can be seen in **Table 3**. Due to these reasons, additional methods of determining which mutagenesis treatment is best should be used. One approach would be to determine the appearance of auxotrophic or antibiotic-resistant mutants (e.g. Rif^r or Str^r). This would be a better indicator than cell viability to assess the degree of mutagenesis (see **Supplemental Information**).

Characteristics of Azaguanine-Resistant (Az^r) Mutants

After the 12 Az^r lead candidate mutants were isolated from the EMS library, we used three different assays to characterize them. Through these assays, we determined the best purine-producing mutant among the total.

1. Maximum Inhibitory Concentration (MIC) Assay

Through this assay we tested mutants Az1 through Az7A, comparing their different level of resistance to azaguanine. From this assay, we were able to conclude that Az2 had the greatest resistance to azaguanine, although all eight mutants tested displayed a greater resistance than the JP5 parent strain. We assumed that the higher the resistance to azaguanine, the better the chance that the mechanism of resistance was overproduction of purines rather than an uptake deficiency or export of the toxic analog. This assumption was partially confirmed since HPLC analysis of Az2 showed a higher level of purine production than Az3, Az4, and Az5 (**Figure 6**).

2. Reverse Halo Assay

As opposed to the MIC assay, the reverse halo assay is specific for identifying mutants that excrete guanine. Moreover, the extent of growth of the indicator, the guanine-dependent strain RM1, was dependent in a dose-dependent way, on the level of guanine, as seen by the intensity of the red vital stain tetrazolium. This was clearly seen in the control experiment when different concentrations of guanine were added to wells containing RM1 (**Figure 5, wells A1-A3**). This clear-cut result was not observed, however, with the Az^r mutants. When viewed under the microscope, almost all the mutants produced a strong red color immediately surrounding the colony with varying degrees of individual RM1 colony growth in the space from the edge of the mutant colony to the outside edge of the MTP well. We assume that this indicated diffusion of guanine from the mutant colonies to the well's edge. Although the growth of the RM1 indicator around all the mutants was more intense that the parental control (JP5), the Az^r mutants that generated the most RM1 colony growth towards the well's edge were Az2 and Az4. That these mutants were also the ones that gave the highest level of azaguanine resistance in the MIC assay, which gave us confidence that higher azaguanine resistance resulted in better guanine production. The *B. subtilis* wild-type strain. PY79, interestingly, gave the strongest growth signal of the RM1 indicator. This was likely due to the absence of the *purB* block. As mentioned before, the *purB* mutation blocks the purine pathway, preventing the formation of adenine and guanine, and the use of Ru5P to proceed through the purine pathway. Without these blocks being present in the cell, guanine can be produced from two sources in the growth medium: from the added adenine via the AMP-IMP-GMP pathway and from glucose via normal carbon flow down the purine pathway.

3. HPLC Analysis

Despite our interpretation of the results of the MIC and reverse halo assays, these assays could not determine the effect of the Az^r mutations on the total purine pool. For that reason, we developed an HPLC assay to identify and quantify purine exogenously produced from the mutants grown in minimal medium shake flask cultures. After we applied this assay to Az2, Az3, Az4, and Az5, we were confident that it was providing sufficient information on the production of purines from these mutants. Consequently, we used this assay exclusively to analyze the second round of mutants, Az8-Az11.

However, we did encounter several technical issues with the HPLC assay. One issue was a reduction of RT of the purine standards over multiple HPLC runs. This could have been caused by the deterioration of the C-14 column and/or perhaps the decomposition of the purine standards. To rectify this issue, we instituted three changes: one, we made fresh purine standards more often; two, we replaced the old column with a new one and designating it for use only for purine analysis; three, we implemented a reconditioning step of the column after every HPLC run, as described by Hou and Ding, 2013. This involved washing the column isocratically with 100% acetonitrile and then 100% water.

The second issue was the co-migration of the authentic purine standards. This phenomenon occurred for the following pair of purines: guanine/hypoxanthine, adenine/inosine, and guanosine/xanthine. The first was the most serious since it made identifying guanine in our strains difficult, specifically, in the last experiment comparing Az2 and Az8. Unfortunately, in this experiment, spiking one or the other purine was not able to completely rectify this issue. This was because control experiments combining authentic guanine and hypoxanthine actually

caused the peaks to migrate closer together (**Figure 1S**). This was further exacerbated if the amount of one compound was much greater than the other: the larger peak would totally engulf the smaller. Consequently, in addition to reporting individual peaks, we also reported the purine production of these mutants as a total of purines, hypoxanthine, and guanosine/xanthine. If guanine was present within these samples, the peak was either not significant (<100 mA) or it was masked by the hypoxanthine peak. In the future, to obtain better separation of these non-polar compounds, we may need to optimize the mobile phases, and/or increase the length of the alkane groups on the silicone resin in the HPLC column from the current C-14 to C-18.

The third issue we faced was the inability to detect phosphorylated purines by HPLC, a key consideration since it is important to know if our Az^r mutants are not only increasing the level of guanine but also GMP, which leads to the formation of the riboflavin precursor GTP. In control experiments, we observed that IMP, GMP, and AMP, eluted early from the column between 2 and 3 min., the same general retention time salts and other polar components of the spent fermentation broth elute from the column (data not shown). So it was not possible to determine if and at what quantities our mutants contain these compounds. One approach to rectify this issue was to treat our culture samples prior to HPLC analysis with perchlorate at high temperature, a chemical approach to dephosphorylate purine compounds (Fukuuchi et al., 2013). However, initial efforts to implement this assay were not successful: perchlorate treatment of authentic GMP did not lead to the release of detectable levels of guanosine or guanine (data not shown). An alternate approach would be to develop an HPLC method specific for detecting phosphorylated purines. Recently, we have come across such a method described by Pedro et al., 2019. We plan to test this protocol in future work.

Finally, to ensure that the strains JP5, Az2, and Az8 were growing similarly during the three-day time period, the optical density of these strains was taken at regular intervals. As observed in our experiments, during the first 25 hours of growth we observed a rapid increase in cell density. This we determined was the exponential growth phase (**Figure 8**). After 25 hours, we observed little change in the optical density to about 75 hours; which we determined as the stationary growth phase. During this phase, cell death and cell growth were occurring at equal rates. However, at the 75-hour interval, we detected a slight decrease in the optical density of the JP5 parent strain, signifying cells lysis at a slightly greater rate. Since cell lysis results in the release of intracellular metabolites into the medium, including intercellular purines, we believe this caused the slight increase in purine levels detected by HPLC at this time interval (T=75 hr). In the future, it may be better not to extend the growth phase to 75 hours.

Despite these technical issues, our results showed that Az8 is the best purine production strain, with Az2 as the next best. Az8 represents an EMS induced mutant, whereas Az2 a spontaneous mutant. Based on the mode of action of EMS, the mutation in Az8 is likely a GC-to-AT transition, whereas the mutation in Az2 could be another nucleotide change. However, the exact location of mutations in Az2 and Az8 that increase purine production, is not known. One possibility is that they reduce the enzyme activity which converts hypoxanthine to xanthine (xanthine oxidase). Another possibility is that the activity of enzymes that converts IMP into GMP via XMP is increased (IMP dehydrogenase and GMP synthetase, respectively). Nevertheless, we plan to use Az8 in the next mutagenesis step to introduce a decoyinine-resistance mutation in order to generate double mutants resistant to Dc and Az.

As discussed in the **Introduction**, the ultimate goal of this project is to make an RB50-like strain that overproduced riboflavin. It is hoped that this strain will overproduce riboflavin to

a similar extent as the original RB50 strain described in Perkins et al. Consequently, after the isolation of the double Az Dc mutant, the addition of a roseoflavin-resistant mutation and possibly other genetic steps are needed. However, as mutagenesis is a black box, random event, the mutations in the original RB50 and the new RB50-like strain should be different except for those that improve riboflavin synthesis. We anticipate that a comparison of the genomic sequences should identify those key mutations, which can be utilized to further increase riboflavin production in *B. subtilis*.

Conclusions

A *purB* mutant strain of *B. subtilis* was mutated with the chemical mutagen EMS and these mutagenized cells were then screened for resistance to the toxic purine analog azaguanine to increase purine production, specifically GTP, the precursor to riboflavin production. In all, we recovered 12 Az^r mutant strains from the mutagenesis and characterized these mutants for purine production through the use of three assays: maximum inhibitory concentration (MIC) plate assay, reverse growth halo assay, and high-performance liquid chromatography (HPLC). Of these 12 mutants, Az8 was determined to have the greatest total purine production (with Az2 as the next best). Az8 will be used in subsequent mutagenesis steps to further increase purine production and ultimately riboflavin production.

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Supplemental Information

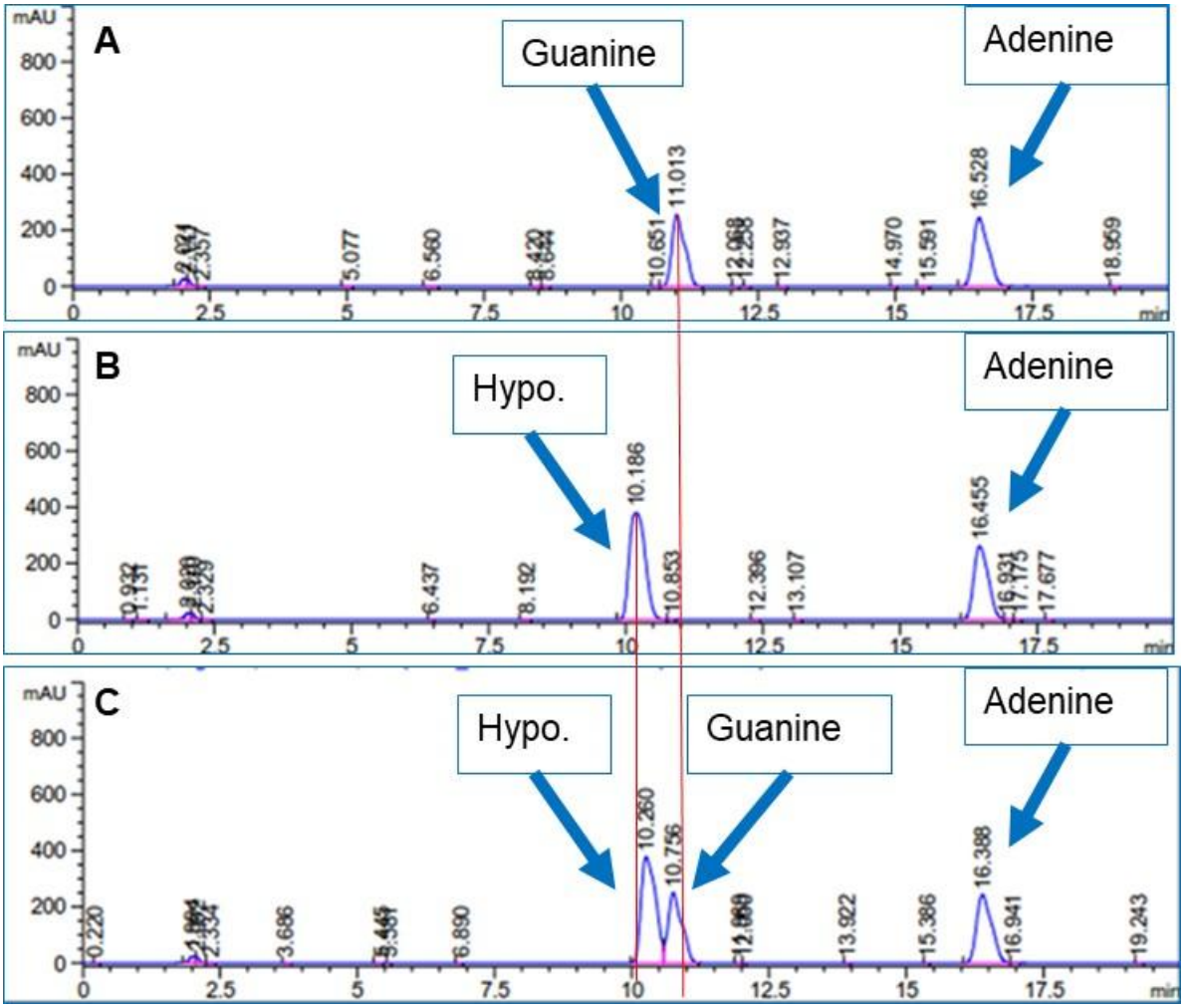
Abbreviations

ADLS	Adenylosuccinate lyase
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Az	Azaguanine
BHI	Brain heart infusion agar
Dc	Decoyinine
DNA	Deoxyribose nucleic acid
EMS	Ethyl methyl sulfonate
ES complex	Enzyme-substrate complex
ETC	Electron Transport Chain
FAD	Flavin dinucleotide
FMN	Flavin mononucleotide
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HPLC	High-performance liquid chromatography
IMP	Inosine 5-monophosphate
MIC	Maximum inhibitory concentration
MTP	Micro titer plate

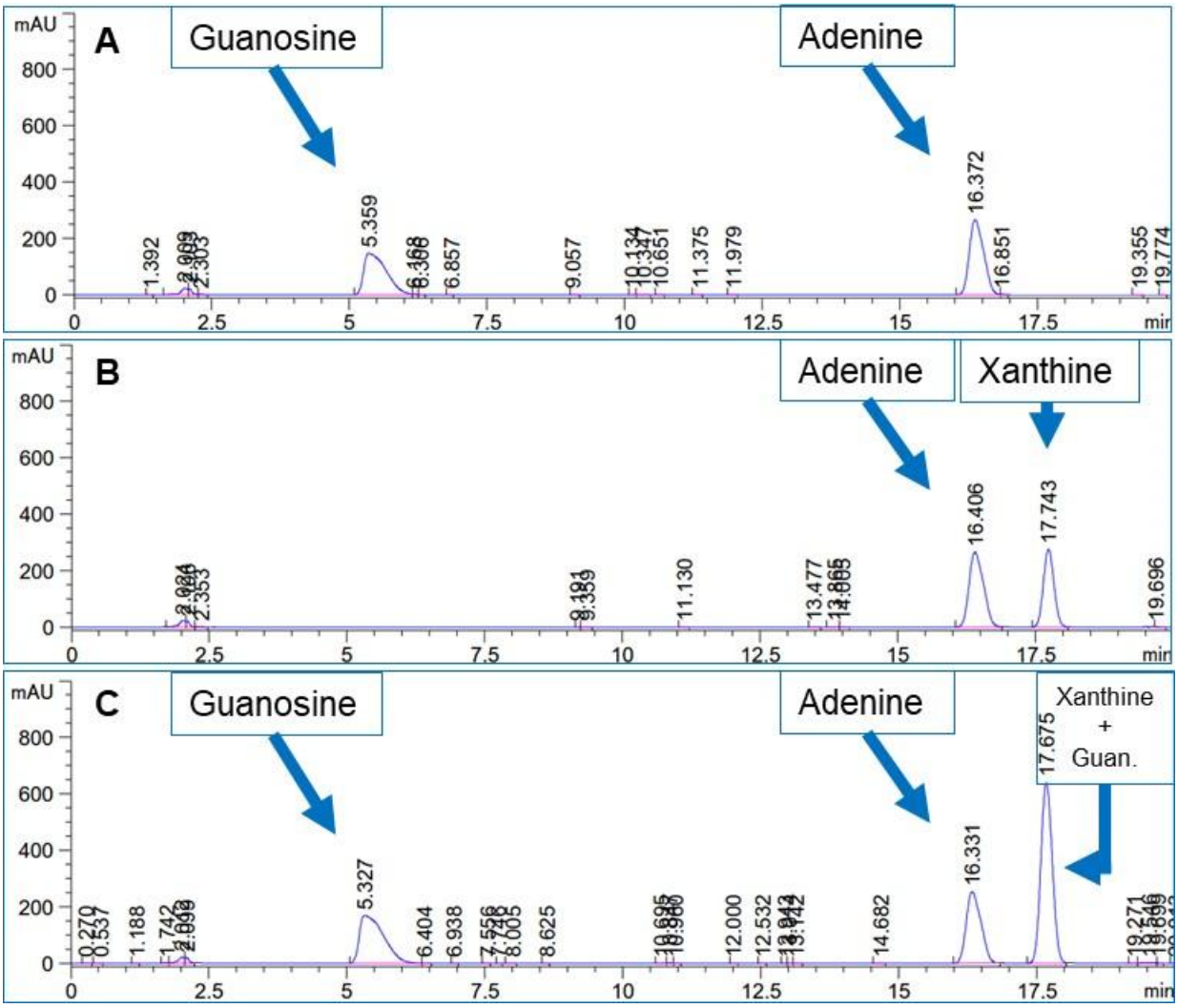
MMS	Methyl methane sulfonate
MS	Methionine sulfate
NADH	Nicotinamide adenine dinucleotide
PCA	Perchloric acid
Riboflavin	Vitamin B_2
RT	Retention time
RoF	Roseoflavin
ROS	Reactive oxygen species
R5P	Ribose-5-phosphate
Ru5P	Ribulose-5-phosphate
SAICAR	(S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate
SS	Spizizen salt minimal media
TMTC	Too many to count

Supplemental **Table 1**. Abbreviations.

HPLC of Authentic Purine Standards

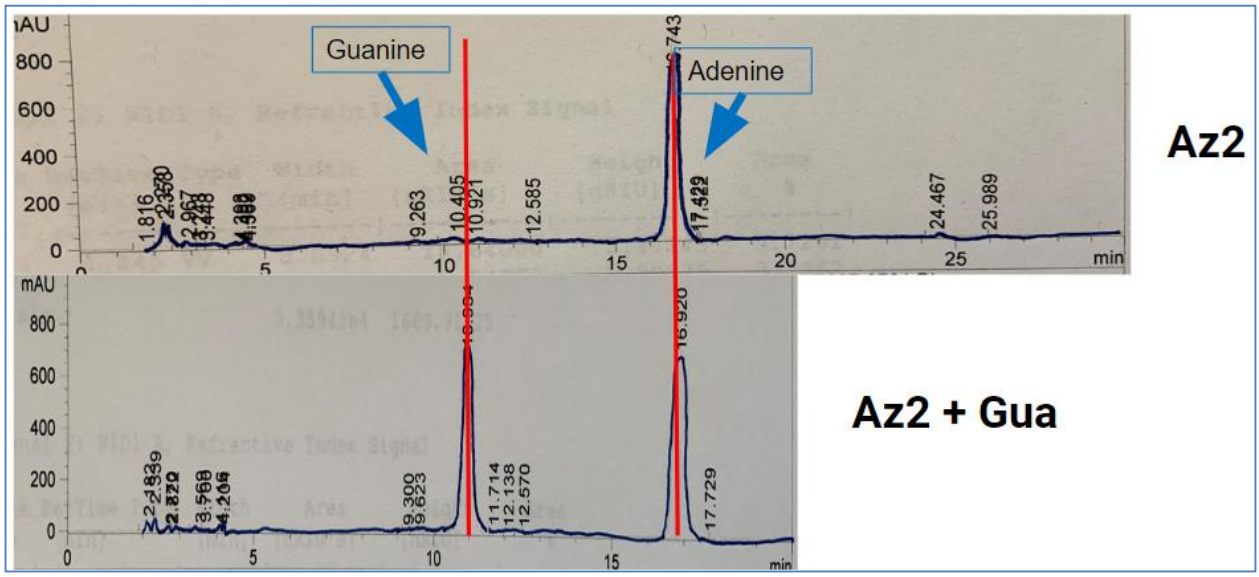


Supplemental Figure 1: Interaction of hypoxanthine and guanine in HPLC. 25 µl of a 0.2% concentration of authentic guanine and hypoxanthine were added separately or together into fresh 1 mL SS Ade medium (50 µg/mL final concentration) and the compounds were separated by HPLC as described in **Materials and Methods**. A, guanine; B, hypoxanthine; C, guanine+hypoxanthine. Red lines are used to help align peaks. An adenine peak is visible in all samples due to its presence in the media at 20 µg/mL. The migration of the purine peaks differ from the data in **Table 5** since this experiment was conducted at a later date using a new C-14 column.



Supplemental Figure 2: Interaction of guanosine and xanthine in HPLC. 25 µl of a 0.2% concentration of authentic guanosine and xanthine were added separately or together into fresh 1 mL SS Ade medium (50 µg/mL final concentration) and the compounds were separated by HPLC as described in **Materials and Methods**. A, guanosine; B, xanthine; C, guanosine+xanthine. An adenine peak is visible in all samples due to its presence in the media at 20 µg/mL. The migration of the purine peaks differ from the data in **Table 5** since this experiment was conducted at a later date using a new C-14 column. Guan., guanosine.

HPLC Spiking Example

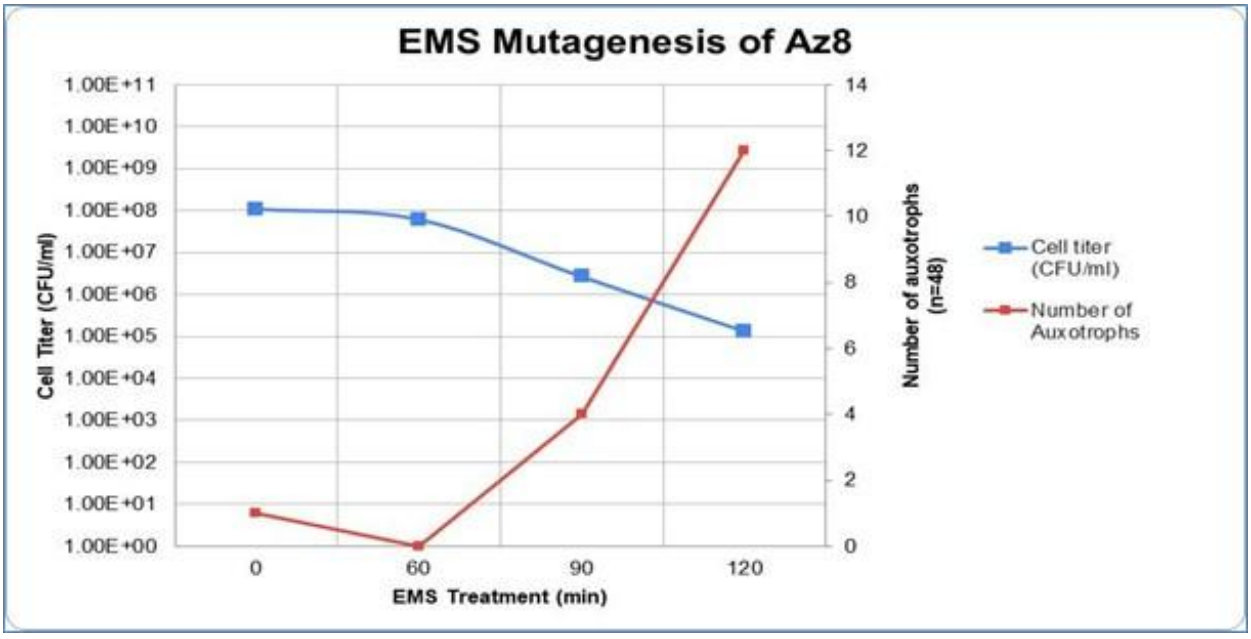


Supplemental Figure 3: Spiking of Az2 sample with authentic guanine. 25 µl of a 0.2% concentration of authentic guanine was added to 1 mL of 72 hour spent fermentation broth of Az2, and the compounds were separated by HPLC. A. Az2 72 hr. spent media alone; B. Az2 72 hr. spent media plus guanine. Red lines are used to help align common peaks.

EMS Mutagenesis of Az8

The first step in producing a double Az^r Dc^r mutant of *B. subtilis* was completed by preparing an EMS mutant library of Az8. To do this, Az8 cells were exposed to EMS following the protocol described in **Materials and Method**, with the exception that the concentration of EMS was reduced to 1.8% and the mutagenesis time course was extended to 120 minutes.

Preparation of EMS mutant libraries of Az8



Supplemental Figure 3: Kill curve of Az-8 mutagenesis EMS mutagenesis. Kill curve resulting from the treatment of Az-8 with EMS over the time interval of 0, 60, 90, and 120 minutes.

As shown in **Figure 3S**, The EMS mutagenesis of Az8 produces a killing of about 2.5-log after 120 minutes of treatment. The 90-minute time period killing of about 2-log also falls within the optimal mutagenesis range. To better assess mutagenesis, as mentioned in the **Discussion** section, we then determine the frequency of non-*purB* auxotrophic mutants that arose

by EMS mutagenesis. To do this, colonies from each treatment were sequentially patched onto minimal medium agar plates containing 20 μg/mL adenine and then BHI agar plates containing 10 μg/mL kanamycin, as the number of colonies that failed to grow on the minimal medium containing adenine was determined. As shown in **Figure 3S**, the number of auxotrophic colonies increased the longer that Az8 was exposed to the mutagen (**red line**), indicating that EMS mutagenesis was successful and that both the 90-minute and 120-minute EMS produced the highest level of mutagenesis. However, due to the low titer of the 120-minute EMS treatment only the 90-minute time interval will be used to select for Dc^r mutants since cell titers of this library should be sufficient to ensure that enough Dc^r mutants can be recovered. Preliminary results indicate that Dc^r mutants could be recovered from the 90 min EMS treatment and at least one produced more purine compounds than Az8 in preliminary HPLC experiments (data not shown).