Implementation of a loss-of-function system to determine growth and stress-associated mutagenesis in *Bacillus subtilis*

Norberto Villegas-Negrete¹, Eduardo A. Robleto², Armando Obregón-Herrera¹, Ronald E. Yasbin³, Mario Pedraza-Reyes¹*

¹ Department of Biology, Division of Natural and Exact Sciences, University of Guanajuato, Guanajuato, Mexico, ² School of Life Sciences, University of Nevada, Las Vegas, Nevada, United States of America, ³ College of Arts and Sciences, University of Missouri—St. Louis, St. Louis, Missouri, United States of America

* pedrama@ugto.mx

Abstract

A forward mutagenesis system based on the acquisition of mutations that inactivate the thymidylate synthase gene (TMS) and confer a trimethoprim resistant (Tmp') phenotype was developed and utilized to study transcription-mediated mutagenesis (TMM). In addition to *thyA*, *Bacillus subtilis* possesses *thyB*, whose expression occurs under conditions of cell stress; therefore, we generated a *thyB* *thyA*+ mutant strain. Tmp' colonies of this strain were produced with a spontaneous mutation frequency of ~1.4 × 10⁻⁹. Genetic disruption of the canonical mismatch (MMR) and guanine oxidized (GO) repair pathways increased the Tmp' frequency of mutation by ~2–3 orders of magnitude. A wide spectrum of base substitutions as well as insertion and deletions in the ORF of *thyA* were found to confer a Tmp' phenotype. Stationary-phase-associated mutagenesis (SPM) assays revealed that colonies with a Tmp' phenotype, accumulated over a period of ten days with a frequency of ~ 60 × 10⁻⁷. The Tmp' system was further modified to study TMM by constructing a Δ*thyA*Δ*thyB* strain carrying an IPTG-inducible P*spac-thyA* cassette. In conditions of transcriptional induction of *thyA*, the generation of Tmp' colonies increased ~3-fold compared to conditions of transcriptional repression. Further, the Mfd and GreA factors were necessary for the generation of Tmp' colonies in the presence of IPTG in B. subtilis. Because GreA and Mfd facilitate transcription-coupled repair, our results suggest that TMM is a mechanism to produce genetic diversity in highly transcribed regions in growth-limited B. subtilis cells.

Introduction

The ability of stressed microbial subpopulations to acquire genetic alterations in response to a persistent non-lethal pressure allowing them to escape from growth-limiting conditions has been termed adaptive or stationary-phase mutagenesis (SPM) [1–3]. This phenomenon promotes genetic diversity and is conserved in prokaryotes and eukaryotes [4–7].
In *Bacillus subtilis*, the mechanisms underlying SPM have been successfully investigated in the strain YB955 bearing the chromosomal auxotrophies hisC952, leuCA27 and metB5 [6]. Using this gain-of-function (reversion) mutagenesis system it has been shown that adaptive mutations arise from error-prone processing of mismatched and chemically modified DNA bases [8–11]. Additional evidence revealed a direct correlation between transcriptional derepression and SPM in non-dividing *B. subtilis* cells [12, 13]. Recent results showed that in growth-limited *B. subtilis* cells, the transcription repair-coupling factor (Mfd) promotes mutagenic events in transcriptionally active genes coordinating error-prone repair events that required nucleotide excision (NER) and base excision (BER) repair components as well as low-fidelity DNA synthesis [14]. Notably, a mutagenic pathway dependent on Mfd, the NER system and the error prone polymerase PolY1 that prevents conflicts between the replicative and transcriptional machineries has been recently described in growing *B. subtilis* cells [15]. In *Escherichia coli*, the elongation factor of the RNA polymerase NusA has been found to be necessary for stress-induced mutagenesis [16]. In addition to Mfd, *B. subtilis* possesses the transcriptional factors NusA and GreA [17, 18]; however, a possible contribution of these factors in modulating transcriptional-mediated mutagenic events in nutritionally stressed, non-growing *B. subtilis*, remains to be elucidated.

Two types of genetic alterations affect organism’s physiology, namely, the gain-of- (i.e., reversion mutagenesis) and the loss-of-function (i.e., forward mutagenesis) mutations; the former may generate a product with an enhanced or a novel function whereas the latter one leads to reducing or abolishing protein function [19]. As noted above, SPM frequencies in *B. subtilis* have commonly calculated from mutation events occurring in strain YB955 containing point mutations in the chromosomal genes, metB5 (ochre), leuCA27 (missense) and hisC952 (amber) [6]. However, evolutionary experiments conducted in distinct microorganisms have revealed that loss-of- or modification-of- are by far more frequent than gain-of-function mutational events [19].

Thymidine synthesis plays an essential role in DNA metabolism. In both, prokaryotes and eukaryotes, thymidylate synthase (TMS) converts dUMP to dTMP using N5N10-methylenetetrahydrofolate as cofactor [20]. Thus, tetrahydrofolate analogs such as aminopterin or trimethoprim that inhibit dihydrofolate reductase (DHFR) also inhibit thymidine synthesis [21, 22]. *B. subtilis* possesses thyA and thyB encoding TMSs [23, 24]. In this bacterium, thymine auxotrophs incorporate this metabolite much more efficiently than the wild type strain and are able to grow in the presence of aminopterin or trimethoprim [20–22]. Thus, loss of TMS function allows selection of TmpR mutants that requires exogenous thymine for growth [20–22].

Here, we developed a loss-of-function mutagenesis system based on the production of trimethoprim resistant colonies (TmpR) resulting from mutations that inactivate TMS as an efficient and more direct method to analyze growth and SPM in *B. subtilis*. The use of this system showed that a null thyB *B. subtilis* strain proficient for thyA accumulated a high frequency of adaptive TmpR colonies. Furthermore, under growth-limited conditions, derepression of a wild type thyA gene in a ΔthyA ΔthyB background revealed a positive correlation between transcription and accumulation of TmpR colonies. Interestingly, the generation of transcription-associated TmpR mutants was dependent not only on Mfd but also on GreA, two proteins known to process RNA polymerase (RNAP) pausing. Thus, our results suggest that under conditions of nutritional stress RNAP backtracking and/or RNAP pausing promote mutagenesis in non-growing *B. subtilis* cells.

**Materials and methods**

**Bacterial strains and culture conditions**

The bacterial strains used in this study are listed in Table 1. *B. subtilis* YB955 is a prophage-“cured” strain that contains the hisC952, metB5, and leuCA27 alleles [6, 25]. The procedures for
Table 1. Strains and plasmid used in this study.

| Strain or Plasmid   | Genotype or descriptiona | Construction or sourceb |
|---------------------|--------------------------|-------------------------|
| **B. subtilis**     |                          |                         |
| YB955               | hisC952 metB5 leuC 427 xin-1 sp_ SENS | [6]                     |
| PERM573             | YB955 ΔytkD::neo ΔmutM:: tet ΔmutY:: sp Neo' Spc' Tet' | [9]                     |
| PERM739             | YB955 ΔmutSL::neo Neo' | [10]                    |
| YB9800              | YB955 Δmtfd::tetTet' | [12]                    |
| PERM1000b           | YB955 ΔthyA::neo Neo' | This study              |
| PERM1037b           | YB955 ΔthyB::em Em' | pPERM1013→ YB955        |
| PERM1074b           | YB955 ΔthyA::neo ΔthyB:: em Neo' Em' | pPERM1013→ PERM1000a  |
| PERM1075b           | YB955 ΔthyA::neo ΔthyB:: em with a Phs construct inserted into amyE locus; Neo' Em' Sp' | This study              |
| PERM1100b           | YB955 ΔthyA::neo ΔthyB:: em with a Phs-thyA construct inserted into amyE locus; Neo' Em' Sp' | pPERM1099→ PERM1074a  |
| PERM1171b           | YB955 ΔthyA::neo ΔthyB::em Δmtfd:: tet with a Phs-thyA construct inserted into amyE locus; Neo' Em' Tat' Sp' | YB9800 → PERM1100     |
| PERM1192b           | YB955 ΔthyA::neo ΔthyB:: em ΔgreA:: cm with a Phs-thyA construct inserted into amyE locus; Neo' Em' Cm' Sp' | pPERM1191→ PERM1171   |
| PERM1491            | YB955 ΔthyB::em ΔytkD:: neo ΔmutM:: tet ΔmutY::sp Em' Neo' Tet' Sp' | PERM573→ PERM1037     |
| PERM1565            | YB955 ΔthyB::em ΔmutSL::neo Em' Neo' | PERM153→ PERM1037      |
| **Plasmids**        |                          |                         |
| pBEST               | PGEM4 containing the neomycin resistance cassette under control of repU promoter of B. subtilis | [28]                    |
| pdr-111-amyE-Phyperspank | bla spec carrying Phyperspank promoter | [13]                    |
| pMUTIN4             | Integrational lacZ fusion vector; Amp' Em' | [29]                    |
| pMUTIN4-Cat         | pMUTIN4-Cat; Cm' | [11]                    |
| pPERM1013           | pMUTIN4 with a 357-bp HindIII/BamHI PCR product from inside of the 5' end of thyB ORF; Amp' | This study              |
| pPERM1014           | pBEST with a 493-bp BamHI/Sacl PCR product from the 5' end of thyA and with a 663-bp BamHI/Sacl PCR product from the 3' end of thyA ORF; Amp' | This study              |
| pPERM1098           | pJET1.2/blunt carrying a 1,194-bp SalI/Sphl PCR fragment from thyA ORF; Amp' | This study              |
| pPERM1099           | pdr-111-amyE-Phyperspank carrying a 1,194-bp SalI/Sphl fragment from pPERM1098; Sp' | This study              |
| pPERM1191           | pMUTIN4 with a 222-bp EcoRI/BamHI PCR fragment from gneA ORF; Cm' | This study              |

a“Em”, Erithromycin; Amp, Ampicillin; Neo, Neomycin; Sp, Spectinomycin; Tet, Tetracycline; Cm, Chloramphenicol.
b“X”→“Y” indicates that “strain Y” was transformed with DNA from “source X”.

The background for this strain is YB955.

https://doi.org/10.1371/journal.pone.0179625.t001

...transformation and isolation of chromosomal and plasmid DNA were as described previously [26–28]. Liquid cultures of B. subtilis strains were routinely grown in Penassay broth (PAB) (antibiotic A3 medium; Difco Laboratories, Sparks, MD). When required, neomycin (Neo; 10 μg ml⁻¹); tetracycline (Tet; 10 μg ml⁻¹); spectinomycin (Sp; 100 μg ml⁻¹); erythromycin (Em; 1 μg ml⁻¹ or 5 μg ml⁻¹); chloramphenicol (Cm; 5 μg ml⁻¹); kanamycin (Kan; 50 μg ml⁻¹); trimethoprim (Tmp; 10 μg ml⁻¹), or IPTG (0.07 mM) was added to the medium. E. coli cultures were grown in Luria-Bertani (LB) medium supplemented with ampicillin to a final concentration of 100 μg ml⁻¹. The PCR products were generated with Vent DNA polymerase (New England BioLabs, Ipswich, MA) and the set of homologous oligonucleotide primers described in Table 2.

Strains construction

Knockouts in the genes of interest were constructed by marker exchange between the chromosome- and plasmid-borne alleles. A plasmid to disrupt thyA was constructed as follows, a 493-bp fragment from the 5´-thyA region (−300 to +164 relative to thyA start codon) and a
473-bp fragment from the 3'-thyA region (+600 to +1073 relative to thyA start codon) were PCR amplified using chromosomal DNA from *B. subtilis* 168. The set of oligonucleotide primers 3, 4 and 5, 6 (Table 2) were used for amplification of the 5'-thyA and 3'-thyA fragments, respectively. The amplified 5'-thyA and 3'-thyA fragments were cloned between the SphI/SalI and BamHI/SalI sites of pBEST502 [29]. The resulting construction pPERM1014 was replicated in *E. coli* XL10-GOLD Kan' (Stratagene, Cedar Creek, TX) and employed to transform the strain *B. subtilis* YB955 to neomycin resistance, thus generating strain *B. subtilis* PERM1000 (Table 1).

A ΔthyB strain was generated by amplifying a fragment from position 38 to 675 from the thyB open reading frame was amplified by PCR using chromosomal DNA from *B. subtilis* YB955 and the oligonucleotide primers 5 and 6 (Table 2). The PCR product was cloned between the HindIII and BamHI sites of the integrative plasmid pMUTIN4 [30]. The resulting construct was designated pPERM1013 and used to transform *B. subtilis* YB955 and PERM1000, thus generating strains PERM1037 (ΔthyB; Em'') and PERM1074 (ΔthyA ΔthyB; Neo'' Em'''), respectively (Table 1). Genetic inactivation of error prevention GO system (*ytkD*, *mutM* and *yfhQ*) from *B. subtilis* YB955 was achieved by transforming the strain ΔthyB (PERM1037; Table 1) with genomic DNA isolated from *B. subtilis* PERM573 (ΔytkD::Neo ΔmutM::Tet ΔmutY::Spc). This procedure generated the strain *B. subtilis* PERM1491 (ΔthyB GO; Em' Cm' Tet' Spc'), respectively (Table 1). The thyB mutSL mutant in the YB955 background was generated by transforming strain PERM151 (ΔmutSL::Neo) [8] with genomic DNA isolated from *B. subtilis* PERM1037 (ΔthyB), thus generating strain PERM1565 (ΔthyB mutSL; Em' Neo'') (Table 1). Disruptions of the appropriate chromosomal genes were confirmed by PCR.

The strain containing the thyA gene under the control of an IPTG-inducible promoter was constructed as follows. The open reading frame (ORF) of thyA was PCR amplified from *B. subtilis* 168 chromosomal DNA and the oligonucleotide primers 7 and 8 (Table 2). The PCR product (1,194 bp) was purified from a low-melting-point agarose gel and cloned between the SalI and SphI sites of the *amyE* integrative vector pdr111 (a gift from David Rudner), immediately downstream of the IPTG-inducible Phyperspank promoter (*Phs*). The resulting plasmid

| Oligonucleotide | (Sequence 5' to 3') | Restriction site (underlined) |
|-----------------|---------------------|------------------------------|
| 1) thyA FW      | CGGCATGCCGTATACACACACCTGAC | SphI |
| 2) thyA RV      | GCCTGACCGAACCTCTGATTTGTC | SalI |
| 3) thyA FW      | CGGGATCCCGCATGATTGCTAAGTGCAC | BamHI |
| 4) thyA RV      | GCAGACTGATGATGCTCAAGTGCAC | SalI |
| 5) thyB FW      | CGAAGCTTGGACATGTTGAGAAAGG | HindIII |
| 7) ORF thyA FW  | GCCTGACCGAACCTCTGATTTGTC | BamHI |
| 8) ORF thyA RV  | GCCTGACCGAACCTCTGATTTGTC | SphI |
| 9) greA FW      | GCGAATTCCCGGAAGCTTCGAGACC | EcoRI |
| 10) greA RV     | GCGGATCCCGGTTCGAGATCSTA | BamHI |
| 11) thyART FW   | GGGACTCAGATGGAACCC | - |
| 12) thyART RV   | CCAATATGGACGCCCATC | - |
| 13) veg FW      | TGCCGAGACGTTTGGCGATATTA | - |
| 14) veg RV      | CGGCCACCGTTTGGCTTTAAC | - |

a F, forward; R, Reverse
Stress-associated *B. subtilis* Tmp\(^r\) mutagenesis

(pPERM1099) was replicated in *E. coli* XL10-GOLD (Stratagene). This construct was transformed and integrated into the *amyE* locus of *B. subtilis* PERM1074 (Δ*thyA thyB*) to generate the strain *B. subtilis* PERM1100 (Δ*thyA ΔthyB; amyE::Phs-thyA; Neo\(^r\) Em\(^r\) Spc\(^r\)) (Table 1). As an experimental control, the empty pdr111 vector was also recombined in the *amyE* locus thus generating the strain *B. subtilis* PERM1075 (Δ*thyA ΔthyB; amyE::Phs; Neo\(^r\) Em\(^r\) Spc\(^r\)) (Table 1).

To disrupt *mfd*, competent cells of strain *B. subtilis* PERM1100 were transformed with chromosomal DNA of *B. subtilis* YB9800 (Δ*mfd::Cm*) [12] to generate the strain *B. subtilis* PERM1171 (Δ*amyE ΔamyB Δmfd; amyE::Phs-thyA; Neo\(^r\) Em\(^r\) Cm\(^r\) Spc\(^r\)) (Table 1). An integrative plasmid designed to inactivate *greA* was constructed as follows. A fragment of *greA* was first amplified by PCR using chromosomal DNA from *B. subtilis* 168 and the set of oligonucleotide primers 9 and 10 (Table 2). The 222-bp PCR product, extending from position 115-bp to 318-bp downstream of the *greA* start codon was cloned between the EcoRI-BamHI sites of the integrative plasmid pMUTIN4-Cat [11]. The resulting construct designated pPERM1191 was used to transform *B. subtilis* PERM1100, generating strain PERM1192 (Δ*amyE ΔamyB ΔgreA; amyE::Phs-thyA; Neo\(^r\) Em\(^r\) Cm\(^r\) Spc\(^r\)) (Table 1). The single- or double-crossover events leading to inactivation of the appropriate genes and integration of transcriptional cassettes were corroborated by PCR with specific oligonucleotide primers.

**Stationary-phase mutagenesis soft-agar overlay assays**

Cultures were grown in flasks containing antibiotic A3 medium with aeration (250 rpm) at 37°C to 90 min after the cessation of exponential growth (designated T\(_{90}\)). Growth was monitored with a spectrophotometer measuring the optical density at 600nm (OD\(_{600}\)). The cultures were centrifuged at 10,000 × g for 10 min and resuspended in 10 ml of 1X Spizizen Minimal Salts (SMS) [31]. Aliquots of 0.1 ml were then spread plated on Spizizen minimal medium (SMM) containing 1X SMS, 0.5% (w/v) dextrose, 50 μg isoleucine ml\(^{-1}\), 50 μg glutamate ml\(^{-1}\), 1.5% (w/v) agar (BD, Bioxom), 50 μg histidine ml\(^{-1}\) of and a trace amount (200 ng ml\(^{-1}\)) of leucine and methionine (amino acids from Sigma-Aldrich, St. Louis, MO), with or without 0.07 mM IPTG. The initial titer was determined from the 10-ml culture. Starting from 48 h of incubation, a set of plates was overlaid with soft agar (0.7% (w/v) agar and prewarmed at 42°C lacking histidine and containing 0.07 mM IPTG, 50 μg thymine ml\(^{-1}\), 10 μg Tmp ml\(^{-1}\), 50 μg methionine and leucine ml\(^{-1}\). Of note, adjustment of the final concentrations for IPTG, methionine and leucine considered the volume and IPTG concentration in the medium dispensed previous to performing the overlay. The plates were incubated for two days, and the number of Tmp\(^r\) colonies was scored. The initial titers were used to normalize the cumulative number of resistant colonies per day to the number of CFU plated. Assays were replicated three times, and the experiment was repeated at least twice. The viability of the non-revertant cell background was assessed every other day as follows. Using a sterile Pasteur pipette, a plug of agar was removed from the non-revertant background of each of five plates corresponding to one type of selection (no leucine and methionine without IPTG or no leucine and methionine with IPTG). The plugs were combined in 0.4 ml of 1X SMS, serially diluted, and plated in triplicate on SMM containing 50 μg ml\(^{-1}\) of the required amino acids. Colonies were counted after 48 h of incubation.

**Phenotypic analysis of *B. subtilis* thy mutants**

Trimethoprim resistance of the strains of interest was analyzed on solid SMM containing 1X SMS, 0.5% (w/v) dextrose, 50 μg ml\(^{-1}\) isoleucine, glutamate, histidine, methionine, leucine and thymine. All plates contained thymine at 50 μg ml\(^{-1}\) and the concentration (μg/ml) of
trimethoprim indicated in Table 3. When necessary, the media was supplemented with IPTG to a final concentration of 0.1 mM. Growth was scored after 24 hr at 37˚.

### Analysis of mutation frequencies to Tmp<sup>r</sup> and Rif<sup>r</sup>

Essentially, the appropriate strains were propagated for 12 h at 37˚C in A3 medium with proper antibiotics. For Tmp<sup>r</sup>, mutation frequencies were determined by plating aliquots on six LB plates containing 10 μg ml<sup>-1</sup> trimethoprim and 50 μg ml<sup>-1</sup> thymidine, and the trimethoprim-resistant (Tmp<sup>r</sup>) colonies were counted after 2 days of incubation at 37˚C. The same procedure was employed to determine the Rif<sup>r</sup> phenotype, except that mutant colonies were selected in LB plates containing 10 μg ml<sup>-1</sup> rifampin. The number of cells used to calculate the frequency of mutation to Tmp<sup>r</sup> or Rif<sup>r</sup> was determined by plating aliquots of appropriate dilutions on LB plates without antibiotics and incubating the plates for 24 to 48 h at 37˚C. These experiments were repeated at least three times.

### Total RNA extraction

Cultures were grown to saturation in 1X SMS containing 0.5% (w/v) dextrose; 50 μg ml<sup>-1</sup> of Ile, Glu, methionine, histidine, and leucine; Ho-Le trace elements; 5 mM MgSO<sub>4</sub>; and 1 mM IPTG. Samples were removed at mid-exponential (approximately OD<sub>600nm</sub> = 0.5) and stationary (150 min after onset of the stationary phase; T<sub>150</sub>) growth phases, centrifuged at 5,000 × g and 4˚C for 10 min and frozen at -20˚C. RNA was extracted from the pellets using a Tri reagent (Molecular Research Center, Inc. Cincinnati, OH). After DNase I treatment, the samples were analyzed by PCR to confirm the absence of genomic DNA with Vent DNA polymerase and the set of primers 11 and 12 described in Table 2. The RNA content in the samples was quantitated using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Dubuque, IA).

### Reverse transcription and quantitative real-time PCR

One microgram of RNA was reverse transcribed using an ImPromII reverse transcriptase kit (Promega), as directed, with random hexamers (0.5-μg final concentration). No-reverse transcriptase (NRT) controls were included for all examples. Master mixes for real-time PCR contained Absolute QPCR SYBR green Mix (Thermo Fisher Scientific) and a 70 mM final concentration of the oligonucleotide primers thyART FW and thyART RV (191-bp amplicon) or of veg FW and veg RV (82-bp amplicon) described in Table 2. Three replicates from each culture condition containing 4 μl of cDNA were assayed and normalized to the expression of the internal control gene veg [32, 33]. Two replicates were assessed for NRT and no-template controls. Quantitative real-time PCR was run on a Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA), using the manufacturer’s suggested protocol and an

### Table 3. Phenotypes of B. subtilis thy mutants.

| Strain   | Genotype   | Resistance to Trimethoprim 10 μg ml<sup>-1</sup> |
|----------|------------|-----------------------------------------------|
| YB955    | thy<sup>A</sup>-thy<sup>B</sup> | – |
| PERM1000 | thy<sup>A</sup>-thy<sup>B</sup> | – |
| PERM1037 | thy<sup>A</sup>-thy<sup>B</sup> | – |
| PERM1074 | thy<sup>A</sup>-thy<sup>B</sup> | ++ |
| bPERM1100| thy<sup>A</sup>-thy<sup>B</sup>; amyE::Phs-thyA | – |

<sup>a</sup>Strains were grown on solid Spizizen Minimal Medium (SMM) as described in Materials and Methods. ++, full growth; +, weak growth; –, no growth.  
<sup>b</sup> For this strain, solid SMM was supplemented with 0.1mM IPTG.
annealing temperature of 57˚C, followed by a melting profile and assessment of amplicon size on an agarose gel. Results were calculated by the $2^{-\Delta\Delta CT}$ (where $C_T$ is threshold cycle) method for relative fold expression [34].

DNA sequencing
Colonies with a Tmp′ phenotype were independently propagated in liquid A3 medium supplemented with 10 μg/ml trimethoprim and 50 μg ml$^{-1}$ thymidine and subjected to DNA isolation [27]. The thyA open reading frame of each colony was amplified by PCR using high fidelity and specific oligonucleotide primers (Table 2). Sequencing services were carry out by Functional Biosciences, Inc. (Madison, WI).

Results and discussion
Spontaneous mutation frequencies to trimethoprim resistance in growing B. subtilis cells
To implement a loss-of function system to study mutagenesis in B. subtilis, the thyA gene that codes for TMS was considered as a possible target [23, 24]. In B. subtilis the presence of ThyA prevents the incorporation of exogenous thymine into DNA synthesis [20]; therefore, strains deficient for this activity incorporate this metabolite more efficiently than ThyA-proficient strains [20]. Of note, thyA mutants of B. subtilis are able to grow in medium supplemented with DHFR inhibitors such as trimethoprim; if thymine is present in the culture medium [20], spontaneous colonies with a Tmp′ phenotype can be selected [20, 24, 35]. This phenomenon has been described in several bacteria, including Escherichia coli [35–37]. B. subtilis possesses two TMS encoding genes; i.e., thyA and thyB respectively [20]. It has been reported that thyB expression takes place when B. subtilis is grown under temperatures below 37˚C contributing with only 5–8% of the total TMS activity present in this bacterium [23]. Since thyB is the first cistron of the thyB-dfrA-ypkP operon, we employed a gene construct (pPERM1013; Table 1) to only disrupt thyB and left dfrA-ypkP under control of the IPTG-inducible Pspac promoter. Under our experimental conditions, no significant differences were observed in the growth rates of this mutant with respect to those of the parental strain YB955. Thus, in A3 medium, in the absence or presence of 0.1 mM IPTG the thyB strain grew with similar doubling time values (i.e., 31 ± 2, 30 ± 2.5 and 31 ± 1.8 min, respectively); furthermore, this strain was incapable of growing in minimal medium supplemented with trimethoprim and thymidine (Table 3).

The ΔthyB strain was first used to analyze the spontaneous appearance of colonies resistant to trimethoprim in B. subtilis cells. As shown in Fig 1, colonies with a Tmp′ phenotype appeared, in solid minimal medium supplemented with thymidine, with a spontaneous mutagenesis frequency of ~1.4 ± 0.11 × 10$^{-9}$. These results are in good agreement with the mutation frequencies reported for the strain B. subtilis YB955 using the rpoB gene as a marker of mutagenesis [10, 14]. Previous reports, employing the Rif′ phenotype demonstrated that inactivation of the canonical mismatch (MMR) and guanine-oxidized (GO) repair systems increased, two and three orders of magnitude, respectively, the spontaneous mutagenesis in growing B. subtilis cells [8, 9]. As shown in Fig 1, the genetic inactivation of the GO system, which in B. subtilis is composed of the MutY, MutM and YtkD proteins [9, 38] increased the mutation frequency to Tmp′ in the ThyB-deficient B. subtilis strain around 1000 times. Furthermore, our results revealed that disruption of the mutSL operon, encoding the MMR system, increased the frequency of mutation to trimethoprim resistance in the ΔthyB strain around 30 times. As shown in Fig 1, the mutation frequencies as determined with the rpoB marker in the mutSL
and GO-deficient strains were similar to those determined with the thyA gene. Therefore, the loss-of-function mutagenesis system described in this report is robust and can be successfully employed to study the spectrum of mutagenic events that occur in thyA in a population of B. subtilis cells.

Mutational spectra of thyA in colonies exhibiting a Tmp^r phenotype

We further investigated the mutagenic events associated with the loss of TMS function in the thyB-deficient strain. It was recently shown, that the heterologous expression of thyP3, a homologue of thyA, from the ITPG-inducible Pspac promoter, resulted in the production of mutations in the -5 region of Pspac conferring trimethoprim resistance in actively growing B. subtilis cells [39]. In this study, we were interested in identifying mutations affecting the function of ThyA under conditions of native thyA expression; therefore, the thyA gene of 40 colonies exhibiting a Tmp^r phenotype was PCR amplified with high-fidelity DNA polymerase. All the samples produced amplification DNA bands of the expected size for the thyA ORF (namely, 837 bp), which were subsequently subjected to DNA sequencing to identify the type of mutations conferring the Tmp^r phenotype. A wide spectrum of frameshift mutations and
Base substitutions were detected in the sequenced thyA mutants, predominating the insertions/deletions over the base substitutions in a proportion of 60% to 40% (Table 4 and Figs 2 and 3). Among the substitutions, 63% corresponded to transversions and 37% to transitions events, predominating the A→T (~29%) and A→G (~17%) changes (Table 4). Of note, three of the base changes generated non-sense mutations producing truncated ThyA proteins (Table 4 and Fig 2). It was found that most of the missense mutations in thyA resulted in non-conserved amino acid changes, moreover, two of the substitutions changed an alanine residue for the secondary structure-disrupting amino acid proline and one of them switched an isoleucine for the bulky aromatic residue phenylalanine (Table 4 and Fig 2). Importantly, 14 of the 17 missense mutations took place in codons encoding amino acids conserved in both thymidylate synthases (Table 4 and Fig 2). However, additional mutations in residues non-conserved in E. coli ThyA, were identified in the enzyme from B. subtilis, including, Ala₆₈→Asp, Trp₃₁→Arg and Ala₆₈→Pro (Fig 2). Thus, in addition to Arg₁₈₁ previously reported as essential for ThyA activity in E. coli [40], our results identified additional residues necessary for the proper function of ThyA in B. subtilis.

As noted above, insertion/deletions were also detected in the thyA sequence of colonies exhibiting a Tmp<sup>+</sup> phenotype. As shown in Fig 3, the insertion events took place between the positions 553–562 of the thyA ORF and consisted of single base additions, including, 4 adenines 2 thymines or 1 guanine. On the other hand, the frameshift deletions that took place between the positions 455–458 of the thyA ORF, involved the single loss of 7 thymines or the dinucleotide guanine-adenine (Fig 3). Notably, the insertion and deletions occurred in the base repeats GGGAAA and GAATT, respectively, and gave rise to truncated non-functional ThyA proteins. Thus, replication errors in these sequences that escaped the action of the mismatch repair machinery may be involved in generating these types of mutations. In support of this notion, the genetic inactivation of the MMR system (MutSL) in the ΔthyB strain increased by about two orders of magnitude the mutation frequency to trimethoprim resistance (Fig 1). Altogether, our results revealed that a wide spectrum of mutagenic events may lead to loss of

### Table 4. Base substitutions in thyA alleles from Tmp<sup>+</sup> colonies.

| Position(s) of mutation in the ORF of thyA (bp) | Number of Thy<sup>+</sup> colonies bearing the mutation | Type of mutation | *DNA change (Codon involved) | Result of mutation |
|-----------------------------------------------|-------------------------------------------------------|------------------|-------------------------------|-------------------|
| 205                                           | 2                                                     | Transversion     | A→T (69)                     | Ile→Phe           |
| 535                                           | 2                                                     | Transversion     | A→T (179)                    | Arg→Stop          |
| 779                                           | 1                                                     | Transversion     | A→T (260)                    | Asp→Val           |
| 243                                           | 1                                                     | Transversion     | T→A (81)                     | Asn→Lys           |
| 473                                           | 1                                                     | Transversion     | T→A (158)                    | Leu→Stop          |
| 406                                           | 2                                                     | Transition       | A→G (136)                    | Asn→Asp           |
| 407                                           | 1                                                     | Transition       | A→G (136)                    | Asn→Ser           |
| 779                                           | 1                                                     | Transition       | A→G (260)                    | Asp→Gly           |
| 202                                           | 1                                                     | Transversion     | G→C (68)                     | Ala→Pro           |
| 468                                           | 1                                                     | Transversion     | G→C (156)                    | Met→Ile           |
| 469                                           | 1                                                     | Transversion     | G→C (157)                    | Ala→Pro           |
| 91                                            | 1                                                     | Transition       | T→C (31)                     | Trp→Arg           |
| 473                                           | 1                                                     | Transition       | T→C (158)                    | Leu→Ser           |
| 113                                           | 1                                                     | Transversion     | C→A (38)                     | Ala→Glu           |

*aPosition of the base substitutions detected in the ORF of thyA composed by 279 amino acids. The codon number that was modified by the base change is shown between parentheses.*

https://doi.org/10.1371/journal.pone.0179625.t004
ThyA function corroborating that the Tmp\(^r\) system informs on the genetic events underlying stress-associated mutagenesis in *B. subtilis*.

### Analysis of stationary-phase associated mutagenesis to trimethoprim resistance in nutritionally stressed *B. subtilis* cells

We employed the ΔthyB strain to investigate whether loss-of thymidylate synthase (ThyA) function leading to Tmp\(^r\) resistance can be employed to measure adaptive mutagenesis in *B. subtilis*. To this end, *B. subtilis* cells of the ThyB-deficient strain collected from the stationary-phase of growth were extensively washed to eliminate residual nutrients and plated in selective Spizizen...
Minimal Medium (SMM) as described in Materials and Methods. The number of colonies that acquired a Tmp\textsuperscript{r} phenotype under growth-limited conditions was scored every two days for a ten days period. The results revealed that Tmp\textsuperscript{r} colonies from the ΔthyB thyA\textsuperscript{+} accumulated over a period of ten days with a frequency of $\sim 65 \pm 11 \times 10^{-7}$ (Fig 4A), demonstrating the feasibility of stress-associated \textit{B. subtilis} Tmp\textsuperscript{r} mutagenesis

---

**Fig 3.** Frame-shift mutations in the \textit{thyA} ORF from \textit{B. subtilis} colonies with a Tmp\textsuperscript{r} phenotype. The black arrow depicts the open reading frame of \textit{thyA} in kbp. Nucleotide insertions or deletions at each position are shown above or below the \textit{thyA} ORF, respectively. Oligonucleotide deletions and insertions as well as its positions are shown between brackets. I, insertions; D, deletions.

https://doi.org/10.1371/journal.pone.0179625.g003

---

**Fig 4.** (A). Frequencies of stationary-phase accumulation of Tmp\textsuperscript{r} colonies of strain \textit{B. subtilis} PERM1037 (\textit{thyA}\textsuperscript{+} \textit{thyB}) were determined as described in Materials and Methods. Data were normalized to initial cell titers ± SD and represent counts averaged from three separate tests. (B). Ability of strain PERM1037 to survive Met-Leu\textsuperscript{−}. Data were collected from plugs removed from three plates and titers were plated on media containing all essential amino acids every other day for testing of viability of non-revertant background cells (see Materials and Methods for details). Data is represented as the number of CFU per plug. Error bars represent 1 standard error of the mean.

https://doi.org/10.1371/journal.pone.0179625.g004
of employing this loss-of-function system to study mutagenesis in non-growing *B. subtilis* cells. Notably, the increase in the number of Tmp<sup>r</sup> colonies took place without significant changes in the viable cell count number providing thus additional support for this assumption (Fig 4B).

**Transcription-associated mutagenesis analysis in growth-limited *B. subtilis* cells acquiring a Tmp<sup>r</sup> phenotype**

Previous studies have reported on the role of transcription in stationary-phase mutagenesis in *B. subtilis* [12]. It has been shown that a combination of transcriptional derepression and error-prone repair events in stress conditions can modulate the generation of mutations in highly transcribed DNA regions [13, 41].

To investigate whether acquisition of Tmp<sup>r</sup> resistance can be used to study transcription-associated mutagenesis (TAM) in nutritionally stressed bacteria we engineered a double *thyA thyB* knock out strain that overexpressed a wild type copy of *thyA* from the IPTG-inducible Phyperspank (Phs) promoter; this strain was termed *B. subtilis* PERM1100 (Table 1). As specified in Table 3, the *thyA thyB* mutant was able to grow in SMM supplemented with Tmp and thymidine. However, IPTG-induction of *thyA* expression abrogated the growth of the strain PERM1100 in this medium. Further, the lack of two amino acids (methionine and leucine) in the incubation agar media prevented the growth of cells. Therefore, the strain’s properties and the double amino acid starvation allowed us to inquire whether derepression of *thyA* in growth-limited cells promote mutagenic events influencing the production of Tmp<sup>r</sup> colonies. As shown in Fig 5A in reference to the non-induced condition, derepression of the Phs-*thyA* construct resulted in a 3-fold increase in the production of colonies with a Tmp<sup>r</sup> phenotype.

Our loss-of function mutagenesis system indicate that increased transcription levels of *thyA* correlated with an increased production of colonies with a Tmp<sup>r</sup> phenotype in the Δ*thyAthyB amyE::Phs-thyA* strain. Of note, these results are in good agreement with former studies showing a positive correlation between derepression of a *leuC* allele and production of Leu<sup>+</sup> prototrophs in *B. subtilis* [13]. Furthermore, viability of the strain PERM1100 did not significantly change in the absence and/or presence of the inducer IPTG (Fig 6A); therefore, the increase in the number of Tmp<sup>r</sup> can be uncoupled from the growth of the sensitive strain in the plate. To confirm that accumulation of Tmp<sup>r</sup> mutants were due to increases in transcription levels of

---

**Fig 5.** Frequencies of stationary-phase accumulation of Tmp<sup>r</sup> colonies of strains *B. subtilis* PERM1100 (A), PERM1171 (B) and PERM1192 (C) under repressed (–IPTG; white symbols) or induced (+ IPTG; black symbols) were determined as described in Materials and Methods. Data represent counts from three plates averaged from three separate tests, normalized to initial cell titers.

https://doi.org/10.1371/journal.pone.0179625.g005
thyA, the mRNAs of this gene were quantified by qRT-PCR in stationary-phase cells of strain PERM1100 under induced and non-induced conditions. The results of this analysis showed that the mRNA levels of thyA increased over 3-fold (i.e. from 0.5 ± 0.06 to 4.5 ± 0.4) when IPTG was added to the medium. Therefore, the loss-of-function system implemented here can be successfully employed to investigate the mutagenic processes occurring in transcriptionally active DNA regions that allow cells to escape from non-proliferating conditions. Of note, the discovery of missense and nonsense mutations affecting B. subtilis ThyA function (Fig 2), will allow to adapt this system to determine the production of thyA⁺ revertants in nutritionally stressed thyA⁻thyB⁻ cells overexpressing point-mutated alleles of thyA.

A recent study revealed that Mfd, a protein that couples DNA repair with transcription, promotes mutagenic events that increase the production of His⁺, Met⁺ and Leu⁺ revertants in starved cells of strain B. subtilis YB955 [12, 13]. Here, we inquired whether the Mfd requirement for transcription-associated SPM can be detected with the loss-of-function Tmp r mutagenesis system. To this end, the gene mfd was genetically inactivated in the ΔthyA thyB strain. The number of Tmp r colonies produced by this strain under starving conditions was determined over a period of ten days under conditions where the thyA gene was repressed or derepressed for transcription. As shown in Fig 5B, in reference to the strain harboring an intact copy of Mfd, the number of adaptive Tmp r colonies produced by the Mfd-deficient strain did not significantly differ under conditions of repression or derepression for the thyA gene. Therefore, employing a system that overexpresses a different gene; namely, thyA, our results provide further support for the concept that Mfd is a factor required for TMM in growth-limited B. subtilis cells. As noted above, NusA, the elongation factor of the RNA polymerase has been found to be necessary for stress-induced mutagenesis in Escherichia coli [16]. Remarkably, disruption of greA in the thyA thyB strain abrogated the production of Tmp r colonies under conditions that induced thyA expression. Thus, as shown in Fig 5C, the levels of Tmp r mutagenesis in this strain were almost similar under conditions that induced or repressed thyA. Importantly, these results took place in the absence of growth since viability of the GreA-deficient strain did not significantly change in the absence and/or presence of the

https://doi.org/10.1371/journal.pone.0179625.g006
inducer IPTG (Fig 6C). Based on these results is feasible to speculate that in nutritionally stressed \textit{B. subtilis} cells, processing of paused or backtracked RNAP-DNA complexes promote mutagenic events that allow cells to escape from growth-limited conditions. Interestingly, in \textit{E. coli}, both Mfd and GreA are speculated to prevent UvrD-dependent RNAP backtracking and repair during transcription [42]. However, the consequences of such transcriptional events on growth and stress-associated mutagenesis remain to be elucidated.

In summary, the mutagenesis method implemented here and the contribution of GreA to TMM in \textit{B. subtilis} cells add further elements to our understanding on how bacteria develop beneficial mutations, including antibiotic resistance, under stressful conditions.

### Acknowledgments

The authors are grateful for the excellent technical assistance of Karina Olmos-López and Norma Ramirez-Ramirez.

### Author Contributions

**Conceptualization:** Norberto Villegas-Negrete, Eduardo A. Robleto, Mario Pedraza-Reyes.

**Data curation:** Norberto Villegas-Negrete, Eduardo A. Robleto, Armando Obregón-Herrera, Ronald E. Yasbin, Mario Pedraza-Reyes.

**Formal analysis:** Norberto Villegas-Negrete, Armando Obregón-Herrera, Mario Pedraza-Reyes.

**Funding acquisition:** Eduardo A. Robleto, Mario Pedraza-Reyes.

**Investigation:** Norberto Villegas-Negrete.

**Methodology:** Norberto Villegas-Negrete, Eduardo A. Robleto, Mario Pedraza-Reyes.

**Resources:** Eduardo A. Robleto, Mario Pedraza-Reyes.

**Supervision:** Eduardo A. Robleto, Mario Pedraza-Reyes.

**Validation:** Norberto Villegas-Negrete, Armando Obregón-Herrera, Mario Pedraza-Reyes.

**Visualization:** Norberto Villegas-Negrete, Mario Pedraza-Reyes.

**Writing – original draft:** Norberto Villegas-Negrete, Mario Pedraza-Reyes.

**Writing – review & editing:** Norberto Villegas-Negrete, Eduardo A. Robleto, Armando Obregón-Herrera, Ronald E. Yasbin, Mario Pedraza-Reyes.

### References

1. Bridges BA. The role of DNA damage in stationary phase (“adaptive”) mutation. Mutat Res. 1998; 408: 1–9. PMID: 9678058

2. Rosenberg SM, Thulin C, Harris RS. Transient and heritable mutators in adaptive evolution in the lab and in nature. Genetics. 1998; 148: 1559–1566. PMID: 9560375

3. Robleto EA, Yasbin R, Ross C, Pedraza-Reyes M. Stationary phase mutagenesis in \textit{Bacillus subtilis}: a paradigm to study genetic diversity programs in cells under stress. Crit Rev Biochem Mol Biol. 2007; 42:327–338. https://doi.org/10.1080/1040923070197717 PMID: 17917870

4. Cairns J, Overbaugh J, Miller S. The origin of mutants. Nature. 1988; 335: 142–145. https://doi.org/10.1038/335142a0 PMID: 3045565

5. Kasak L, Horak R, Kivisaar M. Promoter-creating mutations in \textit{Pseudomonas putida}: a model system for the study of mutation in starving bacteria. Proc Natl Acad Sci USA. 1997; 94: 3134–3139. PMID: 9086358
6. Sung HM, Yabin RE. Adaptive, or stationary-phase, mutagenesis, a component of bacterial differentiation in Bacillus subtilis. J Bacteriol. 2002; 184: 5641–5653. https://doi.org/10.1128/JB.184.20.5641-5653.2002 PMID: 12270822

7. Halas A, Baranowska H, Policinska Z. The influence of the mismatch repair system on stationary phase mutagenesis in the yeast Saccharomyces cerevisiae. Curr Genet. 2002; 42:140–146. https://doi.org/10.1007/s00294-002-0334-7 PMID: 12491007

8. Pedraza-Reyes M, Yabin RE. Contribution of the mismatch DNA repair system to the generation of stationary-phase-induced mutants of Bacillus subtilis. J Bacteriol. 2004; 186: 6485–6491. https://doi.org/10.1128/JB.186.19.6485-6491.2004 PMID: 15375129

9. Vidales LE, Cardenas LC, Robleto E, Yabin RE, Pedraza-Reyes M. Defects in the error prevention oxidized guanine system potentiate stationary-phase mutagenesis in Bacillus subtilis. J Bacteriol. 2009; 191:506–513. https://doi.org/10.1128/JB.01210-08 PMID: 1901023

10. Deora BN, Vidales LE, Ramirez R, Ramirez M, Robleto EA, Yabin RE, et al. Mismatch repair modulation of MutY activity drives Bacillus subtilis stationary-phase mutagenesis. J Bacteriol. 2011; 193:236–245. https://doi.org/10.1128/JB.00940-10 PMID: 20971907

11. Barajas-Oroelias RDC, Ramirez-Guadiana FH, Juarez-Godinez R, Ayala-Garcia VM, Robleto EA, Yabin RE, et al. Error-prone processing of apurinic/apyrimidinic (AP) sites by PolX underlies a novel mechanism that promotes adaptive mutagenesis in Bacillus subtilis. J Bacteriol. 2014; 196:3012–3022. https://doi.org/10.1128/JB.00168-14 PMID: 24914186

12. Ross C, Pybus C, Pedraza-Reyes M, Sung HM, Yabin RE, Robleto E. Novel role of mdt effects on stationary-phase mutagenesis in Bacillus subtilis. J Bacteriol. 2006; 188:7512–7520. https://doi.org/10.1128/JB.00980-06 PMID: 16950921

13. Pybus C, Pedraza-Reyes M, Ross CA, Martin H, Ona K, Yabin RE, et al. Transcription-associated mutation in Bacillus subtilis cells under stress. J Bacteriol. 2010; 192:3321–3328. https://doi.org/10.1128/JB.00354-10 PMID: 20435731

14. Gómez-Marroquin M, Vidales LE, Debora BN, Obregón-Herrera A, Robleto EA, Pedraza-Reyes M. Role of Bacillus subtilis DNA glycosylase MutM in counteracting oxidative-induced DNA damage and in stationary-phase-associated mutagenesis. J Bacteriol. 2015; 197:1963–1971. https://doi.org/10.1128/JB.00147-15 PMID: 25825434

15. Millon-Weaver S, Samadpour NA, Moreno-Hable DA, Nugen P, Brinacher MJ, Weiss E, et al. An underlying mechanism for the increased mutagenesis of lagging-strand genes in Bacillus subtilis. Proc Natl Acad Sci USA. 2015; 112:E1096–E1105. https://doi.org/10.1073/pnas.1416651112 PMID: 25713353

16. Cohen SE, Walker GC. The transcription elongation factor NusA is required for stress-induced mutagenesis in Escherichia coli. Curr Biol. 2010; 20:80–85. https://doi.org/10.1016/j.cub.2009.11.039 PMID: 20036541

17. Ishikawa S, Oshima T, Kurokawa K, Kusuya Y, Ogasawara N. RNA polymerase trafficking in Bacillus subtilis cells. J Bacteriol. 2010; 192: 5768–5787. https://doi.org/10.1128/JB.00489-10 PMID: 20817769

18. Kusuya Y, Kurokawa IK, Ishikawa S, Ogasawara N, Oshima T. Transcription factor GreA contributes to resolving promoter-proximal pausing of RNA polymerase in Bacillus subtilis cells. J Bacteriol. 2011; 193: 3090–3099. https://doi.org/10.1128/JB.00866-11 PMID: 21515770

19. Behe MJ. Experimental evolution, loss-of-function mutations, and “The first rule of adaptive evolution”. Q Rev Biol. 2010; 85: 419–445. PMID: 21243963

20. Wilson MC, Farmer JL, Rothman F. Thymidylate synthesis and aminopterin resistance in Bacillus subtilis. J Bacteriol. 1966; 92:186–196. PMID: 4957432

21. Quinlivan EP, McPartlin J, Weir DG, Scott J. Mechanism of the antimicrobial drug trimethoprim revisited. FASEB J. 2000; 14:2519–2524. https://doi.org/10.1096/fj.99-1037com PMID: 11099470

22. Hawser S, Lociuro S, Islam K. Dihydropolate reductase inhibitors as antibacterial agents. Biochem Pharma. 2006; 71:941–948.

23. Fox KM, Maley F, Garibian A, Changchien LM, Van Roey P. Crystal structure of thymidylate synthase A from Bacillus subtilis. Protein Sci. 1999; 8:538–544. https://doi.org/10.1110/ps.8.3.538 PMID: 10091656

24. Neuhard J, Price AR, Schack L, Thomassen E. Two thymidylate synthetases in Bacillus subtilis. Pro Natl Acad Sci USA. 1978; 75:1194–1198.

25. Yabin RE, Fields PI, Andersen BJ. Properties of Bacillus subtilis 168 derivatives freed of their natural prophages. Gene. 1980; 12:155–159. PMID: 6783474

26. Boylan RJ, Mendelson NH, Brooks D, Young FE. Regulation of the bacterial cell wall: analysis of a mutant of Bacillus subtilis defective in biosynthesis of teichoic acid. J Bacteriol. 1972; 110:281–290. PMID: 4629900
27. Cutting SM, Vander Horn PB. Genetic analysis. In Harwood CR, Cutting SM (ed), Molecular biological methods for Bacillus. John Wiley & Sons, Sussex, England; 1990. pp.27–24.
28. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y; 1989.
29. Itaya M, Kondo K, Tanaka T. A neomycin resistance gene cassette selectable in a single copy state in the Bacillus subtilis chromosome. Nucleic Acids Res. 1989; 17:4410. PMID: 2500645
30. Vagner V, Dervyn E, Ehrlich SD. A vector for systematic gene inactivation in Bacillus subtilis. Microbiology. 1998; 144:3097–3104. https://doi.org/10.1099/00221287-144-11-3097 PMID: 9846745
31. Spizizen J. Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonuclease. Proc Natl Acad Sci USA. 1958; 44:1072–1078. PMID: 16590310
32. Ollington JF, Haldenwang WG, Huynh TV, Losick R. Developmentally regulated transcription in a cloned segment of the Bacillus subtilis chromosome. J Bacteriol. 1981; 147:432–442. PMID: 6790515
33. Fukushima T, Ishikawa S, Yamamoto H, Ogasawara N, Sekiguchi J. Transcriptional, functional and cytochemical analyses of the veg gene in Bacillus subtilis. J Biochem. 2003; 133:475–483. PMID: 12761285
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2\(^{-\Delta\Delta CT}\) method. Methods. 2001; 2:402–408.
35. Okada T, Yanagisawa K, Ryan FJ. Elective production of thymine-less mutants. Nature. 1960; 188:340–341. PMID: 13730568
36. Okada T, Yanagisawa K, Sonohara M. Improved method for obtaining thymineless mutants of Escherichia coli and Salmonella typhimurium. J Bacteriol. 1962; 84:602–603. PMID: 13939760
37. Farmer JL, Rothman F. Transformable thymine-requiring mutant of Bacillus subtilis. J Bacteriol. 1965; 89:262–263. PMID: 14255676
38. Castellanos-Juárez FX, Álvarez-Alvarez C, Yasbin RE, Setlow B, Setlow P, Pedraza-Reyes M. YtkD and MutT protect vegetative cells but not spores of Bacillus subtilis from oxidative stress. J Bacteriol. 2006; 188:2285–2289. https://doi.org/10.1128/JB.188.6.2285-2289.2006 PMID: 16513759
39. Sankar ST, Wastuwidyantygas BD, Dong Y, Lewis SA, Wang JD. The nature of mutations induced by replication-transcription collisions. Nature. 2016; 535:178–181. https://doi.org/10.1038/nature18316 PMID: 27362223
40. Michaels ML, Kim CW, Matthews DA, Miller JH. Escherichia coli thymidylate synthase: Amino acid substitutions by suppression of amber nonsense mutations. Proc Natl Acad Sci USA. 1990; 87:3957–3961. PMID: 2187197
41. Ambriz-Aviña Y, Yasbin RE, Robleto EA, Pedraza-Reyes M. Role of base excision repair (BER) in transcription-associated mutagenesis of nutritionally stressed non-growing Bacillus subtilis cell subpopulations. Curr Microbiol. 2016; 73:721–726. https://doi.org/10.1007/s00284-016-1122-9 PMID: 27530626
42. Kamarathapu V, Nudler E. Rethinking transcription coupled DNA repair. Curr Opin Microbiol. 2015; 24:15–20. https://doi.org/10.1016/j.mib.2014.12.005 PMID: 25596348