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Role of Base Excision Repair (BER) in Transcription-associated Mutagenesis of Nutritionally Stressed Nongrowing *Bacillus subtilis* Cell Subpopulations

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Abstract Compelling evidence points to transcriptional processes as important factors contributing to stationary-phase associated mutagenesis. However, it has not been documented whether or not base excision repair mechanisms play a role in modulating mutagenesis under conditions of transcriptional derepression. Here, we report on a flow cytometry-based methodology that employs a fluorescent reporter system to measure at single-cell level, the occurrence of transcription-associated mutations in nutritionally stressed *B. subtilis* cultures. Using this approach, we demonstrate that (i) high levels of transcription correlates with augmented mutation frequency, and (ii) mutation frequency is enhanced in nongrowing population cells deficient for deaminated (Ung, YwqL) and oxidized guanine (GO) excision repair, strongly suggesting that accumulation of spontaneous DNA lesions enhance transcription-associated mutagenesis.

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Introduction

Transcription has been proposed to be the link between physiology and mutation occurrence [7, 14, 19]. Two mechanisms have been proposed to explain why transcription increases mutation rates. First, the nontranscribed strand is unprotected in the transcription bubble and is thus significantly more vulnerable to mutations than the transcribed strand. Second, transcription drives localized supercoiling which facilitates secondary structures formation, thus favoring lesions on exposed bases [23, 25]. In nonreplicating *E. coli* cells, genome instability propitiated by DNA/RNA hybrids and nicks, and triggers Mfd-dependent mutations and amplifications [24]. A mutagenic process dependent on Mfd, excision repair, and translation synthesis that prevents conflicts between transcription and replication have been recently described in growing *B. subtilis* cells [13]. The occurrence of stationary-phase-associated mutations (SPM) in the Gram-positive bacteria, *B. subtilis* has been previously demonstrated [18, 20]. In addition to suppressed or reduced DNA repair, error-prone repair of lesions has been proposed to contribute to the generation of mutations in nondividing *B. subtilis* cells [3, 5, 8, 21]. Experimental evidences in mouse embryonic fibroblasts (MEF) and *Saccharomyces cerevisiae* have revealed that BER deficiencies promote transcription-associated mutations (TAM) [7, 14]. However, the role of spontaneous lesions, including deaminated and oxidized bases in modulating this process in *B. subtilis,* is currently unknown. Results have demonstrated that in a bacterial culture, there is cell heterogeneity [6, 16], and that the batch methods employed for physiologic characterization are limited, since the contribution of small populations could be overlooked [2, 9]. Since mutation rates are low, even in the stressful starving conditions of stationary phase,

a method of single-cell analysis such as flow cytometry (FCM) may be better suited for measuring mutations with the appropriate reporter system. Here, we describe the use of *gfp* as a reporter gene to evaluate at the single-cell level transcription-associated mutation events in nondividing stationary-phase *B. subtilis* cells. Nonfunctional point mutant alleles of the *gfp* gene were combined with FCM analysis of cells displaying gain-of-function phenotypes to show that a positive correlation between transcriptional derepression and the mutation frequency was enhanced in nongrowing *B. subtilis* cells deficient in base excision DNA repair.

Materials and Methods

Bacterial strains and growth conditions. Strains used are listed in Table S1 and were grown in antibiotic medium no.3 (A3) (Difco Laboratories, Sparks, MD) supplemented with 1× HoLe trace elements [20].

Reporter System Construction for Analysis of *gfp* Transcription Levels

The open reading frame (ORF) of the *gfpmut3a* allele from pAD123 [4] amplified by PCR was cloned between the NheI and SphI sites of plasmid pMPR025 [11]. This construct contained the *s-box-gfp*, where *gfp* expression is under positive transcriptional control with an IPTG-inducible hyperspank promoter (Phs) and under negative transcriptional control, by methionine attenuation through an *s-box* riboswitch (s-box) [12]. The *s-box-gfp* (pPERM1082) construct and an empty *s-box* (pMPR025) were independently recombined into the *amyE* locus to obtain the reporter *amyE*::*sbox-gfp* (PERM1087) and control *amyE*::*s-box* (PERM1114) strains (Fig. 1). Two-point-mutated *gfp* alleles encoding nonfunctional Gfp proteins were designed as follows. In the first one (gfp_{ns}), the third base of the 57th TGG codon was replaced for adenine, resulting in a TGA stop codon. For the second one, the 66th tyrosine codon, essential for Gfp fluorescence was mutated to a histidine codon, generating a full-sized nonfluorescent product, named $gfp_{Tyr–His}$. The *gfpmut3a* gene was used as a template for introducing the gfp_{ns} and $gfp_{Tyr–His}$ mutations using a set of mutagenic oligonucleotide primers and the QuickChange II system (Agilent Technologies; Santa Clara, CA). Once corroborated by DNA sequencing, the nonfunctional phenotype of the point-mutated alleles was determined by fluorescence microscopy with cells expressing the nonsense or missense *gfp* alleles from the IPTG-inducible Hyperspank promoter (Results not shown).

Evaluation of Transcription-associated Mutations

Strains were propagated in A3 medium at 37 °C with vigorous aeration; cell samples collected at an OD_{600nm} of 1 or 90 min after cessation of exponential growth were washed and resuspended in Spizizen Minimal Medium (SMM) [15] supplemented or not with 0.1 mM IPTG or methionine (50 μg mL^{-1}). After 2 h of incubation at 37 °C, the cells were collected and resuspended in 0.2-μm-filtered phosphate buffered saline (PBS) for further analysis.

Microscopy and Flow Cytometry Analysis

Suspensions of cells of interest were processed for epifluorescence microscopy analysis as it was previously described [17]. Alternatively, bacterial suspensions were analyzed in a Flow cytometer MoFlo XDP (Beckman Coulter, Brea, CA, USA), equipped with a 488 nm laser for excitation. Bacterial cells stained with hexidium iodide (10 μg mL^{-1}), washed, and resuspended in PBS were used for adjusting appropriate settings to discriminate debris and electronic noise. Control strains bearing an empty *s-box* construct were used for adjusting appropriate voltage settings in the FL1 filter (515/510 nm BP filter) in logarithmic scale, to establish the negative population, and for determination of background fluorescence. To discriminate singlet events, the events located in a lineal region in a dot-plot Forward Scatter (FS) logA vs FS logH were

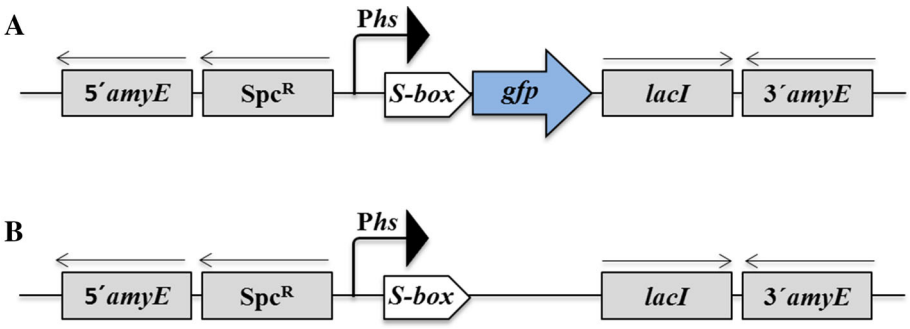

Fig. 1 Diagram of the reporter (**a**) and control (**b**) constructs integrated in the *amyE* locus. *Arrows* above the *boxes* indicate the orientation of the genes

considered. At least, 3 million of singlet's were acquired for *gain-of Gfp* function analysis.

Statistical Analysis

Where indicated, values presented in plots are averages from three triplicate experiments, with variations between experiments of $\leq$10 %. A nonparametric Mann–Whitney test was carried out without adjusting for ties. Minitab Express (Minitab Inc., State College, PA, USA) for Mac and Statistica 7 for Windows (StatSoft, Inc., Tulsa, OK) softwares were employed. Differences with P values $\leq$0.05 were considered statistically significant.

Results

Reporter System to Analyze TAM at a Single-cell Level

The reporter *amyE*::*sbox-gfp* strains (Fig. 1) cultured with 0.1 mM IPTG but lacking methionine and analyzed by microscopy, exhibited a high level of fluorescence (Fig. 2c). However, the presence of methionine in the medium induced a dramatic decrease in the Gfp-associated fluorescence (Fig. 2b), close to the level of the auto-fluorescence that is naturally exhibited by *B. subtilis* cells, carrying only the empty construction (Fig. 2a, e). Spectrofluorometric quantitation showed that under induction conditions, in three triplicate independent experiments, the fluorescent intensity of the cell bearing the *s-box-gfp* construct increased $\sim$10 times with respect to the attenuated condition (i.e., 52 ± 4.5 vs. 5.1 ± 0.8, respectively). Moreover, as shown in Fig. 2c, f, the trace amount of tryptophan added to limit growth did not affect the synthesis of Gfp.

The results shown in Fig. 2 analyzed by FCM to quantitate the cell population exhibiting a Gfp-associated fluorescence phenotype allowed us to establish two levels of *gfp* transcription, (i) attenuation, when methionine was supplemented in the medium (Fig. 2e), and (ii) induction, when cultures were supplemented with IPTG and lacked methionine (Fig. 2f). Therefore, these levels of control for expression of the *gfp* reporter allowed us to inquire whether levels of transcription influences mutagenesis in non-growing bacterial populations (SPM), and whether base excision repair pathways play a role in this cellular process.

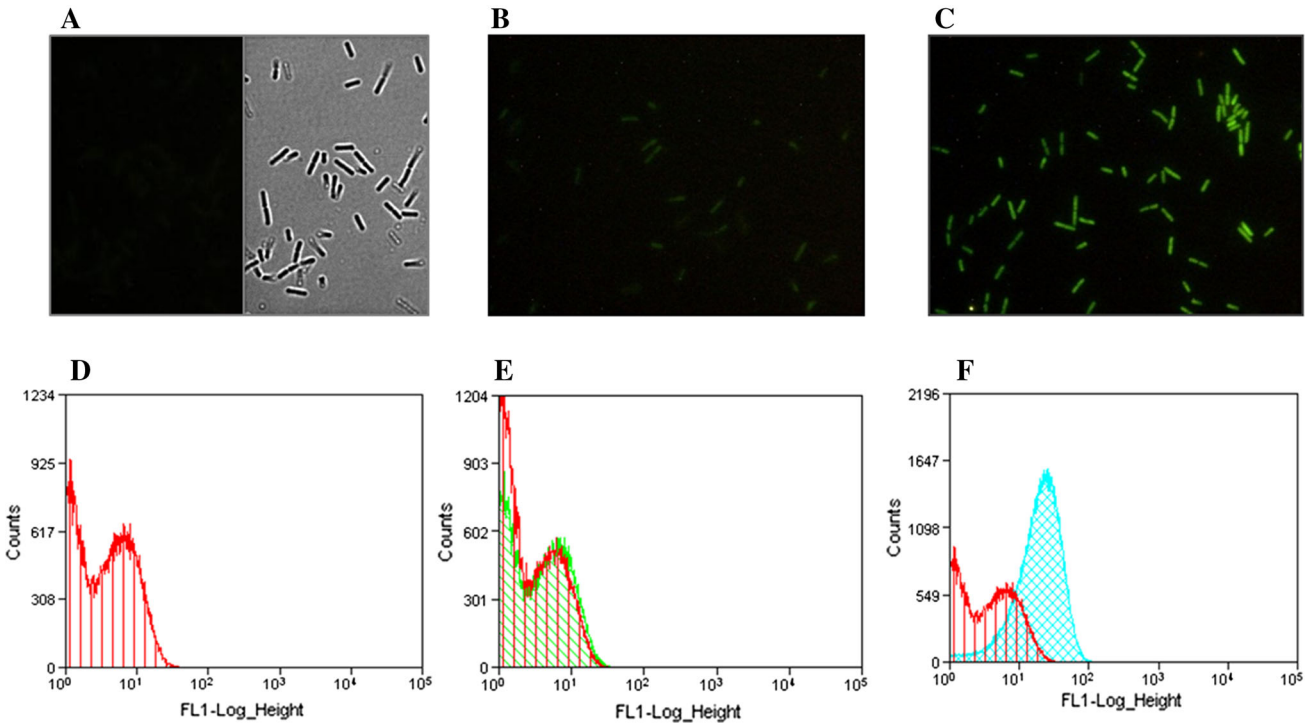

Fig. 2 Control of *gfp* expression by IPTG induction and methionine attenuation. The control (*amyE*::*s-box*) (**a**, **d**) and reporter (*amyE*::*s-box-gfp*) (**b**, **c**, **e**, and **f**) strains were grown to exponential growth collected and incubated under conditions of attenuation (**b**, **e**) or induction (**c**, **f**) and analyzed by epifluorescence microscopy (**a**–**c**) and by flow cytometry (**d**–**f**). In **a** a bright-field image of cells of the control strain is also shown. (**d**–**e**): histogram of control strain in induction conditions (**d**), and overlay of reporter strains with the control strain in attenuation (**e**; *green histogram*) and induction (**f**; *cyan histogram*) conditions, control strain histograms shown in red. A total amount of 2500,000 singlet events were analyzed. In **d**–**f**, *Y*-axis = number of events; *X*-axis = fluorescent intensity detected in FL1 as log height (510–520 nm)

FCM Analysis of TAM by Gain of Gfp Function

To determine whether transcriptional levels have an impact in the population of cells that acquired a fluorescent phenotype, stationary-phase cells carrying the gfp_{ns} and $gfp_{Tyr\text{-}His}$ point-mutated constructs were analyzed by FCM. A positive correlation between transcriptional induction and the number of fluorescent cells was found in cells of the parental strain carrying both mutant alleles (Fig. 3). However, it was evident that transcriptional derepression generated a higher amount of fluorescent cells in the *s-box-*gfp_{ns} strain than in the strain bearing the *s-box-*$gfp_{Tyr–His}$ construction (Fig. 3 and Table 1).

Role of Base Excision Repair in Promoting TAM

FCM analysis was conducted on stationary-phase cultures of strains deficient for deaminated (Δ*ung ywqL*) [10] or guanine oxidized (ΔGO) [22] systems in genetic backgrounds that contained the *s-box-*gfp_{ns} or the *s-box-*$gfp_{Tyr\text{-}His}$ constructs, respectively. As shown in Fig. 3a, in cells that overexpressed the $gfp_{Tyr\text{-}His}$ allele, the absence of base deamination repair proteins (Ung, YwqL) significantly increased the fluorescent population in comparison with cells proficient for these repair proteins. Remarkably, the FCM analysis conducted under derepression of the nonsense *gfp* mutant allele identified a significantly higher proportion of cells with a fluorescent phenotype in the strains lacking the deaminated and guanine oxidized repair systems (Fig. 3b).

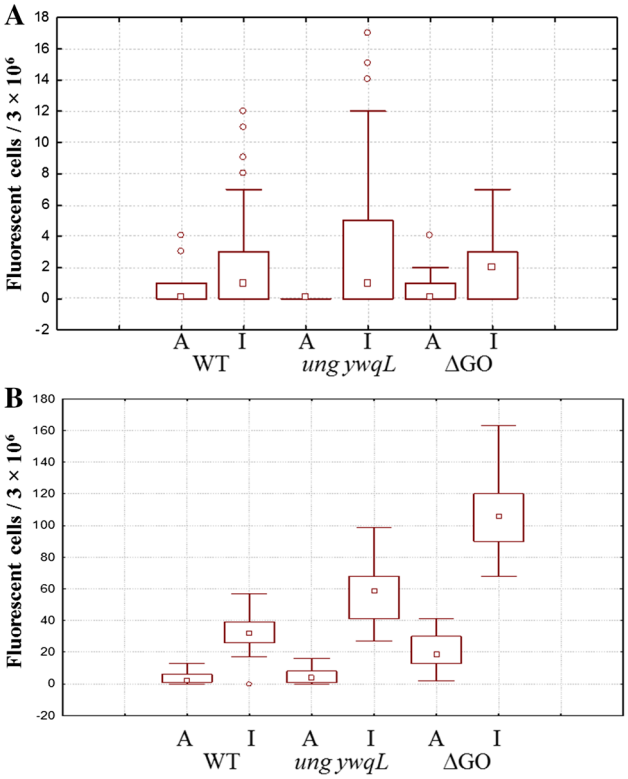

Fig. 3 Analysis of gain-of-function of mutant *gfp* alleles by FCM. Cells carrying $gfp_{Tyr–His}$ missense (**a**) and gfp_{ns} nonsense (**b**) and the respective control cells were recovered from stationary phase cultures and incubated under conditions of attenuation (*A*) or induction (*I*). 3×10^6 cells were analyzed by FCM as described above. *Box* and *Whisker* plot, representing the number of fluorescent cells of 3×10^6 of singlet events analyzed. The *box* represents the 25–75 % interquartile range, the *whiskers* represent the nonoutlier range, the squares represent the median and circles represent the outliers' values

Stationary-phase Subpopulations with a Gain-of-*gfp*-function Phenotype also Exhibited a Hyper-mutagenic Rif^r Phenotype

It has been proposed that adaptive mutations may arise in a hyper-mutagenic subpopulation of nutritionally stressed nongrowing, bacterial cells [8, 18, 20]. To provide support for this hypothesis, a stationary-phase IPTG-induced culture of the strain *B. subtilis* P*hs-sbox-*gfp_{ns} was analyzed by FCM to identify the subpopulation of cells that gained a fluorescent phenotype. The mutation frequency to Rif^r of

Table 1 Stationary-phase mutation frequencies determined by gain-of *gfp* function

Genetic background	Transcription condition	Mutation frequency with $gfp_{Tyr\text{-}His}$ allele	Mutation frequency with gfp_{ns} allele
168	Attenuated	$<3.3 \times 10^{-7}$	6.67×10^{-7}
	Induced	3.3×10^{-7}	103.3×10^{-7}
ung, ywqL	Attenuated	$<3.3 \times 10^{-7}$	13.3×10^{-7}
	Induced	6.7×10^{-7}	196.7×10^{-7}
GO	Attenuated	$<3.3 \times 10^{-7}$	63.3×10^{-7}
	Induced	3.3×10^{-7}	353.3×10^{-7}

Stationary-phase mutation frequencies with point mutated defective *gfp* alleles represent the Median number of fluorescent cells/Total number of singlet cells analyzed

(Number of fluorescent cells was determined by Overton's subtraction and the total number of cells analyzed was 3×10^6)

When the median value of fluorescent cells in 3 million resulted 0, the mutation rate was expressed as $<3.33 \times 10^{-7}$

Table 2 Mutation frequencies (Rif^r) in sorted *B. subtilis* stationary-phase subpopulations exhibiting or not a gain-of Gfp function phenotype

	Cell subpopulation (Phenotype)			
	Nonfluorescent (Gfp^-)		Fluorescent (Gfp^+)	
	Exp1	Exp2	Exp1	Exp2
Mutation frequency (Rif^r colonies $\times 10^{-6}$)[a]	41	66	123.8	176

Fluorescent and nonfluorescent cell subpopulations from an induced stationary-phase culture of strain *B. subtilis Phs-sbox-gfp$_{ns}$* were independently sorted and plated on solid LB supplemented with Rif

[a] The number of Rif^r colonies among the total number of sorted cells was used to calculate the mutation frequency to Rif^r. These experiments were repeated at least two times

cells sorted from this subpopulation and of those separated from a nonfluorescent region was determined as detailed above. The results shown in Table 2 revealed that the mutation frequencies levels to Rif^r, in both subpopulations, were similar to those obtained with the gain-of function nonsense *gfp* allele (Table 1). However, the fluorescent cell subpopulation was ~3 times more mutagenic than the subpopulation of nonfluorescent cells (Table 2).

Discussion

Here, we described a FCM-based approach to study TAM that offers the possibility of directly detecting in bacterial cultures, at single-cell level, the population of cells that gained or lost a fluorescent phenotype owing to mutational events occurring in a *gfp* reporter gene. After corroborating by fluorescence microscopy, the nonfunctional phenotype of the point mutated *gfp* alleles employed we focus our analysis on those mutations occurring in nutritionally stressed nongrowing bacteria using tryptophan auxotrophic isogenic strains carrying a defective *trpC2* allele [1]. Accordingly, viable counts of the strains collected from stationary-phase cultures and starved for tryptophan did not significantly change during a period of 2 h (Results not shown). Overall with the gain-of-function system implemented, it was found that although transcriptional derepression promoted mutations that increased the number of cells with a functional Gfp phenotype, such events were more frequent in stationary-phase cultures of the WT strain bearing the *gfp$_{ns}$* allele. As shown in Table 1, under conditions of derepression, the mutation frequencies in this allele were approximately 31-fold higher than those produced by the missense alleles. Thus, due to the nonessential character of the Trp_{57} residue, it is not unreasonable to speculate that a broad range of mutagenic events can revert the *gfp* mutation in this stop codon.

Previous reports have suggested that in nongrowing *B. subtilis* cells, the accumulation of spontaneous genetic lesions saturate the repair capacity of DNA repair systems promoting transcriptional-associated mutagenic events [19, 20]. Our results supported this suggestion as the genetic inactivation of the base deamination [10] and guanine oxidized excision repair pathways [22] enhanced the population of fluorescent cells in stationary-phase cultures that overexpressed the missense and nonsense *gfp* alleles. However, compared with the reversion frequency of the missense allele, under conditions of derepression, the mutation frequencies in the nonsense allele were approximately 60-fold for the *ung ywqL* mutant and 107-fold for the GO deficient strain (Table 1). Taken collectively, these results strongly suggest that deamination and oxidation events taking place in the TGA codon 57 promote true reversions, as well as compensatory amino acid changes in this *gfp$_{ns}$* allele. Importantly, under growth-limiting conditions processing of damaged DNA bases can proceed in an error-prone manner promoting adaptive mutagenesis in *B. subtilis* [3, 5, 10, 22]. Furthermore, components of the BER pathway, including the 8-oxoguanine glycosylase (Ogg1) from MEF, the GO system and Mfd have been involved in modulating TAM in eukaryotes and bacteria [5, 11, 14, 15, 24].

We employed the *rpoB* gene as a different target to determine mutagenesis in sorted stationary-phase cell subpopulations from derepressed cultures of *B. subtilis* carrying the nonsense *gfp* allele. The mutation frequencies to Rif^r obtained (Table 2) not only paralleled those obtained with the gain-of-function nonsense *gfp* allele (Table 1) but also were in agreement with SPM reversion levels of the *hisC952*, *metB5,* and *leuC427* alleles reported in strain *B. subtilis* YB955 [5, 20, 22]. Interestingly in reference to a sorted cell subpopulation that did not acquired a Gfp functional phenotype, the Rif^r mutagenesis levels were three times higher in the Gfp^+ cell subpopulation (Table 2). These results, together with the increased mutagenesis levels exhibited by *B. subtilis* cell populations deficient for BER (Table 1), support the concepts that (i) adaptive mutations arise in nutritionally stressed growth-limited cell subpopulations with suppressed or saturated DNA repair systems and (ii) that such type of mutations are enhanced by transcriptional derepression. Finally, it must be pointed that although TAM is not limited to cells with a limited growth [13, 19], results from a FCM analysis measuring the loss of function of a wild-type *gfp* gene revealed that a minor proportion of growing cells lost the fluorescent phenotype in comparison with cells collected from nongrowing stationary-phase cultures

suggesting that TAM are most frequent in nutritionally stressed cells (Data not shown).

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Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflict of interest.

References

1. Albertini AM, Galizzi A (1999) The sequence of the Trp operon of *Bacillus subtilis* 168 (*trp*C2) revisited. Microbiology 145:3319–3320
2. Ambriz-Aviña V, Contreras-Garduño JA, Pedraza-Reyes M (2014) Applications of flow cytometry to characterize bacterial physiological responses. Biomed Res Int. doi:10.1155/2014/461941
3. Barajas-Ornelas RC, Ramírez-Guadiana FH, Juárez-Godínez R, Ayala-García VM, Robleto EA, Yasbin RE, Pedraza-Reyes M (2014) Error-prone processing of apurinic/apyrimidinic (AP) sites by PolX underlies a novel mechanism that promotes adaptive mutagenesis in *Bacillus subtilis*. J Bacteriol 196:3012–3022
4. Cormack BP, Valdivia RH, Falkow S (1996) Facs-optimized mutants of the green fluorescent protein (Gfp). Gene 173:33–38
5. Debora BN, Vidales LE, Ramírez R, Ramírez M, Robleto EA, Yasbin RE, Pedraza-Reyes M (2011) Mismatch repair modulation of MutY activity drives *Bacillus subtilis* stationary-phase mutagenesis. J Bacteriol 193:236–245
6. Fraser D, Kaern M (2009) A chance at survival: gene expression noise and phenotypic diversification strategies. Mol Microbiol 71:1333–1340
7. Jinks-Robertson S, Bhagwat AS (2014) Transcription-associated mutagenesis. Annu Rev Genet 48:341–359
8. Kivisaar M (2003) Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. Environ Microbiol 5:814–827
9. Lidstrom ME, Konopka MC (2010) The role of physiological heterogeneity in microbial population behavior. Nat Chem Biol 6:705–712
10. López-Olmos K, Hernández MP, Contreras-Garduño JA, Robleto EA, Setlow P, Yasbin RE, Pedraza-Reyes M (2012) Roles of endonuclease V, uracil-DNA glycosylase, and mismatch repair in *Bacillus subtilis* DNA base-deamination-induced mutagenesis. J Bacteriol 194:243–252
11. Martin HA, Pedraza-Reyes M, Yasbin RE, Robleto EA (2011) Transcriptional de-repression and *mfd* are mutagenic in stressed *Bacillus subtilis* cells. J Mol Microbiol Biotechnol 21:45–58
12. McDaniel BA, Grundy FJ, Artsimovitch I, Henkin TM (2003) Transcription termination control of the s box system: direct measurement of s-adenosylmethionine by the leader RNA. Proc Natl Acad Sci USA 100:3083–3088
13. Millon-Weaver S, Samadpour NA, Moreno-Hable DA, Nugen P, Briinacher MJ, Weiss E, Hayden HS, Miller SI, Liachko I, Merrikh H (2015) An underlying mechanism for the increased mutagenesis of lagging-strand genes in *Bacillus subtilis*. Proc Natl Acad Sci USA 112:E1096–E1105
14. Morreall JF, Petrova L, Doetsch PW (2013) Transcriptional mutagenesis and its potential roles in the etiology of cancer and bacterial antibiotic resistance. J Cell Physiol 228:2257–2261
15. Pybus C, Pedraza-Reyes M, Ross CA, Martin H, Ona K, Yasbin RE, Robleto E (2010) Transcription-associated mutation in *Bacillus subtilis* cells under stress. J Bacteriol 192:3321–3328
16. Rainey PB, Beaumont HJ, Ferguson GC, Gallie J, Kost C, Libby E, Zhang XX (2011) The evolutionary emergence of stochastic phenotype switching in bacteria. Microb Cell Fact 10(Suppl 1):S14
17. Ramírez-Guadiana FH, Barajas-Ornelas RC, Ayala-García VM, Yasbin RE, Robleto E, Pedraza-Reyes M (2013) Transcriptional coupling of DNA repair in sporulating *Bacillus subtilis* cells. Mol Microbiol 90:1088–1099
18. Robleto EA, Yasbin R, Ross C, Pedraza-Reyes M (2007) Stationary Phase Mutagenesis in *B. subtilis:* a paradigm to study genetic diversity programs in cells under stress. Crit Rev Biochem Mol Biol 42:327–339
19. Robleto E, Martin H, Vallin C, Pedraza-Reyes M, Yasbin R (2012) Transcription-mediated mutagenic processes. In: Mittelman D (ed) Stress induced mutagenesis. Springer Verlag, New York. p 41-58, ISBN 978-1-4614-6280-4
20. Sung HM, Yasbin RE (2002) Adaptive, or stationary-phase, mutagenesis, a component of bacterial differentiation in *Bacillus subtilis*. J Bacteriol 184:5641–5653
21. Sung HM, Yeamans G, Ross CA, Yasbin RE (2003) Roles of yqjH and yqjW, homologs of the *Escherichia coli* umuC/dinB or Y superfamily of DNA polymerases, in stationary-phase mutagenesis and UV-induced mutagenesis of *Bacillus subtilis*. J Bacteriol 185:2153–2160
22. Vidales LE, Cárdenas Ll C, Robleto E, Yasbin RE, Pedraza-Reyes M (2009) Defects in the error prevention oxidized guanine system potentiate stationary-phase mutagenesis in *Bacillus subtilis*. J Bacteriol 191:506–513
23. Williams AB, Foster PL (2012) Stress-induced mutagenesis. Ecosal Plus 5(1)
24. Wimberly H, Shee C, Thornton PC, Sivaramakrishnan P, Rosenberg SM, Hastings PJ (2013) R-loops and nicks initiate DNA breakage and genome instability in non-growing *Escherichia coli*. Nat Commun 4:2115. doi:10.1038/ncomm53115
25. Wright BE (2000) A biochemical mechanism for nonrandom mutations and evolution. J Bacteriol 182:2993–3001