Transcriptional De-Repression and Mfd Are Mutagenic in Stressed *Bacillus subtilis* Cells

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Key Words

Stationary-phase mutagenesis • Stress-induced mutagenesis • *Bacillus subtilis*

Abstract

In recent years, it has been proposed that conflicts between transcription and active chromosomal replication engender genome instability events. Furthermore, transcription elongation factors have been reported to prevent conflicts between transcription and replication and avoid genome instability. Here, we examined transcriptional de-repression as a genetic diversity-producing agent and showed, through the use of physiological and genetic means, that transcriptional de-repression of a *leuC* defective allele leads to accumulation of Leu⁺ mutations. We also showed, by using riboswitches that activate transcription in conditions of tyrosine or methionine starvation, that the effect of transcriptional de-repression of the *leuC* construct on the accumulation of Leu⁺ mutations was independent of selection. We examined the role of Mfd, a transcription elongation factor involved in DNA repair, in this process and showed that proficiency of this factor promotes mutagenic events. These results are in stark contrast to previous reports in *Escherichia coli*, which showed that Mfd prevents replication fork collapses. Be-

cause our assays place cells under non-growing conditions, by starving them for two amino acids, we surmised that the Mfd mutagenic process associated with transcriptional de-repression does not result from conflicts with chromosomal replication. These results raise the interesting concept that transcription elongation factors may serve two functions in cells. In growing conditions, these factors prevent the generation of mutations, while in stress or non-growing conditions they mediate the production of genetic diversity.

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Introduction

Our laboratory examines mechanisms that produce genetic diversity in cells under stress or non-dividing conditions. This phenomenon is known as stationary-phase mutagenesis, stress-induced mutagenesis and adaptive mutagenesis. These types of mechanisms are important because they provide novel views in the evolutionary process and are implicated in the formation of mutations that affect all organisms. In single-cell species, these mechanisms may generate changes that confer antibiotic resistance and metabolic expansion [Han et al., 2008; Petrosino et al., 2009; Wright, 2000]. However, in

multi-cellular species, these genetic programs may cause beneficial changes, such as antibody diversity, as well as detrimental ones, such as genome instability and unorganized growth [Hastings, 2007; Wright et al., 2004].

In the *Escherichia coli* plasmid-borne FC40 *lacI* system [Cairns and Foster, 1991], stress-induced mutagenesis is promoted by activation of regulons that respond to distinct environmental insults. In addition to the SOS response (activated by DNA damage and disruption of replication) [McKenzie et al., 2001], studies have shown that the generation of adaptive mutations is dependent on the general stress response [Lombardo et al., 2004] and, most recently, the envelope-stress response [Gibson et al., 2010]. Key factors in the generation of point-mutated Lac⁺ revertants are recombination functions and DinB, an error-prone DNA polymerase in the Y-superfamily of proteins [Galhardo et al., 2009; McKenzie et al., 2001]. This phenomenon is further facilitated in stressed cells by deficiencies in general repair systems such as the methyl-directed mismatch repair system [Feng et al., 1996; Harris et al., 1997]. A model has been postulated to explain how cells hypermutate in times of stress in which double-stranded breaks in DNA, occurring in a subpopulation of cells, are repaired via recruitment of DinB and recombination functions [reviewed in Galhardo et al., 2007]. The genetic variants produced are then subject to selective processes that permit cells to escape stress, carbon source starvation in this case. This model is supported by recent in vivo reports that show competition amongst DNA polymerases at sites where acts of repair of double-stranded breaks take place [Hastings et al., 2010]. Another stress-induced process that generates Lac⁺ variants via amplification of the *lac*⁺ allele marker has also been proposed [Hastings et al., 2004; reviewed in Hastings, 2007]. The generation of amplifications is DinB independent, but requires the generation of double-stranded breaks, the activation of the stress regulon and the activity of PolI (the Okazaki fragment polymerase). The Lac⁺ variants produced by these two processes differ in stability as measured by subsequent growth under nonselective conditions. One other model, based on growth-dependent amplification, which gives rise to Lac⁺ variants has also been described [for a review, see Roth et al., 2006].

Evidence has also been presented that demonstrated the influence of transcriptional de-repression on the generation of stress-induced mutations [Robleto et al., 2007; Wright, 2000]. The process of transcription has been amply shown, since the 1970s, to increase the rate of spontaneous mutations in growing cells [Brock, 1971; Davis, 1989; Fix et al., 2008; Hendriks et al., 2008; Herman and

Dworkin, 1971; Hudson et al., 2003]. This concept has also been examined in the context of conflicts between transcription and replication [Hendriks et al., 2010; Kim et al., 2007; Srivatsan et al., 2010] or as affected by the stringent response [Reimers et al., 2004; Rudner et al., 1999; Wright, 1996; Wright and Minnick, 1997]. Observations from those studies suggest that encounters between replication and transcription may cause genome instability, particularly in the absence of transcription elongation factors mediating the removal of paused RNAP complexes [Pomerantz and O'Donnell, 2010; Teyranchi et al., 2010]. However, recent reports also indicate that transcription elongation factors are elements that promote mutagenesis. In the case of *Bacillus subtilis*, Mfd, also known as the transcription-repair coupling factor (TRCF), mediates the generation of base substitution mutations in three chromosomal markers [Ross et al., 2006]. Because the TRCF-dependent mutagenic process is biased towards the generation of true revertants rather than extragenic suppressors, it was speculated that this process was associated with transcription of the genes under selection. Subsequent studies showed that the Mfd effect was exclusive to stationary-phase cells and suggested that Mfd deficiency impairs transcription of genes under selection [Pybus et al., 2010].

Here, we examined further the concept of transcription-associated mutagenesis and the role the TRCF plays in this process. The mutagenic effects of transcriptional de-repression of a defective *leuC* allele were examined using cell physiology and genetics and resulted in three different levels of transcription. The levels of *leuC* transcript accumulation correlated with levels in accumulation of mutations at *leuC* and were dependent on TRCF. Because this phenomenon was exclusive to stationary-phase cells, these results led to speculation that TRCF prevents or potentiates the production of mutations. In growing conditions, TRCF preserves genome integrity, while in non-growing conditions this factor promotes the production of genetic diversity.

Results

This study examines the hypothesis that transcriptional de-repression in cells under conditions of stress increases the accumulation of mutations in genes under selection. Experiments placed cells under conditions of single or double amino acid starvation and measured the accumulation of reversions in a *leuC427* point-mutated allele prototrophy. These experiments are conducted in cells subject

Table 1. Fold increase in *leuC* expression as affected by transcriptional de-repression effectors

Strain	Repressed condition	De-repressed condition	<i>leuC</i> transcription fold increase
YB955	50 µg/ml isoleucine	200 ng/ml isoleucine	4
HAM202 (s-box'- <i>leuC427</i>)	50 µg/ml tyrosine	no tyrosine	13
HAM104 (t-box'- <i>leuC427</i>)	50 µg/ml methionine	no methionine	58

Values to estimate fold increase were calculated using the $2^{-\Delta\Delta C_t}$ method. The *veg* gene was used as a control.

to culture conditions in which the test allele is transcriptionally de-repressed or in genetically altered cells in which transcriptional de-repression of the test allele responds to unrelated starvation stimuli. The role of Mfd in transcription-associated mutagenesis is also tested.

Low Levels of Isoleucine and Slight Increases in Transcriptional De-Repression Correlate with Slight Increases in the Accumulation of Leu⁺ Reversions

Table 1 shows fold increases in transcription levels in different culture conditions. Results from RT-PCR of *leuC427* showed that in the presence of 0.0002 g/l of isoleucine (de-repressed), stationary-phase *B. subtilis* cells increased transcript levels 4-fold when compared to cells subjected to 0.050 g/l (repressed condition) of the same amino acid. Stationary-phase mutagenesis assays in cells exposed to the same conditions coincided with slight significant differences in the accumulation of leucine revertants between the cells under repressed and de-repressed conditions of transcription by the 10th day of incubation (fig. 1a). Cells subjected to growth-limiting levels of isoleucine reached significantly higher levels of Leu⁺ events by day 9. Cell viability did not change in the test conditions (fig. 1b). Although the differences in transcriptional de-repression are slight (4-fold), so were the differences in mutagenesis at day 9 of starvation; we cautiously interpreted these results as suggestive of a correlation between transcriptional de-repression and stationary-phase mutagenesis. These observations prompted examination of stationary mutagenesis of the *leuC* allele under conditions of high transcriptional de-repression.

High Transcriptional De-Repression Precedes High Accumulation of Leu⁺ Reversions

To effectively decouple transcription from selection, we constructed two versions of the *leuC427* allele that respond to either tyrosine or methionine starvation. This was accomplished by placing the *leuC427* allele under the

transcriptional control of riboswitches that respond to cellular concentrations of tRNA^{tyr} or s-adenosylmethionine [Grundy and Henkin, 2003]. To eliminate context effects of the *ilv-leu* promoter, we recombined these alleles into the chromosomal *amyE* location. With this genetic arrangement, which includes two defective *leuC427* alleles that are differently controlled for their transcription (normal regulation and riboswitch regulation), these experiments (a) further separate transcriptional de-repression from selection, (b) generate different levels of expression in *leuC427*, and (c) allow control of transcription by antitermination mechanisms.

Table 1 shows that in the absence of exogenous tyrosine or methionine, *leuC427* relative transcript levels were increased by either 13- or 58-fold, respectively, compared to conditions in which tyrosine or methionine were present in our stationary-phase assay. Cells subjected to these conditions were starved for leucine and one other amino acid (methionine for the experiments that used the t-box construct and histidine for the experiments that used the s-box), which negates any selective (growth) advantage conferred by the generation of Leu⁺ mutations, for different times. After different periods of incubation, cells were supplemented with a soft-agar overlay that contained methionine (t-box) or histidine (s-box) and examined for their ability to accumulate Leu⁺ revertants. Figures 2 and 3 summarize the accumulation of Leu⁺ revertions for the t-box and s-box strains and show a significant increase at day 10 of incubation. Interestingly, incubation times from 0 to 6 days of stress resulted in no significant differences in accumulation of mutations between strains or conditions. However, after 8 and 10 days of incubating cells in nutritional stress, transcriptional de-repression was directly correlated to the ability of cells to produce mutations; a 13-fold increase in transcriptional de-repression of the *leuC427* coincided with a 6-fold increase in Leu⁺ revertions, whereas conditions in which transcription of *leuC427* was increased 58-fold resulted in 12

Fig. 1. Stationary-phase mutagenesis in *B. subtilis* strain YB955 as affected by isoleucine media amendments. **a** Mean and standard deviations of accumulation of Leu⁺ revertants in Spizizen minimal media amended with 0.05 g/l of isoleucine (ile+) or none

(ile-). **b** Mean and standard errors of survival counts of non-revertant cells. Results are based on four replicates and are representative of at least three repetitions. See 'Experimental Procedures' for more details.

times as many revertants as in conditions in which *leuC427* was transcriptionally repressed (fig. 2a, 3a). The differences in the accumulation of revertants were not due to differences in cell viability (fig. 2b, 3b). We also measured mutations at neutral markers in the t-box and s-box strains, and found that there were no differences among strains or between conditions of repression or de-repression. The accumulation of Met⁺ and His⁺ for the t-box and s-box strains (fig. 2c, 3c) did not differ significantly, which further indicates a specific effect of transcriptional de-repression on Leu⁺ events.

The rates of Leu⁺ mutation in actively growing cultures were also examined to determine whether transcriptional de-repression-mediated mutagenesis was exclusive to cells under stress. The results of fluctuation tests for the occurrence of Leu⁺ and His⁺ in the strains carrying the s-box and t-box gene arrangements are shown in table 2. The rates of histidine prototrophy varied less than 8-fold among strains and showed no overall trend that suggested that transcriptional de-repression influenced the accumulation of His⁺ mutations. The rate of leucine prototrophy mutations for the s-box strains ranged between 1.1×10^{-9} and 5.7×10^{-9} per cell generation amongst strains and transcription conditions. Similar results were observed in the strains carrying the s-box constructs (HAM201 and HAM202). Leu⁺ colonies were tested on minimal media, devoid of either methionine or histidine, for the presence of secondary mutations

at the *metB5* and *hisC952* alleles, respectively. In the strain with s-box constructs, only histidine reversion was tested. Consistent with previously reported results, no additional Met⁺ or His⁺ phenotypes were observed [Ross et al., 2006; Sung and Yasbin, 2002]. In summary, the results of these experiments suggest that transcriptional de-repression in cells under nutritional stress promote the formation of mutations. Furthermore, this mutagenic response was independent of selection and was not observed in actively growing cells or in neutral alleles.

Leucine revertant colonies from HAM104 (s-box'-*leuC427*) were tested in order to assess their phenotype on minimal medium lacking leucine and in conditions of transcriptional repression. While all the Leu⁺ colonies that arose in the repressed condition grew robustly in the presence of methionine, the vast majority of Leu⁺ derivatives from the de-repressed condition grew very faintly in the presence of methionine. This suggests that most of the mutations in the de-repressed condition mapped to the s-box'-*leuC427*. Sequence analysis of Leu⁺ revertants further corroborated the location of the mutations and pointed to AGA→AAA changes at position 427. Since previous reports showed that most Leu⁺ mutations at the *ilv-leu* operon are AGA→GGA [Ross et al., 2006; Sung and Yasbin, 2002], we tested the possibility that the G→A change confers growth only in conditions of high transcription by transforming YB955 (Leu⁻) with DNA from AAA revertants (s-box'-*leuC427*) and selected for leucine

prototrophy. To determine whether the integration took place at the *ilv-leu* or the *amyE* loci (confers spectinomycin resistance), *Leu*⁺ transformants were screened for spectinomycin sensitivity, which indicates integration into the *ilv-leu* operon. Transformation with the revertant s-box'-*leuC427* allele yielded *Leu*⁺ revertants that were Sp^r and Sp^s, indicating integration at both chromosomal loci. Sequence of the *ilv-leu* locus confirmed our results that the AAA codon at the *ilv-leu* operon produced leucine prototrophic cells. Altogether, these observations suggest that the changes to *Leu*⁺ mediated by transcriptional de-repression are not an artifact of the generation of an enzyme with reduced activity that confers growth because of high expression levels.

Stationary-Phase Mutations Are Generated via an Mfd-Dependent Pathway

To determine the molecular mechanism(s) responsible for the generation of mutations in *B. subtilis* cells in con-

ditions of stress, we examined the effects of Mfd on the accumulation of mutations at the *amyE*'-*leuC* (fig. 4) locus. Mfd is a transcriptional factor involved in transcription elongation and transcription-coupled repair, previously shown to decrease the occurrence of *Leu*⁺ mutations and the cell's ability to transcribe *leuC* in conditions of nutritional stress [Pybus et al., 2010; Ross et al., 2006]. Figure 4 shows the accumulation of mutations in stationary cells and implies that for the generation of adaptive *Leu*⁺ reversions Mfd is required. There were no significant differences in cell survival in the strains tested, and the mutagenesis effect is specific to stationary phase because there were no differences in the rates of mutation in exponential growth (table 2). These results suggest that Mfd is a genetic factor that promotes the formation of point mutations and is necessary for the accumulation of *Leu*⁺ stationary-phase mutations.

Fig. 3. Effect of methionine starvation and transcriptional de-repression on *Leu*⁺ stationary-phase mutagenesis in *B. subtilis*. **a** The accumulation of *Leu*⁺ mutations responds to tyrosine starvation. Strains HAM103 and HAM104 carry the *leuC427* mutation at the *ilv-leu* operon. Strain HAM104 contains an *s-box*⁺-*leuC427* transcriptional fusion integrated into *amyE*; HAM103 contains the same *s-box* construct with no *leuC427*. Transcriptional control was established by adding none (de-repressed) or 0.05 g/l of methionine (repressed) to Spizizen minimal medium (see table 1 for fold increase in *leuC427* transcription). **b** Survival of non revertants was not differentially affected in strains or transcriptional conditions. **c** Stationary-phase mutagenesis at other markers (*His*⁺ events) is not affected by transcriptional de-repression. Results are based on four replicates and are representative of at least three repetitions. See 'Experimental Procedures' for more details.

Fig. 4. Effect of Mfd on transcriptional de-repression on *Leu*⁺ stationary-phase mutagenesis in *B. subtilis*. **a** The accumulation of *Leu*⁺ mutations responds to methionine starvation. Strains HAM104 and HAM106 carry the *leuC427* mutation at the *ilv-leu* operon. Strain HAM104 contains an *s-box*⁺-*leuC427* transcriptional fusion integrated into *amyE*; HAM106 also contains a tetracycline resistance cassette within the *mfd* gene. Transcriptional

control was established by adding none (de-repressed) or 0.05 g/l of methionine (repressed) to Spizizen minimal medium (see table 1 for fold increase in *leuC427* transcription). **b** Survival of non-revertants was not differentially affected in strains or transcriptional conditions. Results are based on four replicates and are representative of at least three repetitions. See 'Experimental Procedures' for more details.

Table 2. Spontaneous His⁺, Met⁺ and Leu⁺ reversion rates for HAM103, HAM104, HAM201 and HAM202

Revertant allele	Strain	Condition	Rate ^a	Error (±)
Histidine	HAM103 (s-box')	repressed	1.35E-09	1.12E-09
Histidine	HAM103	de-repressed	4.47E-09	1.33E-09
Histidine	HAM104 (s-box'- <i>leuC427</i>)	repressed	1.73E-09	8.10E-10
Histidine	HAM104	de-repressed	6.34E-10	5.90E-11
Leucine	HAM103	repressed	1.06E-09	9.80E-10
Leucine	HAM103	de-repressed	5.68E-09	9.80E-10
Leucine	HAM104	repressed	1.75E-09	1.12E-09
Leucine	HAM104	de-repressed	3.49E-09	1.12E-09
Histidine	HAM201 (t-box')	repressed	9.41E-10	9.80E-10
Histidine	HAM201	de-repressed	3.61E-09	5.90E-11
Histidine	HAM202 (t-box'- <i>leuC427</i>)	repressed	1.21E-09	8.10E-10
Histidine	HAM202	de-repressed	1.5E-09	8.10E-10
Leucine	HAM201	repressed	2.07E-10	7.10E-11
Leucine	HAM201	de-repressed	1.39E-10	7.10E-11
Leucine	HAM202	repressed	3.03E-09	8.10E-10
Leucine	HAM202	de-repressed	1.47E-09	9.80E-10

Error is representative of the 95% confidence value. Leucine rates of reversion have been previously reported for strains deficient in Mfd [Ross et al., 2006].

^a Expressed as per cell generation.

Discussion

Recent studies from our laboratory provided evidence that in conditions of stress genes under selection and under transcriptional de-repression accumulate more mutations than in conditions of transcriptional repression [Pybus et al., 2010]. Here, we further advance the concept that high transcription levels alone mediate the generation of mutations in cells subjected to stress by conducting experiments that alter or decouple transcription from selection. First, we altered transcription of the *ilv-leu* operon using isoleucine as a transcriptional effector to reduce transcription levels of a point-mutated *leuC427*, which renders cells auxotrophic for leucine synthesis, and showed that modest increases in transcription levels at *leuC427* did result in a slight increase in the accumulation of adaptive Leu⁺ reversions (fig. 1). We also conducted experiments in which transcription of *leuC427* did not respond to leucine starvation but to either tyrosine or methionine nutritional stress, and demonstrated that increases in transcription levels directly correlated with significant increases in the accumulation of mutations that confer leucine biosynthesis (fig. 2, 3).

Based on the observations that transcriptional de-repression resulted in significant increases in adaptive mutations and the well-documented deficiencies in DNA re-

pair that take place in conditions of stress in *B. subtilis* [Debora et al., 2011; Pedraza-Reyes and Yasbin, 2004; Vidales et al., 2009], it is reasonable to speculate that genes experiencing high levels of transcription are subject to more DNA lesions than those regions that are not transcribed or transcribed at low levels. Interestingly, in conditions of active replication, there were no significant effects of transcriptional de-repression on leucine reversion (table 2). This is in contrast to previous observations in *E. coli* and other organisms where high levels of transcription correlate with increases in spontaneous rate of mutations, particularly in the context of conflicts with replication [Datta and Jinks-Robertson, 1995; Hendriks et al., 2008; Kim et al., 2007; Reimers et al., 2004; Rudner et al., 1999; Wright and Minnick, 1997]. However, it should be noted that in our case rates of mutation were calculated using cells grown in defined media, which, in *B. subtilis*, has been shown to reduce genome instability events compared to when cells are grown in rich medium conditions [Srivatsan et al., 2010]. Then, it appears the mutagenic processes in stressed *B. subtilis* cells are independent of chromosomal replication or reassembly of replication forks, and are mediated by events directly associated with transcriptional de-repression.

The results of the experiments conducted here complement and further advance results previously presented

in *B. subtilis*, where transcription was controlled by using IPTG [Pybus et al., 2010], in four novel ways. One, transcriptional de-repression responded to two different molecule effectors that are independent of selection pressure. Two, there were three levels of transcriptional de-repression of the *leuC427* and three different levels in the accumulation of Leu⁺ mutations; 4-, 13- and 58-fold increases in expression of *leuC427* resulted in 1.5-, 6- and 12-fold increases in the number of revertants at day 10, respectively (table 1). Three, the constructs used here rely on the mechanism of anti-termination and suggest that the ability to initiate transcription does not have a role in the accumulation of stationary-phase mutations. Lastly, Mfd, the transcription coupling repair factor, acts epistatically onto this process (fig. 4).

This discussion then leads to a scenario in which stationary-phase mutations in *B. subtilis* are the result of the combined effects of repair deficiencies or misrepair and misprocessing transcriptional elongation complexes at sites where RNAP is stalled. Concomitant with this idea, activity of YfhQ (the *E. coli* MutY homolog that removes A residues in OG-A pairs) promotes the formation of Leu⁺ mutations by possibly competing with a deficient mismatch repair system [Debora et al., 2011]. The misprocessing of transcriptional elongation complexes may come via transcription-coupled repair, as mediated by Mfd, and the recruitment of low-fidelity DNA polymerases, which are actively expressed in stationary-phase conditions or by cells undergoing the sporulation development pathway [Duigou et al., 2004; Rivas-Castillo et al., 2010]. It should be noted that in *E. coli* Mfd, or its functional homologue in mammalian cells, gets recruited to pauses that are caused by different events and that this factor may interact with different repair systems [Bregeon et al., 2003, 2009; Clauson et al., 2010; Smith and Savery, 2008; Tornaletti et al., 2004]. Because Mfd mediates nucleotide excision repair, it would be interesting to determine whether stationary-phase mutations are dependent on Uvr proteins and PolI, which fills in the DNA gap created during nucleotide excision repair. A variant of transcription-coupled repair, mediated by the transcriptional elongation factor NusA and the low fidelity polymerase DinB, has been implicated in the formation of stress-induced mutations in *E. coli* in the FC40 system as part of DNA damage tolerance mechanisms [Cohen et al., 2009, 2010; Cohen and Walker, 2010].

An alternate mechanism that may explain how transcriptional de-repression mediates the formation of mutations is by promoting transcriptional bypass, the process in which RNAP generates a mutated mRNA

by misinsertion of ribonucleotides during transcription [Bregeon et al., 2003; Liu et al., 1995; Liu and Doetsch, 1998; Saxowsky and Doetsch, 2006]. This process may give rise to transient phenotypes that license stressed cells back into replication which may or may not lock the potential to escape stress into the DNA of a cell; this has been denoted as transcriptional mutagenesis and is part of cell-selfish modes of evolution [Doetsch, 2002; Holmquist, 2002]. Also, it has been shown that Mfd, in addition to dissociating RNAP from DNA lesions in the transcribed strand and mediating transcription-coupled repair [Selby and Sancar, 1993], has the ability to translocate stalled elongation complexes and realign the DNA template and the active site of RNAP [Borukhov et al., 2005; Park et al., 2002; Roberts and Park, 2004]. It was then hypothesized that Mfd could rescue paused elongation complexes back into active transcription and potentiate transcriptional bypass by RNAP [Svejstrup, 2002]. CSB, the mammalian counterpart of Mfd, has been shown to mediate transcriptional bypass of oxidative DNA lesions in vitro [Charlet-Berguerand et al., 2006]; however, in *E. coli* Mfd prevents transcriptional bypass [Bregeon et al., 2003] and does not affect stationary-phase mutagenesis [Bridges, 1995]. In these studies, the effect of transcriptional bypass was not directly measured; however, our assays relied on two selection pressures, which eliminate transient fitness advantages engendered by mutated transcripts.

In summary, the experiments presented here demonstrated that transcriptional de-repression in *B. subtilis* cells under conditions of nutritional stress mediated the formation of adaptive mutations. Mechanistically, it was also genetically demonstrated that Mfd played a central role in this process. Mfd is likely to be influencing the generation of stress-induced mutations during pauses of transcription elongation and facilitating faulty repair. In *E. coli*, stress-induced Lac⁺ mutations are generated by a subpopulation of stressed cells that switches to low fidelity replication [Ponder et al., 2005], or by a variant of transcription-coupled repair mediated by NusA and DinB as part of DNA damage tolerance strategies [Cohen et al., 2010; Cohen and Walker, 2010]. The Mfd and NusA pathways provide cellular strategies that connect aspects of transcription and mutagenic events to escape stress without compromising the integrity of genomic regions that are not under selective pressure. One other interesting concept that is raised by these observations is that transcription elongation factors have a dual function in the cells: protecting genome integrity and preventing conflicts between replication and transcription in growing

conditions, or producing genetic diversity in conditions of stress. Because transcription itself and the factors involved in transcription-mediated mutagenesis are well conserved through the three domains of life, this phenomenon has important implications in the evolutionary process. Further, aspects of transcription-mediated mutagenesis have been associated with diverse life processes that include the formation of cancers [Rodin et al., 2002; Wright et al., 2002], antibiotic resistance [Han et al., 2008] and antibody diversity [Wright et al., 2004].

Experimental Procedures

Bacterial Strains, Media and PCR Conditions

Strain YB955 is a prophage-‘cured’ derivative of *B. subtilis* strain 168 containing point mutations in three alleles, *metB5* (ochre), *hisC952* (amber), and *leuC427* (missense) [Sung and Yabin, 2002]. *B. subtilis* strains were routinely isolated on tryptic blood agar base (Acumedia Manufacturers, Inc., Lansing, Mich., USA), and liquid cultures were grown in Penassay broth (PAB; antibiotic medium 3, Difco Laboratories, Sparks, Md., USA) supplemented with 1× Ho-Le trace elements [Gerhardt et al., 1994]. Antibiotics were added to a final concentration of 0.1 g/l (spectinomycin) when appropriate. *E. coli* strain XL1-Blue, used for plasmid subcloning and transformation was maintained on Luria-Bertani agar containing 0.1 g/l ampicillin. All solid media for maintenance and tests were grown at 37°C in a humidified incubator. Liquid cultures were aerated at 250 rpm at 37°C. Strains and their characteristics are further described in table 3.

The PCR reactions were prepared as follows: 2× Promega Master Mix (Madison, Wisc., USA), 200 nM of each primer, 250 ng of DNA, and nuclease-free water to a final volume of 25 µl. The PCR reactions were amplified as follows: an initial denaturing at 95°C for 2 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at temperature-specific to primer set for 30 s, extension at 72°C and a final extension at 72°C for 5 min.

Construction of Riboswitch Strains

To decouple transcription from selection, we constructed *leuC427* alleles controlled by riboswitches. A riboswitch is referred to the 5' region of a gene transcript that mediates the formation of a full transcript by sensing the cellular concentration of effector molecules of low molecular weight. Examples of such molecules are charged tRNAs or biosynthetic intermediates. Here, we use the t-box and s-box riboswitches that sense charged tRNA^{tyr} and S-adenosylmethionine, respectively [Grundy and Henkin, 2003; McDaniel et al., 2003]. To clone the s-box, a 430-bp DNA fragment immediately preceding the start codon of the *yitJ* gene was PCR amplified from genomic DNA of *B. subtilis* YB955 with Vent DNA polymerase (New England Biolabs, Beverly, Mass., USA) and the oligonucleotide primers 5'-GGCGTCGACGTGCAGGATGATCAAGTGCG-3' (forward) and 5'-CGCGCTAGCTGTCTGCCTCCTTTATTCAC-3' (reverse). The set of primers were designed to insert *SalI* and *NheI* restrictions sites (underlined), respectively, into the amplified DNA fragment. The t-box was obtained from *B. subtilis* YB955 by amplifying a 446-bp nucleotide fragment located immediately upstream of the *tyrS*

ORF using the oligonucleotide primers 5'-AAAGATAAGCTTCCGAAAACCAGAA-3' (forward) and 5'-CGGTCGACAAGATAAAGCTCCTTTTTTA-5' (reverse). The primers inserted *HindIII* and *SalI* restriction sites (underlined), respectively, into the cloned product. Both, the s-box and t-box-containing PCR fragments were ligated into pCR-BluntII-TOPO (Invitrogen, Carlsbad, Calif., USA) and replicated in *E. coli* XL10-GOLD Kan^r (Stratagene; Cedar Creek, Tex., USA).

The s-box and t-box containing constructs were purified from *E. coli* cells and treated with *SalI/NheI* and *HindIII/SalI*, respectively; the released products were independently ligated into the vector pHyperspank treated with the corresponding restriction enzymes. The ligation products were introduced by transformation into competent cells of *E. coli* XL10-GOLD Kan^r (Stratagene; Cedar Creek, Tex., USA) generating plasmids pMPR006 and pMPR024, respectively.

The s-box-*leuC427* construct was engineered as follows. The *leuC* ORF from strain *B. subtilis* YB955 was PCR amplified with Vent DNA polymerase and the set of primers 5'-CGCGCTAGCATGATGCCTCGAACAATCATC (forward; *NheI* site, is shown underlined) and 5'-CGCGCATGCCCTGTTCTTTCAATCCTCTTC (reverse; *SphI* site, is shown underlined). The amplified *leuC* gene was ligated into pCR-BluntII-TOPO as described above and the cloned into the *NheI/SphI* sites of pMPR024; this construct termed pMPR026 was propagated in *E. coli* XL10-GOLD Kan^r. To obtain the t-box-*leuC427* construct, a similar approach was followed except that the forward primer used to amplify the *leuC* gene contained a *SalI* restriction site. In this way, the *leuC* gene was cloned into the *SalI/SphI* sites of pMPR006, thus generating plasmid pMPR008.

Plasmid DNA was extracted from transformants using the Qiagen Mini-Prep Kit (Carlsbad, Calif., USA) and verified by size and restriction digestion. The plasmids were then transformed into YB955 as previously described. Briefly, YB955 was grown to 90 min after the transition from exponential to stationary phase (T90), in GM1 broth (0.5% dextrose, 0.1% yeast extract, 0.2% casein hydrolysate, essential amino acids 0.05 g/l, 1× Spizizen salts, SMS) and then diluted 10-fold into GM2 broth (GM1 broth plus 50 µM CaCl₂, 250 µM MgCl₂). After 1 h of incubation at 37°C with aeration, plasmid DNA is added. After plasmid addition, the culture was incubated for 1 h supplemented with 0.1 ml of 10% yeast extract and incubated for an additional hour. Cultures were then plated onto tryptone blood agar base containing 0.1 g/l spectinomycin. Transformants were verified by PCR using the primers amyEF, GATCAAAAGCGGAACCATTCTTC, and amyER, AATGGGGAAGAGAACCGC. These sets of transformations gave rise to the following *B. subtilis* YB955 derivatives: HAM101 (*amyE::s-box*), HAM102 (*amyE::s-box'-leuC*), HAM201 (*amyE::t-box*) and HAM202 (*amyE::t-box'-leuC*).

Because YB955, the background strain used in this study, is Met⁻ and the s-box riboswitch system demands methionine prototrophy, we transformed HAM101 and HAM102 with DNA from a Met⁺ strain (YB007) [Luinetti, unpubl.] and created HAM103 and HAM104. To do this, genomic DNA was isolated from the methionine prototrophic strain, YB007. The sample was grown overnight in 2.5 ml of PAB. Cells were harvested by centrifugation, washed with 1.0 ml of lysis buffer (50 mM EDTA, 100 mM NaCl, pH 7.5), and resuspended in 0.3 ml of lysis buffer. Cell suspensions were treated with lysozyme by adding and 0.1 ml of lysozyme (10 g/l in lysis buffer), delivered in a dropwise manner,

Table 3. Strains, plasmids and primers used in this study

Strains	Characteristics	Reference
<i>E. coli</i>		
XL1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIqZΔM15</i> Tn10 (Tet ^r)]	Stratagene
DH5α	<i>recA1, endA1</i>	Invitrogen
MON1	<i>endA1 recA1 mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15ΔlacX74 Δ(ara,leu7697 ara Δ139 galU galK nupG rpsL F- λ-</i>	Monserat Biotech
<i>B. subtilis</i>		
RUB818	Met ⁺ , Lys ⁺ , Trp ⁺ BR151 derivative	Yasbin et al. [1973]
YB890	Met ⁻ , phage-cured strain	BGSC
YB007	Leu ⁺ , Met ⁺ , His ⁺ derivative of YB890	this study
YB955	Leu ⁻ , Met ⁻ , His ⁻ ; parental strain used in these studies	Sung and Yasbin [2003]
YB955 <i>mfd::Neo^R</i>	Km ^R , YB955 derivative with <i>mfd</i> disrupted by antibiotic cassette insertion	this study
HAM101	YB955 derivative with the s-box construct integrated into <i>amyE</i>	this study
HAM102	YB955 derivative with the s-box'- <i>leuC427</i> construct integrated into <i>amyE</i>	this study
HAM103	Met ⁺ YB955 derivative with the s-box construct integrated into <i>amyE</i>	this study
HAM104	Met ⁺ YB955 derivative with the s-box'- <i>leuC427</i> construct integrated into <i>amyE</i>	this study
HAM106	Met ⁺ YB955 derivative with the s-box'- <i>leuC427</i> construct integrated into <i>amyE, mfd::Neo</i>	this study
HAM201	YB955 derivative with the t-box construct integrated into <i>amyE</i>	this study
HAM202	YB955 derivative with the t-box'- <i>leuC427</i> construct integrated into <i>amyE</i>	this study
Plasmids		
pUC18	<i>E. coli</i> cloning vector, ColE1 replicon, Amp ^R	Yanisch-Perron et al. [1985]
pUC18mfd	Amp ^R , pUC18 derivative containing <i>mfd</i>	this study
pUC18mfd::Neo	Amp ^R , Neo ^R , pUC18 derivative containing an insertional disruption in <i>mfd</i>	this study
pBest502	pUB110 derivative, Neo ^R	Itaya et al. [1989]
pHyperspank	Spc ^R , <i>amyE</i> integrative plasmid; used to integrate constructs into <i>B. subtilis</i> in single copy	gift from D. Rudner
pHyperspank- <i>leuC427</i>	Spc ^R , Hyperspank derivative carrying the <i>leuC427</i> allele	Pybus et al. [2010]
MPR006	pHyperspank derivative containing the <i>tyrS</i> promoter and its untranslated region for terminator and antiterminator structures (t-box)	this study
MPR008	pHyperspank derivative containing t-box'- <i>leuC427</i> transcriptional fusion	this study
MPR024	pHyperspank derivative containing the <i>yitJ</i> promoter and its untranslated region for terminator and antiterminator structures (s-box)	this study
MPR026	pHyperspank derivative s-box'- <i>leuC427</i> transcriptional fusion	this study
Primers		
yitJsboxF	ACCCAAGCTTATTATTGTCTTTATTTTCGGTAAATTTGGAGAAA – amplifies s-box riboswitch	this study
yitJsboxR	TAGAGTCGACTGTCTGCCTCCTTTATTACATCA – amplifies s-box riboswitch	this study
DirTB-TyrS	AAAGATAAGCTTCCGAAAACCAGAA – amplifies t-box riboswitch	this study
RevTB-TyrS	CGGTCGACAAGATAAAGCTCCTTTTTTA – amplifies t-box riboswitch	this study
metBf	GCGAGAGAACACCTTGACG – used to amplify <i>metB</i>	this study
metBr	AAACCCCGGCTTCCTTT – used to amplify <i>metB</i>	this study
SalI mfd For	CGGTCGACGCCCATGACAG – amplifies <i>B. subtilis mfd</i>	this study
XbaI mfd Rev	AATCTAGAATCCGCTTTCCGC – amplifies <i>B. subtilis mfd</i>	this study
leuC RT F	GACCCGGGCGCTGTTTACG – <i>leuC</i> primer for RT-PCR	Pybus et al. [2010]
leuC RT R	GTTAATGCCCATGTCAACCATAGG – <i>leuC</i> primer for RT-PCR	Pybus et al. [2010]
veg RT F	TGGCGAAGACGTTGTCCGATATTA – veg primer for RT-PCR	Pybus et al. [2010]
veg RT R	CGGCCACCGTTTGCTTTAAC – veg primer for RT-PCR	Pybus et al. [2010]

BGSC = Bacillus Genetic Stock Center.

vortexing after each drop, and incubating at 37°C for 15 min. Proteinase K (1 g/l) is added to the sample and incubated at 37°C for 30 min, combined with 30 μl of 20% SDS, mixed and incubated at 37°C for 5 min. The nucleic acids were extracted with equal volumes of buffer-saturated 25 phenol:24 chloroform:1 isoamyl alcohol until the aqueous-phenol interface was clear, then extracted once with 24 chloroform:1 isoamyl alcohol. The DNA was precipitated with the addition of 45 μl of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 95% ethanol. The sample was invert-

ed several times, spun down, and the remaining pellet was rinsed with 95% ethanol, air dried, and resuspended in 0.1 ml of 10 mM Tris HCl (pH 8.0), 1 mM EDTA.

This DNA was then transformed into HAM101 and HAM102 as described above without the addition of 10% yeast extract. The cells were plated onto minimal plates lacking methionine (containing 1× SMS, 0.5% glucose, and a concentration of 0.05 g/l of leucine, histidine, isoleucine, and glutamic acid). Transformants were verified by sequencing the *metB* gene, which was amplified

with primers, metBf, GCGAGAGAACACCTTGACG and metBr, AAACCCCGGCTTCCTTT. The PCR fragments were purified with the Qiagen PCR clean-up kit. Sequencing was performed by Biotechnology (Madison, Wisc., USA). Analysis of the sequences indicated that HAM103 and HAM104 had acquired the *metB*⁺ allele from YB007.

To investigate the role of Mfd in transcription-associated mutagenesis, we constructed riboswitch strains that lacked Mfd, the transcription-coupled repair factor. These strains were constructed by transforming HAM103 and HAM104 with DNA from a Mfd⁻ strain (YB9800) and creating HAM105 and HAM106. The same method was used as described above.

Stationary-Phase Mutagenesis Assay

The procedure for the stationary-phase mutagenesis assay has been previously described [Sung and Yasbin, 2002]. Strains were grown overnight in 2 ml of PAB (BD) in a 37°C shaking incubator. An aliquot of 0.6 ml of overnight cultures was transferred into 10 ml of PAB with 10 µl of Ho-Le trace elements [Gerhardt et al., 1994] into 125 ml neploflasks. Growth was tracked using a Klett-Summerson colorimeter (No. 66 filter; Klett MFG Co., Inc.; 1 kU 106 CFU/ml). When cells reached T90, the cells were harvested by centrifugation and resuspended in 1× SMS. The cell suspensions (0.1 ml) were plated onto minimal medium containing 1× SMS, 0.5% glucose and either 0.05 g/l or 0.0002 g/l of the required amino acid and 0.05 g/l of both isoleucine and glutamic acid. Over 9 days, the plates were incubated at 37°C and scored for revertant colonies. The initial amount of cells used in the assay is determined by serially diluting and plating an aliquot of the cell suspension onto medium containing all the required amino acids. This experiment was replicated at least five times.

Survival rates of the non-revertant background cells were determined by removing agar plugs from areas of agar that contained no colonies and plating on medium containing all the essential amino acids. These plates were incubated at 37°C for two days and then counted.

Stationary-Phase Mutagenesis Assays Using Amino Acids to Repress Transcription

To first examine whether altering the rate of transcription of the *leuC427* allele that is controlled by its native promoter, located in the *ilv-leu* operon, affects the accumulation of mutations in cells under stress, we used regulation of gene expression features idiosyncratic to the *ilv-leu* promoter region in stressed cells. That is, the *ilv-leu* promoter responds to several effectors and transcription altering proteins that gauge the energetic state of the cell. It is well established that TnrA, CcpA and CodY, proteins that sense the nutritional state of the cell, interact with the promoter region of the *ilv-leu* operon. Moreover, CodY interacts with GTP and branch chain amino acids to enhance repression of this operon [Shivers and Sonenshein, 2004, 2005; Tojo et al., 2005, 2004]. Thus, to alter the transcription of *leuC427*, we conducted our stationary-phase assays (described above) in the presence of 0.05 g/l (repressed condition – sufficient for growth of auxotrophs) or 0.0002 g/l (de-repressed condition – limiting for growth of auxotrophs) of isoleucine. A separate experiment also tested additions of isoleucine and valine to modulate transcription of the *ilv-leu* operon. Transcript accumulation was measured as described elsewhere in this paper.

Stationary-Phase Mutagenesis Soft Agar Overlay Assays Using Riboswitches to Repress Transcription

This assay is similar to the one described above and modified to include the use of soft agar overlays containing methionine or tyrosine as effector molecules. Cell suspensions from cultures grown as previously described [Pybus et al., 2010], were spun down, resuspended in 1× SMS, and added to 3 ml semi-solid overlay, which consisted of Spizizen minimal media supplemented with 0.5% glucose and trace amounts of leucine and histidine (0.002 g/l). To control for transcription de-repression in HAM103, HAM104, and HAM106, 0.002 g/l (de-repressed) or 0.05 g/l (repressed) of methionine, was used to amend the overlay. For the experiments involving HAM201 and HAM202, transcription of *leuC* was controlled by amending with the experimental medium with 0.05 g/l (repressed) or 0.0002 g/l (de-repressed) of tyrosine.

Starting 2 days after the initial overlay was added and every other day from then until the 10th day, a top overlay, which allowed for growth of leucine prototrophs, was added. This semi-solid overlay consisted of Spizizen minimal media supplemented with 0.5% glucose and 0.05 g/l of histidine. Revertant colonies were counted 2 days after the top overlay was added. This experiment was replicated at least five times. Since cells are starved for two amino acids, there is no selective growth advantage to the acquisition of a Leu⁺ or His⁺ mutation. Further, because *leuC427* transcription responds to tyrosine or methionine starvation, our experimental strategy examines the accumulation of mutations in the absence of growth and as a function of transcriptional de-repression.

Plugs were taken from the minimal plates to determine the viability of the non-revertant background. Agar plugs were diluted 10-fold and plated on media containing all the essential amino acids. Plates were incubated at 37°C for 2 days and then counted.

Fluctuation Test

The growth-dependent reversion frequencies for the His⁻, Met⁻, and Leu⁻ alleles were measured by fluctuation tests which has been previously described [Sung and Yasbin, 2002]. Cultures were grown in the absence or presence of tyrosine (HAM201 and HAM202), or methionine (HAM103, HAM104, and HAM106). Bacterial cultures were grown to saturation (roughly 14–16 h) at 37°C with aeration in PAB. The saturated cultures were then diluted 10⁻⁴-fold in PAB, and 1-ml aliquots were dispensed into 38 18-mm test tubes. Test tubes were incubated to saturation at 37°C with aeration. The cultures were harvested by centrifugation and resuspended in 0.1 ml of 1× SMS. The cells were then plated onto the same selective minimal media described in the stationary-phase mutagenesis assay section. After 48 h of incubation, the plates were scored for revertant colonies. The Lea-Coulson formula, $r/m - \ln(m) = 1.24$, which uses the median (r), was used to estimate number of mutations per culture (m). To determine the total number of CFU plated (N_t), three cultures run in parallel were serially diluted and spread onto minimal agar media containing all the required amino acid. Mutation rates were calculated with the formula $m/2 N_t$.

RNA Extraction and Preparation

RNA was isolated, as previously described [Silvaggi et al., 2005], from experiments in which cells were subjected to conditions of transcriptional de-repression or activation by supplementing cultures with either of the following effector molecules,

tyrosine or methionine. HAM202 was grown in 10 ml of minimal broth (1 × SMS, 0.5% glucose, 0.05 g of the required amino acids per liter, 0.05 g of glutamic acid per liter, 0.005 M MgSO₄, Ho-Le trace elements, and 0.05 g/l of isoleucine, with tyrosine at a concentration of 0.05 g/l to control transcription of the *leuC427* allele. For the experiments with HAM104, 0.05 g/l of methionine was used to repress transcription of *leuC427*. Cells were harvested during mid-exponential phase or at T90 by mixing the culture with an equal volume of methanol and centrifuging for 10 min at 4,000 g at 4°C. The pelleted cells were stored at -20°C to be processed later. Pellets were resuspended in 3.2 ml of LETS buffer (10 mM Tris-HCl pH 8, 50 mM LiCl, 10 mM EDTA, 1% SDS). The cells were lysed and nucleic acids precipitated by vigorously vortexing the sample in vials containing 2 ml of glass beads (Sigma, P-4682, St. Louis, Mo., USA) and 2.4 ml of acid-phenol. An equal volume of chloroform was added to the sample and centrifuged for 10 min at 3,200 g at 4°C. The aqueous phase was added to a fresh tube containing 3.2 ml of 5:1 phenol-chloroform and centrifuged for 10 min at 3,200 g at 4°C. The aqueous phase was divided into 0.7 ml aliquots and added to an equal volume of isopropanol. After a 10-min incubation at room temperature, the samples were centrifuged at 14,000 rpm for 25 min at 4°C. The pellets were then washed with 75% ethanol, centrifuged for 5 min, resuspended in 20 µl of DEPC-treated water and vortexed upon the addition of 1.2 ml of TRIzol reagent. The sample was incubated at room temperature for 5 min, combined with 0.24 ml of chloroform, mixed by inversion for 15 s and kept at room temperature for 2 min. Then, samples were centrifuged at 14,000 rpm for 15 min at 4°C to separate the aqueous phase, which was transferred to isopropanol at a 1:1 (v/v) and allowed to precipitate at -20°C overnight. The RNA pellet was collected by centrifugation at 14,000 rpm for 15 min at 4°C and air dried for 2 min. Lastly, pellets were resuspended in 20 µl of DEPC-treated water, incubated at 55°C for 2 min and stored at -20°C until further use. The resulting RNA was quantified on the General Electric NanoVue Plus Spectrophotometer (Piscataway, N.J., USA) which was graciously provided by the Wing lab.

DNase Treatment

To remove any residual DNA, the Ambion TURBO DNA-free (Austin, Tex., USA) kit was used according to manufacturer's instructions.

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16S rDNA PCR

To verify that RNA samples were free of any contaminating DNA, the 16S rRNA gene was PCR amplified using primers 16S RT F, TCGCAAGACTGAAACTCAAAGGA, and 16S RT R, TCAGAGGATGTCAAGACCTGGTAAG.

cDNA Synthesis/Real-Time PCR

For each RNA sample, we conducted three independent cDNA synthesis reactions. 200 ng of RNA was used in the Quanta Biosciences qScript One-Step SYBR GREEN qRT-PCR Kit (Gaithersburg, Md., USA) to synthesize cDNA and perform real-time PCR. The following primers were used to amplify the *leuC* gene, *leuC* RT F, GACCCGGGCGCTGTTTACG, and *leuC* RT R, GTTAATGCCCCATGTCACCATAGG. The primers used to amplify the housekeeping gene *veg* were *veg* RT F, TGGCGAAGACGTTGTCCGATATTA, and *veg* RT R, CGGCCACCGTTTGCTTTA-AC. Quantitative real-time PCR was performed using a BioRad iCycler iQ Real-Time PCR Detection system (BioRad, Hercules, Calif., USA), using the manufacturer's suggested protocol and an annealing temperature of 57°C, followed by a melting profile and assessment of amplicon size on an agarose gel. Ct values for the *leuC* and the *veg*, used as a control [Fukushima et al., 2003], genes from stationary-phase cultures under conditions of either transcriptional de-repression or transcriptional repression were used to calculate relative fold increase in transcription by the 2^{-ΔΔCt} method [Livak and Schmittgen, 2001].

Test for Slow-Growing Revertants and for Multiple Mutations

Leu⁺ colonies were further tested by patching the colonies on SMM media lacking leucine and in conditions of transcriptional de-repression. Plates were incubated at 37°C and scored after 48 h.

A portion of the colonies from these plates was patched to SMM lacking methionine or histidine and scored similarly.

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