Transcription-Associated Mutation in *Bacillus subtilis* Cells under Stress[▽]

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Adaptive (stationary phase) mutagenesis is a phenomenon by which nondividing cells acquire beneficial mutations as a response to stress. Although the generation of adaptive mutations is essentially stochastic, genetic factors are involved in this phenomenon. We examined how defects in a transcriptional factor, previously reported to alter the acquisition of adaptive mutations, affected mutation levels in a gene under selection. The acquisition of mutations was directly correlated to the level of transcription of a defective *leuC* allele placed under selection. To further examine the correlation between transcription and adaptive mutation, we placed a point-mutated allele, *leuC427*, under the control of an inducible promoter and assayed the level of reversion to leucine prototrophy under conditions of leucine starvation. Our results demonstrate that the level of *Leu*⁺ reversions increased significantly in parallel with the induced increase in transcription levels. This mutagenic response was not observed under conditions of exponential growth. Since transcription is a ubiquitous biological process, transcription-associated mutagenesis may influence evolutionary processes in all organisms.

The generation of mutations has been traditionally ascribed to spontaneous processes affecting actively growing, dividing cells. Nevertheless, by the mid-1950s, several reports describing mutagenesis in nondividing cells of bacteria, plants, flies, and fungi appeared in the scientific literature (reference 36 and references therein). Much of the initial characterization of this process in bacteria took place in the laboratory of Francis Ryan, who observed *Escherichia coli* mutants capable of synthesizing histidine arising from *his* mutant (auxotrophic) cells undergoing prolonged starvation (36) while cell turnover remained undetectable, and DNA replication slowed with increasing time (26). Renewed interest in adaptive mutation was generated when Cairns and coworkers published their work on the generation of *Lac*⁺ reversions in *E. coli* cells unable to use the lactose provided as the sole carbon source in a minimal medium (6). This work demonstrated that adaptive mutations can arise as a result of stress rather than from selection of preexisting mutations. The generation of stress-induced *Lac*⁺ reversions, assayed via a plasmid-borne system, has been studied intensively by several laboratories (reviewed in references 13, 15, and 34; 32) and is dependent on activation of the SOS and/or stress responses. Further studies have also suggested that a subpopulation within the *Lac*[−] stressed cells engage in an exquisitely regulated transient state of hypermutation limited in time and to DNA sites near double-stranded DNA breaks (reviewed in reference 15). Collectively, the results

from studies on this system have provided interesting insights into the acquisition of beneficial mutations and demonstrated the role of several genetic factors in the adaptive mutation phenomenon.

A significant question in the study of adaptive mutagenesis centers on what processes would allow arrested cells under stress to acquire beneficial mutations before the accumulation of deleterious mutations resulted in cell death. One intriguing and seldom explored possibility is the role of transcription in this phenomenon (10). While cells are under stressful conditions, stochastic processes acting upon derepressed genes could bias the accumulation of mutations to highly transcribed alleles. Selection would favor mutations enhancing cell survival while a transcription-associated mutation bias would aid the population in avoiding a lethal genetic load. In support of this idea, several studies on adaptive mutagenesis have noted the appearance of mutations principally in chromosomal alleles under selection (17, 40).

Other experiments have directly illuminated the role of transcription in mutagenic processes. For instance, using actively dividing *E. coli* cultures, Wright and Minnick established a correlation between rates of transcription and of reversion to prototrophy in the *argH* and *leuB* alleles (47). In addition, they demonstrated that mutations in *leuB* decreased in the absence of RelA (an enzyme synthesizing the transcription-modulating alarmone guanosine tetraphosphate) while mutation rates in *argH* increased with increased expression of RelA or when the repressor protein ArgR was absent (29, 47). Such observations led Wright to hypothesize a transcription-mediated mutagenesis model (46): essentially, as a response to stress, a small percentage of the genome is derepressed with respect to its transcription. During the transcription process, the potential

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FIG. 1. Diagram of the *ilv-leu* (A) and *amyE* chromosomal regions carrying *leuC427* (B). The *P_{hyperspank}* vector containing either *leuC427* or no allele (C) under the transcriptional control of IPTG was integrated into the *amyE* locus. The *amyE* gene function is nonessential under conditions of the experimental assays.

exists for single-stranded DNA to supercoil and form secondary stem-loop structures, exposing vulnerable bases to mutagenic processes. Subsequent lack of high-fidelity repair would then generate a heritable mutation. The resulting genetic variants are selected for an adaptive advantage, facilitating escape from arrested growth. This phenomenon is not exclusive to bacteria. Specifically, in growing cells of *Saccharomyces cerevisiae* and mammalian stem cells, it has been hypothesized that high transcription rates promote the formation of mutations (21, 23). So, while the role of transcription in mutagenic processes has been demonstrated under conditions of active DNA replication, whether transcription mediates the formation of mutations in nondividing cells remains largely unknown.

Here, we demonstrate two lines of evidence that support the role of transcription in adaptive mutagenesis. First, a *Bacillus subtilis* 168 strain derivative lacking the Mfd transcription factor, previously shown to be decreased in the accumulation of stationary-phase mutants (33), showed a dramatic decrease in levels of mRNA of a gene under selection. This correlation between transcription and mutagenesis was further investigated by placing a defective *leu* allele under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter and comparing the accumulations of *Leu*⁺ revertants under conditions of transcriptional induction and repression. *Leu*⁺ reversions increased significantly in parallel with the induced increase in transcription levels.

MATERIALS AND METHODS

Bacterial strains and media. 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) (Fig. 1A) (41). In addition to the *leuC427* allele placed at the *ilv-leu* locus (44), a copy of *leuC427* was placed under the control of the IPTG-inducible *Hyperspank* promoter *P_{hyperspank}* (*P_{hs}*) (a gift from David Rudner). This construct was transformed into strain YB955 and integrated into the *amyE* locus by homologous recombination (Fig. 1B) (20). The *amyE* locus is catabolite repressed under the conditions of our study; therefore, its disruption should not alter the results of our assay. As an experimental control, the *P_{hyperspank}* vector was also integrated into this locus (Fig. 1C). So the experimental strains contained *leuC427* at the *ilv-leu* operon locus as background and either an additional copy, transcriptionally controlled by IPTG, or a geneless IPTG promoter. The IPTG constructs were integrated at *amyE*.

B. subtilis strains were routinely isolated on tryptic blood agar base (TBAB) (Acumedia Manufacturers, Inc., Lansing, MI), and liquid cultures were grown in Penassay broth (PAB) (antibiotic medium 3; Difco Laboratories, Sparks, MD) supplemented with 1× Ho-Le trace elements (16). Spectinomycin was added to a final concentration of 100 μ g/ml where appropriate. *E. coli* strain XL1-Blue, used for plasmid subcloning and transformation, was maintained on Luria-

Bertani agar containing 100 μ g/ml 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.

Strain construction. To construct an inducible gene in YB955, the 1,419-bp *leuC427* allele (GenBank accession number Z99118) and its Shine-Dalgarno sequence were amplified by PCR using primers hsluC F SalI (5'-AAAAGTC GACTAGAGGGAGGAAATAAAAGATGATGC-3') and hsluC R SphI (5'-AAAAGCATGCCGCACTCCTTACACA ACTGT-3'); restriction sites are underlined). The PCR mix contained 1.2 units of Vent DNA Polymerase (New England Biolabs, Ipswich, MA), 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, 0.2 mM of the deoxynucleotide triphosphates, and 500 ng of YB955 genomic DNA. To simplify subcloning, the PCR product was ligated into pGEMT Easy (as recommended by the manufacturer; Promega, Madison, WI) by following the manufacturer's protocol, digested with SalI and SphI, gel purified, and ligated to the similarly digested *P_{hyperspank}* vector. The resulting construction was confirmed by PCR and sequenced. The *P_{hs}-leuC427* vector was transformed into strain YB955 by natural competence (2). Integration was confirmed by screening for spectinomycin-resistant colonies and by PCR. IPTG induction of this construct was confirmed by SDS-PAGE (data not shown).

Stationary-phase mutagenesis soft-agar overlay assays. There were two types of experiments that employed soft-agar overlays to detect *Leu*⁺ mutations in the strains carrying the *ilv-leu leuC427* and either the *amyE::P_{hs}* or the *amyE::P_{hs-leuC427}* alleles. In the first type of experiment, strains YB955 *amyE::P_{hs}* and YB955 *amyE::P_{hs-leuC427}* were grown to 90 min past the cessation of exponential growth (*T*₉₀) in 10 ml of PAB with trace elements. Cultures were centrifuged and resuspended in 1× Spizizen minimal salts (SMS) (39) to 10 ml. Aliquots of 100 μ l of this suspension were plated on minimal medium with trace (200 ng/ μ l) leucine and methionine containing either 0.07 mM IPTG or no 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% agar and prewarmed at 42°C) lacking leucine and containing 0.07 mM IPTG and 50 μ g/ml methionine. Of note, adjustment of the final concentrations for IPTG and methionine considered the volume and IPTG concentration in the medium dispensed previous to performing the overlay. The plates were incubated for 2 days, and the number of revertant colonies was scored. For the second type of experiment, we used soft-agar overlays to deliver the test cells onto agar medium with trace (200 ng/ μ l) amounts of leucine and methionine and either 0.07 mM IPTG or no IPTG, instead of spread plating. As described above, at different times of incubation a second overlay, adding IPTG and methionine, was used in a subset of plates. The test cells were initially grown and prepared as described above and added to 3-ml aliquots of soft agar lacking leucine and methionine and containing no IPTG or 0.07 mM IPTG. The viability of nonrevertant cells was assayed as described for the plate assay.

Stationary-phase mutagenesis agar plate assay. As described previously (41), cells from an overnight culture were inoculated into a Nephloflask containing 10 ml of PAB supplemented with trace elements. Cultures were grown to 90 min past the cessation of exponential growth. Measurements were recorded with a Klett-Summerson meter (number 66 filter; Klett Manufacturing Co., Inc., New York, NY). The cultures were centrifuged at 10,000 × *g* for 10 min and resuspended in 10 ml of 1× SMS (39). Aliquots of 0.1 ml were then spread plated in quintuplicate on Spizizen minimal medium (SMM) containing 1× SMS, 0.5% dextrose, 50 μ g/ml isoleucine, 50 μ g/ml glutamate, 1.5% agar (ThermoFisher Scientific, Waltham, MA), 50 μ g/ml of histidine and methionine, and a trace amount (200 ng/ml) of leucine (amino acids from Sigma-Aldrich, St. Louis, MO), with or without 0.07 mM IPTG. The plates were incubated for 9 days. Each day, plates were scored for the appearance of *Leu*⁺ colonies. The initial titer of *B. subtilis* cells was determined by serially diluting the resuspended culture and spread plating on SMM containing 50 μ g/ml of histidine, methionine, leucine, isoleucine, and glutamic acid. Colonies were counted after 48 h of incubation. The initial titers were used to normalize the cumulative number of revertants per day to the number of CFU plated. Assays were replicated three times, and the experiment was repeated at least twice. *Leu*⁺ colonies were further tested for growth in 48 h by patching the colonies on SMM lacking leucine. Plates were incubated at 37°C and scored after 48 h. To determine whether multiple mutations conferring *Met*⁺ or *His*⁺ phenotypes occurred, a portion of the colonies from these plates was patched to SMM lacking methionine or histidine and scored similarly. To determine the level of reversion at the *hisC952* and *metB5* alleles in the presence and absence of inducer, the above methods were employed, except that medium contained a trace amount of histidine or methionine, respectively, and 50 μ g/ml of the remaining four amino acids.

The viability of the nonrevertant cell background was assessed every other day as follows. Using a sterile Pasteur pipette, a plug of agar was removed from the

nonrevertant background of each of five plates corresponding to one type of selection (no leucine without IPTG or no leucine with IPTG). The plugs were combined in 0.4 ml of 1× SMS, serially diluted, and plated in triplicate on SMM containing 50 µg/ml of the required amino acids. Colonies were counted after 48 h of incubation.

Fluctuation test. In brief, 76 overnight cultures of the experimental strains were diluted 1:10⁵ into 28-mm culture tubes containing 2 ml of PAB medium and reincubated overnight in the absence or presence of IPTG. A 1-ml sample was extracted from each culture, resuspended in SMS, and plated on selective medium. Colonies arising on each plate were counted after 48 h of growth at 37°C. Four cultures were used to determine the total number of cells by plating on nonselective agar. Exponential reversion rates were then determined for each strain by the Ma-Sandri-Sarkar maximum-likelihood method instead of the Lea-Coulson formula (31), as previously described.

Test for slow-growing revertants and for multiple mutations. *Leu*⁺ colonies were further tested by patching the colonies on SMM lacking leucine. 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.

RNA extraction and preparation. Cultures were grown to saturation in 1× SMS containing 0.5% dextrose; 50 µg/ml of Ile, Glu, methionine, histidine, and leucine; Ho-Le trace elements; and 5 mM MgSO₄. Samples were removed at mid-exponential (approximately 50 Klett units) and stationary (*T*₁₅₀) growth phases, centrifuged at 5,000 rpm and 4°C for 10 min, and frozen at −20°C. RNA was extracted from the pellets using a RiboPure Bacteria Kit (Ambion, Austin, TX). After being treated with Turbo-DNase (Ambion), the sample was analyzed by PCR and by native agarose gel electrophoresis to confirm the absence of genomic DNA as well as sample integrity. Samples were quantitated using a Nanodrop spectrophotometer (ThermoFisher 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 samples. Master mixes for real-time PCR contained Absolute QPCR SYBR green Mix (ThermoFisher Scientific) and a 70 mM final concentration of leuCRT F (5'-GACCCGGGCGCT GTTTACG-3') and leuCRT R (5'-GTTAATGCCCATGTCACCATAGG-3') primers (78-bp amplicon) or of veg F (5'-TGGCGAAGACGTGTCCGATATT A-3') and veg R (5'-CGGCCACCGTTTGCTTTTAAC-3') (82-bp amplicon). Three replicates from each culture condition containing 4 µl of cDNA were assayed and normalized to the expression of the internal control gene *veg* (14, 27). 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 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^{−ΔΔC_T} (where *C_T* is threshold cycle) method for relative fold expression (24).

RESULTS AND DISCUSSION

General and specific DNA repair systems, mismatch repair, and oxidative damage repair (GO system) have been shown to be repressed or inefficient in cells under conditions of stress in eukaryotic and bacterial systems (19, 25, 28, 45) while error-prone polymerases are active in stationary-phase cells (11, 12, 19, 42, 43). Thus, one can speculate that the combination of transcriptional derepression and DNA repair inadequacies under conditions of nongrowth biases mutations to transcribed regions. Interestingly, results from stationary-phase mutagenesis assays in *B. subtilis* have alluded to the role of transcription in adaptation (33). As with the *E. coli* paradigm, adaptive mutation decreases in the absence of RelA (35). Surprisingly, this also occurs in the absence of the transcription repair coupling factor Mfd (33), which is counterintuitive because Mfd is a repair factor, and its absence would be expected to increase mutations. It should also be noted that this novel property of *mfd* is not observed under conditions of exponential growth (33), and it is expressed in bacterial species other than *B. subtilis* (18). This factor mediates transcription coupling repair and is known to dissociate RNA polymerase stalled at a lesion

FIG. 2. Mutagenesis and level of transcription in stationary-phase cells. (A) Accumulation of *Leu*⁺ colonies in strain YB955 and in the transcription factor *Mfd*[−] isogenic derivative (YB955 *mfd::tet*). Data represent counts from five plates averaged from three separate tests, normalized to initial cell titers. Error bars represent 1 standard error of the mean. (B) Relative fold change in *leuC427* mRNA levels in YB955 and in YB955 *mfd::tet*. Levels of mRNA from cultures in exponential and stationary phases of growth are compared to YB955 mRNA levels in exponential phase. The *veg* gene was used as a control. Error bars represent 95% confidence intervals.

in DNA (1, 38), which would consequently result in repeated attempts at transcription. Thus, we hypothesize that transcription-associated mutagenesis mediates adaptive mutation.

Strain YB955 contains a *leuC427* allele and expresses leucine auxotrophy via a missense mutation at position 427 (41). This strain and its isogenic *mfd::tet* mutant derivative were used in our stationary-phase assay (as described previously [41]). The accumulation of *Leu*⁺ prototrophic colonies for each strain is illustrated in Fig. 2A. Cumulative *Leu*⁺ reversions are essentially absent in the *Mfd*[−] strain. Of note, bacterial colonies appear on our minimal medium containing all essential ingredients in 48 h. Therefore, revertant colonies arising during the first 3 or 4 days of incubation are likely the result of growth-dependent mutation. Colonies arising on day 6 or after are the consequence of mutations in stationary phase. In previous reports, we have established that the colonies growing in the absence of leucine are true revertants and not slow-growing bacteria (33, 41, 42). We have also demon-

strated that the bacterial concentrations of the auxotrophic cells on the plates do not impede the growth of prototrophic revertant colonies (33, 41, 42). Furthermore, the viabilities of the nonrevertant colonies are similar in both strains (33); therefore, differential cell survival cannot account for the difference in mutation frequencies.

After the elimination of growth-related artifacts, we concentrated on the mechanism that would explain the dramatic decline in *Leu*⁺ revertant colonies in the absence of Mfd. One possibility is that Mfd functions in stationary-phase cells by promoting lesion bypass by RNA polymerase. In *E. coli*, the absence of *mfd* promoted bypass (3) but showed no effect on the accumulation of mutations in a tRNA gene involved in tyrosine biosynthesis (5). Interestingly, NusA, a transcription antitermination factor first described to influence transcription of λ genes, has been recently shown to dramatically affect stress-induced mutagenesis in *E. coli* (9). Perhaps, transcription influences adaptive mutation through elongation factors and mechanisms differently in these two model systems.

It is also possible that Mfd in *B. subtilis* mediates the dissociation of stalled RNA polymerase (1), facilitates increased attempts at transcription, and promotes the formation of mutagenic stem-loop structures as discussed above. If Mfd works in this manner, one would predict that the levels of mRNA (and the level of transcription) would decrease in the absence of Mfd. To examine this possibility, we determined the amount of mRNA present during growth and in stationary phase in the parental YB955 strain and its isogenic Mfd-deficient derivative. Absence of Mfd results in a dramatic decrease in *leuC* mRNA levels compared to the parental strain in stationary phase (Fig. 2B). Since the absence of transcription factor Mfd results in both a noticeable drop in the levels of *leuC* mRNA and in the loss of adaptive *Leu*⁺ reversions, these results suggest a strong correlation between transcription and mutation in stationary-phase cells. Consequently, we decided to investigate how transcription influences stationary-phase mutagenesis of a single gene through the use of an inducible genetic *leuC427* construct.

For this assay, we used a derivative of YB955 that contains two identical defective *leuC* alleles: one allele located within the *ilv-leu* operon (normal locus) and another identical allele, transcriptionally controlled by IPTG, recombined into the *amyE* locus (Fig. 1). In this genetic background, mutations that result in *Leu*⁺ revertants may be acquired in either allele; however, mutations within the allele controlled by the *Hyperspank* promoter require the presence of IPTG to be detected (since transcription of this gene is dependent upon this inducible system). By using this two-allele approach, we can differentiate between genetic events occurring during growth and under conditions of nongrowth, as well as those events that are promoted by experimentally increased transcription under stationary-phase conditions. Thus, we compared levels of mutagenesis in the strain containing this construct, designated YB955 *amyE::P_{hs}-leuC427*, with an isogenic strain containing only the inducible promoter (YB955 *amyE::P_{hs}*) on leucine-deficient medium with and without inducer. In the first set of experiments, we spread plated 100- μ l aliquots of cells onto medium where induced and uninduced cells were starved for leucine and methionine and subsequently shifted to conditions that relieved methionine starvation and induced transcription

of *P_{hs}* constructs. In the second set of experiments, induced and uninduced cells were added to soft-agar overlays containing no leucine or methionine and dispensed on top of regular agar medium of the same composition as the soft agar. The initial absence of the essential amino acids methionine and leucine negates any growth advantage of *Leu*⁺ revertant cells while allowing the application of leucine biosynthesis selective pressure as well as the IPTG-controlled induction of the specific allele. For both experiments, and beginning the second day of incubation and daily thereafter, a set of plates for both strains was overlaid with soft agar containing minimal medium with IPTG, methionine, and no leucine.

As shown in Fig. 3A and B, the accumulation of *Leu*⁺ colonies in YB955 *amyE::P_{hs}-leuC427* was increased over 6-fold in the presence of inducer compared to the amount in YB955 *amyE::P_{hs}*. Levels of mutagenesis in YB955 *amyE::P_{hs}* were comparable under induced and noninduced conditions. Since mutagenesis in this strain reflects reversions at the *ilv-leuC427* allele only, it is clear that increased transcription of *leuC427* from *P_{hs}* is linked to the majority of revertants in YB955 *amyE::P_{hs}-leuC427*. Significantly, cell viabilities were similar for all strains (Fig. 3C). While both experiments show the dramatic effects of increased transcription on stationary-phase mutagenesis, the second experiment rules out the possibility that the overlay process spreads cells of microcolonies formed during starvation. Another important consideration was whether the presence of the inducer would affect mutagenesis at other alleles. YB955 contains two additional point-mutated alleles, *metB5* and *hisC952*, which have been previously studied during stationary phase (41). We assayed YB955 *amyE::P_{hs}* and YB955 *amyE::P_{hs}-leuC427* for reversion to methionine and histidine prototrophy in the presence and absence of IPTG. No change was observed in mutation level (Fig. 4) or in cell viability (data not shown). This indicated that the effect of IPTG-based induction was specific to our construct. We also examined the effect of increased transcription on the rate of mutation of *leuC427* in exponential growth and found no striking differences in rates of mutation between the wild type and the strains carrying the inducible constructs in the presence or absence of IPTG (Fig. 5). These results further suggest that the influence of transcription on mutagenesis is exclusive to, or dramatically pronounced under, stationary-phase conditions.

Leucine revertant colonies from each strain were tested in order to assess their phenotype on minimal medium lacking leucine without IPTG. While the colonies from YB955 *amyE::P_{hs}* (*ilv-leuC427* revertants) were opaque and robust within 48 h, growth of close to 90% of colonies from YB955 *amyE::P_{hs}-leuC427* on this medium was impaired, indicating that these colonies arose from adaptive reversion in the *P_{hs}-leuC427* construct and were dependent upon the presence of IPTG (Tables 1 and 2). The possibility of variant and less efficient enzymes that confer a growth advantage to the cells under conditions of transcriptional derepression do not apply to our study since cells were starved for two amino acids (data not shown).

Finally, the *Leu*⁺ colonies were tested on minimal medium devoid of either methionine or histidine for the presence of secondary mutations at the *metB5* and *hisC952* alleles, respectively. Consistent with previously reported results, no additional *Met*⁺ or *His*⁺ phenotypes were observed (33, 41). Taken

FIG. 3. Accumulation of *Leu*⁺ revertants in the stationary phase under conditions of transcriptional repression and activation. Test cells were either spread plated (A) or immobilized with soft agar (B), placed under conditions of starvation for leucine and methionine, and subsequently relieved, using a soft-agar overlay, of methionine starvation, and induced into transcription at different times of incubation. Results represent levels of mutation in YB955 strains containing the *P_{hs}-leuC427* inducible construct or the *P_{hs}* promoter only. Data represent counts from three plates averaged from three separate tests, normalized to initial cell titers. (C) Viability of nonrevertant test cells. Data were collected from plugs removed from three plates of each selective medium and is represented as the number of CFU per plug. Error bars represent 1 standard error of the mean.

collectively, these results further support the hypothesis that transcription mediates a synergistic effect on mutagenic processes in cells under stress. Significantly, the results from the soft-agar overlay experiments also demonstrate that the mutations accumulate, following transcription induction, without being coupled to growth.

These experiments clearly demonstrate an influence of transcription on the adaptive mutation phenomenon. The molec-

FIG. 4. Accumulation of *Met*⁺ and *His*⁺ revertants during the stationary-phase mutagenesis plate assay. Results represent levels of mutation in YB955 strains on selective medium with and without inducer containing the *P_{hs}* promoter (A) or the *P_{hs}-leuC427* inducible construct (B). Data represent counts from five plates averaged from three separate tests, normalized to initial cell titers. Error bars represent 1 standard error of the mean. Viabilities of nonrevertant cells for *P_{hs}* and *P_{hs}-leuC427* were not significantly different and were not affected by the presence of IPTG.

ular mechanisms responsible for this influence still need to be elucidated. As previously mentioned, the formation of stem-loop structures by single-stranded DNA exposed during transcription is one possibility (46). Higher levels of transcription

FIG. 5. Fluctuation test for leucine biosynthesis reversions in strains containing the *P_{hs}* promoter or the *P_{hs}-leuC427* inducible construct in the presence and absence of IPTG. Thirty-five independent cultures per condition were included in the experiment. Rates of reversion for methionine and histidine were also tested, and results showed similar values for the strains carrying the *P_{hs}* or *P_{hs}-leuC427* construct in the presence or absence of IPTG. See Materials and Methods for further details.

TABLE 1. Growth phenotypes of YB955 *amyE::P_{hs}* Leu⁺ revertants on minimal medium lacking leucine

Day after initial selection and condition ^a	Growth phenotype of revertants (no. of revertants with phenotype/no. of revertants tested [%]) ^b	
	Opaque growth	Faint growth
2		
No IPTG	8/8 (100)	0/8 (0)
IPTG	15/15 (100)	0/15 (0)
3		
No IPTG	7/7 (100)	0/7 (0)
IPTG	2/2 (100)	0/2 (0)
4		
No IPTG	7/7 (100)	0/7 (0)
IPTG	9/9 (100)	0/9 (0)
5		
No IPTG	4/4 (100)	0/4 (0)
IPTG	7/7 (100)	0/7 (0)
6		
No IPTG	5/5 (100)	0/5 (0)
IPTG	1/1 (100)	0/1 (0)
7		
No IPTG	8/8 (100)	0/8 (0)
IPTG	10/10 (100)	0/10 (0)
8		
No IPTG	10/10 (100)	0/10 (0)
IPTG	6/6 (100)	0/6 (0)
9		
No IPTG	16/16 (100)	0/16 (0)
IPTG	18/18 (100)	0/18 (0)

^a All cultures lacked methionine and leucine.^b Data are from two experiments.TABLE 2. Growth phenotypes of YB955 *amyE::P_{hs}-leuC427* Leu⁺ revertants on minimal medium lacking Leu

Day after initial selection and condition ^a	Growth phenotype of revertants (no. of revertants with phenotype/no. of revertants tested [%]) ^b	
	Opaque growth	Faint growth
2		
No IPTG	2/34 (5.9)	32/34 (94.1)
IPTG	3/39 (7.7)	36/39 (92.3)
3		
No IPTG	6/26 (23.1)	20/26 (76.9)
IPTG	6/32 (18.8)	26/32 (81.2)
4		
No IPTG	1/9 (11.1)	8/9 (88.9)
IPTG	2/18 (11.1)	16/18 (88.9)
5		
No IPTG	6/29 (20.7)	23/29 (79.3)
IPTG	3/16 (18.8)	13/16 (81.2)
6		
No IPTG	2/24 (8.3)	22/24 (91.7)
IPTG	5/26 (19.2)	21/26 (80.8)
7		
No IPTG	1/21 (4.8)	20/21 (95.2)
IPTG	1/32 (3.1)	31/32 (96.9)
8		
No IPTG	1/23 (4.3)	22/23 (95.6)
IPTG	2/8 (25)	6/8 (75)
9		
No IPTG	3/24 (12.5)	21/24 (87.5)
IPTG	1/16 (6.2)	15/16 (93.8)

^a All cultures lacked methionine and leucine.^b Data are from two experiments.

would further expose bases prone to mutation to mutagenic conditions in the intracellular environment. Wright et al. tested this hypothesis *in silico* using an algorithm predicting the mutability of individual bases (48). They observed that highly mutable bases in the *p53* tumor suppressor gene were associated with the predicted degree of base exposure in stem-loop structures (SLS) associated with a high negative free energy of folding (48). Mechanistically, from observations in *E. coli* and yeast, the presence of lesions during transcription may result in pauses that recruit Mfd and error-prone DNA polymerases (8, 23).

Another transcription-associated process potentiating the generation of mutations is the ability of RNA polymerase to bypass lesions present in DNA, resulting in base misinsertion in the transcript. Spontaneous cellular processes such as deamination, oxidation, and base loss generate uracil, 8-oxo-7,8-dihydroguanine (8-oxoG), and apurinic/apyrimidinic (AP) sites in DNA, respectively (reviewed in reference 37). Of these DNA-insulting processes, those causing oxidative damage have been shown to be significant in *B. subtilis* cells under conditions of starvation (45). The ability of RNA polymerase to bypass these and perhaps other types of lesions may result in base misinsertion in the transcript, generating mutant proteins resulting in the appearance of pseudo-prototrophs in nondivid-

ing cells (4, 22, 37). This process was shown to occur in non-dividing *E. coli* cells, where Bregeon et al. demonstrated that RNA polymerase can efficiently bypass uracil and 8-oxoG lesions engineered into a luciferase reporter gene (3). Analysis of the resulting transcripts indicated that RNA polymerase has the ability to insert adenine opposite to uracil and adenine or cytosine opposite to 8-oxoG (3). Interestingly, lesion bypass during transcription has also been implicated in the generation of mutations in *p53* (30). Further, recent *in vitro* studies have demonstrated that the human Mfd homolog, CSB (Cockayne syndrome group B), and other transcription elongation factors mediate transcriptional bypass in HeLa cells (7). Hence, transcription-associated mutagenesis appears well conserved across the domains of life.

In the strain containing two *leuC427* loci, growth of the majority of leucine revertants was depressed in the absence of IPTG, which suggests that the effect of transcription on stress mutagenesis is *cis* acting (Table 1 and 2). In yeast cells, a similar *cis*-acting effect of transcription on mutagenesis has been observed in assays with growing cells. It is proposed that transcription roadblocks collapse replication forks that are subsequently reassembled by recombination functions and the recruitment of error-prone DNA polymerases (23). Since recombination functions do not influence the generation of

stress-induced mutations in *B. subtilis* (41), the *cis*-acting effect reported here is mediated by a yet-to-be uncovered mechanism. Future experiments studying transcriptional induction in combination with DNA repair systems and error-prone polymerases should further improve our understanding of the mechanisms producing genetic diversity in *B. subtilis* nondividing cells.

In this study, we have demonstrated the association of transcription with mutation in an inducible allele in stationary cells under selective pressure. Such an association could explain why adaptive mutations often appear directed and how cells under these conditions avoid the accumulation of lethal mutations resulting in genetic load, thus resolving this biological conundrum. In summary, transcription-associated adaptive mutation may provide an evolutionary strategy for escape from growth-limiting conditions by supporting the diversification of metabolic profiles, the utilization of xenobiotic compounds, and the development of antibiotic resistance (18). In human cells, however, adaptive mutation in arrested cells could have potentially deleterious effects, where a cell-selfish strategy for growth results in the formation of cancer or the onset of degenerative disease, as well as in resistance to certain cancer treatments (7, 22). Since transcription is a process occurring in all living cells, including resting cells, transcription-associated mutagenesis may well be relevant to the evolutionary process throughout all the domains of life.

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