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Stationary Phase Mutagenesis in *B. subtilis*: A Paradigm to Study Genetic Diversity Programs in Cells Under Stress

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ABSTRACT One of the experimental platforms to study programs increasing genetic diversity in cells under stressful or nondividing conditions is adaptive mutagenesis, also called stationary phase mutagenesis or stress-induced mutagenesis. In some model systems, there is evidence that mutagenesis occurs in genes that are actively transcribed. Some of those genes may be actively transcribed as a result of environmental stress giving the appearance of directed mutation. That is, cells under conditions of starvation or other stresses accumulate mutations in transcribed genes, including those transcribed because of the selective pressure. An important question concerns how, within the context of stochastic processes, a cell biases mutation to genes under selection pressure? Because the mechanisms underlying DNA transactions in prokaryotic cells are well conserved among the three domains of life, these studies are likely to apply to the examination of genetic programs in eukaryotes. In eukaryotes, increasing genetic diversity in differentiated cells has been implicated in neoplasia and cell aging. Historically, *Escherichia coli* has been the paradigm used to discern the cellular processes driving the generation of adaptive mutations; however, examining adaptive mutation in *Bacillus subtilis* has contributed new insights. One noteworthy contribution is that the *B. subtilis*' ability to accumulate chromosomal mutations under conditions of starvation is influenced by cell differentiation and transcriptional derepression, as well as by proteins homologous to transcription and repair factors. Here we revise and discuss concepts pertaining to genetic programs that increase diversity in *B. subtilis* cells under nutritional stress.

KEYWORDS adaptive mutation, transcriptional bypass, genetic programs in cells under stress

INTRODUCTION

Adaptive or stationary-phase mutagenesis occurs in cells under nongrowing conditions or prolonged nonlethal selective pressure, *e.g.*, starvation for an essential amino acid, or a carbon source (Rosenberg *et al.*, 1998; Sung and Yasbin, 2002). This concept is illustrated in Figure 1 and applies to simple and complex habitats of many bacterial species, *Bacillus subtilis* and *Pseudomonas fluorescens*

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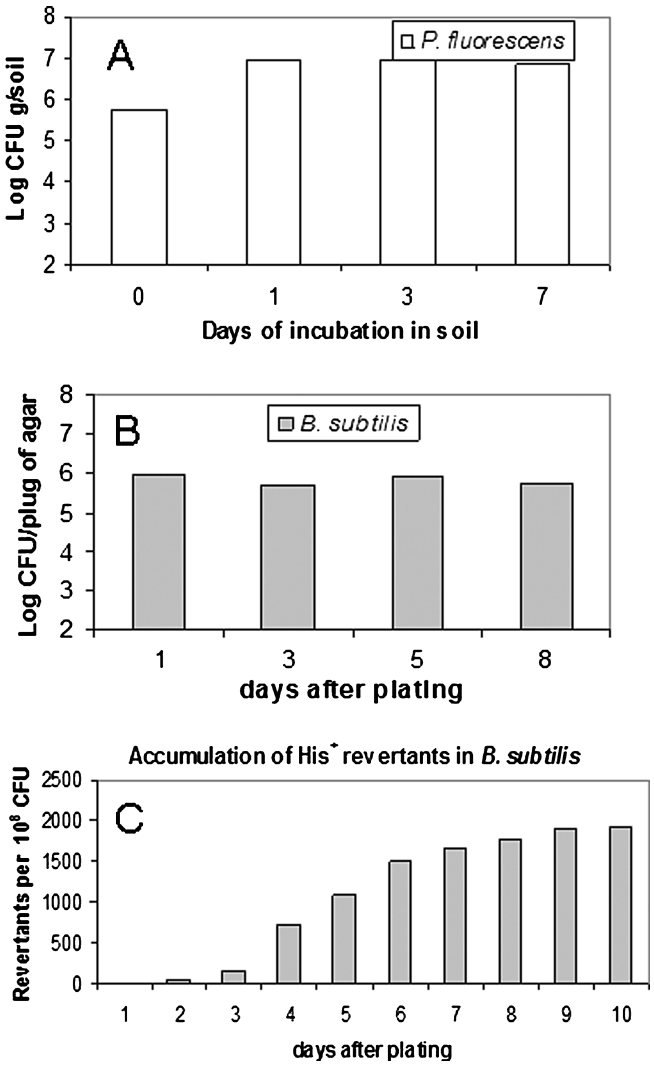

FIGURE 1 **Stationary culture and adaptive mutagenesis. (*A*) Populations of *P. fluorescens* in arid soil (data are representative of population densities presented by [Silby and Levy, 2004]). (*B*) Populations of a *B. subtilis* histidine auxotrophic strain on agar medium lacking histidine. (*C*) Accumulation of *B. subtilis* histidine prototrophic mutants (Ross *et al.*, 2006). Error bars have been omitted for simplicity. Soil populations for days 1 to 7 did not differ signicantly.**

among them. One example of a complex habitat that subjects bacterial cells to conditions of starvation or nongrowing conditions is that of soil (Figure 1A). Similarly, conditions of nutritional stress may be mimicked by using laboratory media lacking a required nutrient (Figure 1B). In these conditions, bacterial populations carrying amino acid auxotrophies held in stationary phase accumulate prototrophic revertant colonies over time (Figure 1C). Since test cultures have been allowed to grow prior to selection, growth dependent mutations (mutations that arise during DNA replication within actively dividing cells) are also observed, evidenced by the appearance of single colonies after two days of incubation. Those colonies arising after prolonged incubation of cells under nongrowing conditions have been denoted adaptive or stationary phase mutants. If mutations are random events arising from growth dependent processes, how is it that a cell under nongrowing conditions acquires a mutation, not to mention a beneficial one? Answering this question improves our understanding of evolution and applies to many diverse processes that encompass cell aging, neoplasia, and prokaryotic resistance to antibiotics and the immune system responses, to name a few phenomena (Foster, 2005).

While most of the research in adaptive mutagenesis has involved *Escherichia coli* model systems, similar observations have been made in other prokaryotes (Bjedov *et al.*, 2003; Kasak *et al.*, 1997; Sung and Yasbin, 2002) as well as in eukaryotic organisms (Halas *et al.*, 2002). In experiments using *E. coli*, the scientific discussion generated by mutagenic processes in nondividing conditions has been spirited and has provided many insights into DNA metabolism in nongrowing conditions. Moreover, the mechanisms by which *E. coli* accumulates mutations in genes under nonlethal selection involve recombination, amplification, error-prone replication, factors involved in stress response, the induction of the SOS system and transcription associated mutagenesis (Foster, 2005; Rosenberg, 2001; Slechta *et al.*, 2003; Wright, 2004; Zinser and Kolter, 2004). Models based on the very well characterized FC40 strain experimental system propose two pathways to the generation of adaptive mutation, amplification and hypermutation (Foster, 2005; Hendrickson *et al.*, 2002; Rosenberg, 2001). While the FC40 +1 frameshift *lacI* system is plasmid-borne and relies on carbon source starvation, the molecular mechanisms governing this process also apply to chromosomal mutagenesis of other genetic markers (Bull *et al.*, 2001). In addition to the mutagenic route models proposed in the FC40 strain system, experiments examining adaptive mutagenesis in other microorganisms and stress conditions suggest that other mechanisms also contribute to this phenomenon (Kivisaar, 2003; Nowosielska and Grzesiuk, 2000; Reimers *et al.*, 2004).

In *Bacillus subtilis*, studies examining the accumulation of adaptive or stationary phase mutations suggest similarities and differences in the molecular mechanisms between *B. subtilis* and *E. coli*, microorganisms used as Gram-positive and Gram-negative models, respectively. For example, error-prone replication and transcription-associated mutagenesis appear common

mechanisms between *E. coli* and *B. subtilis*, whereas the effect of factors mediating cell differentiation on adaptive mutation has, to date, been observed only in the *B. subtilis* paradigm. Here we review current research and concepts that pertain to adaptive mutagenesis in *B. subtilis* and discuss its implications. This paper focuses on describing the cellular environment under non-growing conditions and the increase of genetic diversity as affected by error-prone replication, cell differentiation, and transcription. Of these three cell processes, we will discuss transcription associated mutagenesis in depth.

The amplification and point mutation/hypermutation model mechanisms invoked in adaptive mutation in *E. coli* are discussed at length elsewhere in this issue and will only be referred to when appropriate in this article.

Stationary Phase Cellular Environment

Changes in Gene Expression

It is well established that the onset of stationary phase conditions brings about significant changes in gene expression at the level of transcription. These shifts in gene expression are mediated by global cell regulators and alternate sigma factors. The discussion on changes in gene expression is relevant to the study of adaptive mutation because some of these global regulators and alternate sigma factors have been shown to influence such mutational events and provides insights into the underlying mechanisms. Also, transcriptional activation place stresses on DNA coding for genes under selection.

Low levels of nutrients derepresses transcription of genes involved in amino acid biosynthesis, uptake and catabolism of carbon sources, and in protection against DNA damage (Bernhardt *et al.*, 2003; Chatterji and Ojha, 2001; Jain *et al.*, 2006; Moreno *et al.*, 2001; Pragai *et al.*, 2004; Voelker *et al.*, 1995). One additional layer of gene expression above that mediating carbon and amino acid metabolism in stationary phase cells is that of cell differentiation in *B. subtilis*. Such differentiation programs include the development of competence and the formation of spores. Genomic and proteomic studies during early stationary phase indicate the cessation of synthesis of 400 gene products and the expression of 150 proteins (Bernhardt *et al.*, 2003).

Classical examples of global responses at the cellular level are catabolite derepression and the stringent response (Moreno *et al.*, 2001; Voigt *et al.*, 2007). The depletion of glucose in the growth medium activates alternate systems of carbon catabolism, secondary metabolites, and sporulation. In conditions of glucose abundance, carbon catabolite repression in *B. subtilis* is mediated by CcpA and *cre* (catabolite repression element, 14 bp *cis* acting element) sites (Moreno *et al.*, 2001; Tojo *et al.*, 2005; Zalieckas *et al.*, 1998). CcpA, bound by the HPr corepressor in a phosphorylated state, binds *cre* sites and blocks or terminates transcription (Moreno *et al.*, 2001). This *cis* acting element site has been reported to occur either within the promoter region or well inside transcribed regions of *xylA*, *acsA*, *acu*, *amyE*, *hut*, *gnt*, and *bglPH* (Zalieckas *et al.*, 1998). CcpA and homologues bind *cre* and prevent transcription initiation, in cases where *cre* sites overlap with promoter regions. In cases where *cre* sites are located well within transcribed regions, interaction of CcpA and *cre* may prevent transcription elongation. Evidence that supports this concept was obtained in experiments in which *cre* sites were placed at different positions downstream from the transcriptional start of *tms*, a gene that is not regulated by carbon catabolite repression (Zalieckas *et al.*, 1998). In conditions of glucose depletion *cre* is no longer bound by CcpA, which allows transcription activation and/or elongation. This derepression results in expression of genes for the utilization of C-2 substrates, amino acid and lipid degradation (Voigt *et al.*, 2007).

The stringent response is another fairly well characterized global response at the cellular level that initiates at the cessation of growth (Eymann *et al.*, 2002) or at the onset of stationary phase. The signal molecule mediating the stringent changes in gene expression is guanosine (penta) tetraphosphate, (p)ppGpp. In *E. coli*, this nucleoside polyphosphate interacts with RNAP and acts on stringent promoters. In *B. subtilis*, (p)ppGpp halts replication by inhibiting DNA primase and depletion of GTP results in repression of rRNA promoters (Gourse and Keck, 2007; Krasny and Gourse, 2004; Wang *et al.*, 2007). The overall response at the cellular level is that of repression of genes involved in protein synthesis. Specifically, genes for tRNA, rRNA and ribosomal proteins are significantly repressed (Chatterji and Ojha, 2001; Jain *et al.*, 2006). Concomitantly, genes involved in amino acid biosynthesis are activated in combination with an ATP-activated protease complex that targets unassembled ribosomal proteins (Eymann *et al.*, 2002; Kuroda, 2006). Genetic regions that directly control the amount of (p)ppGpp in *E. coli* are *relA* and *spoT* (*B. subtilis* only contains *relA*). Synthesis of (p)ppGpp is

carried out by RelA and its synthetic activity is greatly enhanced in the presence of stalled ribosomes (Jain *et al.*, 2006).

Genetic manipulation of *relA* has provided findings relevant to the generation of stationary phase mutation in *E. coli* and *B. subtilis*. Using chromosomal amino acid prototrophy markers and genetic backgrounds differing only in RelA proficiency, it was observed that *E. coli* accumulated a higher number of adaptive mutants in *arg* and *leu* defective alleles in the presence of RelA than in its absence (Reimers *et al.*, 2004). Further, the increase in the accumulation of adaptive mutants was correlated with the cell's ability to accumulate transcript levels in the alleles under selection (Reimers *et al.*, 2004). Similarly, a correlation between the stringent response and adaptive mutation has been observed in *B. subtilis* (Rudner *et al.*, 1999). The results of the experiments involving *relA* in *B. subtilis* suggest an influence of transcription on adaptive mutagenesis. This possible association between transcription and mutagenesis is discussed further below.

Another global regulator affecting stationary phase processes is CodY. It influences these processes by regulating transcription of several operons involved in carbon catabolism, amino acid synthesis, and sporulation as well as synthesis of secondary metabolites during the early stages of the stationary phase (Molle *et al.*, 2003; Ratnayake-Lecamwasam *et al.*, 2001). CodY, in combination with AbrB, appears to have multiple functions that include sensing directly the concentration of certain amino acids and signal molecules and binding DNA as either transcription activator or transcription repressor. CodY is highly conserved among many of the low G + C bacteria, which include medically relevant genera, and has been implicated in pathogenicity (Levdikov *et al.*, 2006; Sonenshein, 2005). It has been proposed that CodY competes with RelA for GTP (Sonenshein, 2005). This observation in combination with the experiments that show that the stringent response influences adaptive mutation in *B. subtilis* (Rudner *et al.*, 1999) suggest that CodY may also be affecting the accumulation of mutations in cells under nongrowing conditions. Perhaps CodY influences adaptive mutagenesis by affecting transcript levels of alleles under selection (see discussion on stringent response). With the exception of the genetic studies involving *relA*, there is no experimental data providing a link between the two other global regulators discussed above and stress-induced mutagenesis.

In addition to the global changes in gene expression driven by regulators, *B. subtilis* alters its gene expression patterns in growth-limiting environments through a variety of alternate sigma factors. A sigma factor that operates in resting vegetative cells in Gram-positive bacteria is the alternate sigma factor B, σ^B, the general stress sigma factor. The *E. coli* homolog is σ^S, which becomes active when cells are exposed to a variety of environmental insults that include ethanol, salt, acid, heat, and energy depletion stresses (Patten *et al.*, 2004; Vijayakumar *et al.*, 2004). The general σ^B–dependent expression pattern is that of expression of about 150 proteins conferring general protection against oxidative, heat, acid, and alkaline stresses (Hecker and Volker, 2001; van Schaik and Abee, 2005). In *E. coli*, σ^S influences the generation of adaptive mutagenesis in the FC40 system (Layton and Foster, 2003; Lombardo *et al.*, 2004). What is more, σ^S-deficient strains show a decrease in the accumulation of adaptive mutants arising through amplification or hypermutation and a decrease in levels of Pol IV, an error-prone polymerase of the Y family involved in tranlesion synthesis. These observations, along with the results showing that conjugal transfer functions in the FC40 system also influence adaptive mutation, suggest a model that invokes repair of double strand breaks using the error-prone polymerase. It has been postulated that nicked sites are processed to double strand breaks and repaired by recombination with error-prone replication. This switch to error-prone DNA replication polymerases appears to be mediated by σ^S, which confines hypermutation in time and DNA locations (Ponder *et al.*, 2005). This model also implies that some cells under nutrient limiting conditions can initiate replication and that hypermutation may only occur in a subpopulation of cells (Godoy *et al.*, 2000; Rosche and Foster, 1999; Torkelson *et al.*, 1997). Contrastingly, in *B. subtilis* adaptive mutation in chromosomal markers is not affected by σ^B or RecA, but does appear to involve one of two Y error-prone polymerases, which suggests a different path from that taking place in the *E. coli* FC40 system (Sung and Yasbin, 2002; Sung *et al.*, 2003).

It should be noted that our discussion on gene expression has focused on regulons as if they were isolated. However, it is well established that regulons under the control of σ factors operating in stationary phase conditions or under the control of global regulators have significant overlap, are juxtaposed or have hierarchy among them (Britton *et al.*, 2002; Dagkessamanskaia *et al.*, 2004; Phillips and Strauch, 2002; Tojo *et al.*, 2005).

At the operon and at the specific stimulus level in *B. subtilis*, it has also been shown that attenuation or transcription termination significantly modulates transcript levels in genes for amino acid biosynthesis. Moreover, the combined effects of promoter activation and transcription termination may result in as much as a 200-fold difference on transcript levels for amino acid biosynthesis genes between repressed and fully induced conditions (Henkin and Yanofsky, 2002).

In summary, examination of gene expression networks operating in stationary phase conditions suggest that *B. subtilis* cells respond in different ways that include cell differentiation into competence and spore formation, as well as acquisition of resources, and protection against damaging agents. Interestingly, some stationary phase regulons include genes that activate components of the SOS system (Yasbin *et al.*, 1993). Hence, two aspects to the cellular changes taking place at the onset of stationary phase should be considered: First, genes under selective pressure are transcriptionally derepressed, and second, it is conceivable that cells have evolved mechanisms, in addition to those that procure resources and protection in conditions of stress, that promote routes of genetic escape from stressful conditions such as that of adaptive mutation.

DNA Damage and Repair in Conditions of Stationary Phase

In addition to global changes at the transcription level, there is substantive evidence indicating that cells under arrested growth experience high levels of DNA damage, which would be conducive to the formation of mutations. General DNA repair systems that maintain DNA fidelity become limiting or repressed (the mismatch repair system) whereas those that rely on error-prone repair become activated; both systems have been implicated in adaptive mutagenesis (Foster, 2005; Hersh *et al.*, 2004; McKenzie *et al.*, 2000; McKenzie *et al.*, 2001; Pedraza-Reyes and Yasbin, 2004).

In the case of the mismatch repair system, it is hypothesized that this system is repressed in stationary-phase conditions or becomes limiting due to increased levels of DNA damage. Repression of this system in conditions of stress may facilitate homeologous recombination and acquisition of potential advantageous alleles (Kivisaar, 2003). Alternatively, this repair system may become limiting during this state as recombinant alleles are repaired. Overproduction of mismatch repair proteins decreased the occurrence of mutants under nondividing conditions but not under growth conditions for the *B. subtilis* model (Pedraza-Reyes and Yasbin, 2004). More interestingly, stationary phase mutagenesis was reduced only when MutS was overproduced. Thus, the mismatch repair system, in particular MutS, is either reduced in amount or in activity, or otherwise disabled (*e.g.*, through damage) in cells responsible for the observed during stationary phase mutagenesis of *B. subtilis*. Overexpression of the *mutSL* operon, however, did not completely suppress the production of stationary phase induced mutations. It is possible that the overexpression construct used in that study did not generate enough mismatch repair proteins or additional mechanisms are involved in the generation of stationary phase mutations. The mismatch repair system has also been shown to be involved in adaptive mutagenesis in the *F′-lac* reversion system of *E. coli* (Harris *et al.*, 1997), but the results in *B. subtilis* suggest that the mechanism might be different. While the loss of function of MutL was proposed to be responsible for a mismatch repair deficiency during the stationary phase of *E. coli* (Harris *et al.*, 1997), the evidence in *B. subtilis* strongly suggests that the MutS homolog is the non-functional component of the mismatch repair system for the production of stationary phase induced mutants.

In addition to the depression in mismatch repair, other systems may be decreased or titrated by DNA lesions. Oxidative damage repair has been proposed to be high in stationary phase conditions. This is supported by numerous studies in different bacterial and eukaryotic systems that show that cells under nondividing conditions express genes that protect against reactive oxygen species (Heeren *et al.*, 2004; Kawasaki and Aguirre, 2001; Merkamm and Guyonvarch, 2001; Nachin *et al.*, 2005; Zeller and Klug, 2006). Preliminary experiments examining mutagenesis in *B. subtilis* wild type or genetic backgrounds deficient in three 8-oxo-G repair systems showed that this type of damage is significant in stationary phase (manuscript in preparation). The strains included in these experiments were deficient in two DNA glycosylases (Fromme and Verdine, 2003; Zeibell *et al.*, 2007) and one nucleoside triphosphatase (Xu *et al.*, 2004; Castellanos-Juarez *et al.*, 2006). The strains deficient in 8-oxo-G repair were 500 times more mutagenic than strains deficient in mismatch repair system, a general repair system (Pedraza-Reyes and Yasbin, 2004). Taken collectively, the evidence from studies examining the contribution of the mismatch and

oxidative repair systems to adaptive mutation suggest that *B. subtilis* cells experience high levels of DNA damage. Analysis is continuing to determine if other types of DNA damage accumulate in stationary phase cells.

The SOS repair system has been shown to contribute significantly to the accumulation of adaptive mutations in the *E. coli* FC40 system (He *et al.*, 2006; McKenzie *et al.*, 2000; McKenzie *et al.*, 2001). RecA, a major factor mediating homologous recombination and repair events, and LexA, the SOS repressor, are positioned at the top of a regulatory cascade that transcriptionally activates the SOS regulon. The generation of *E. coli* adaptive mutants arising via amplification requires a functional RecA (Slack *et al.*, 2006), but no SOS components (McKenzie *et al.*, 2001). On the other hand point mutations or recombination dependent mutations require RecA, recombinatorial repair proteins and error-prone polymerases (McKenzie *et al.*, 2001). In *B. subtilis,* this regulon consists of roughly 60 genes and encompass processes including translesion synthesis, recombinatorial repair, excision repair, error-prone replication, filamentation, and other functions with no clear connection to processing high levels of DNA damage (Au *et al.*, 2005; Groban *et al.*, 2005). In this bacterium, the generation of adaptive mutations in chromosomal markers is influenced by YqjH, a hypothetical error-prone DNA polymerase, but does not require a functional RecA protein (Sung and Yasbin, 2002). RecA-independent processes influencing the generation of adaptive mutants have also been shown in Gram-negative model systems (Kivisaar, 2003; Wright, 2000).

Adaptive Mutation in B. subtilis via *the Development of Competence in Subpopulations of Cells*

The cascade of events required for the development of cells with the ability to bind, take up and incorporate DNA from the environment, *i.e.*, competence, is multilayered and its overall result is that approximately 10% of the total population of cells becomes competent. All the cells in the population have the genetic and physiological potential for the development of competence. However, the formation of the K state (effective competence) in only a fraction of the population is dependent on a threshold level of accumulation of the ComK transcriptional activator. The accumulation of ComK depends on a positive feedback loop mechanism. The activation of this loop varies from cell to cell and is determined by several protein interactions and intrinsic (transcription and translation) and extrinsic (post-translational modification) "noise" for each of the interactive components (Maamar and Dubnau, 2005; Smits *et al.*, 2005).

Transcription factors such as ComA and ComK influence the production of stationary phase induced mutants (Sung and Yasbin, 2002). ComA and ComK activate transcription of early and late competence genes, respectively (Claverys *et al.*, 2006). In addition, evidence has revealed that a null mutation in *yqj*H, which encodes one of two *B. subtilis* homologs of the error-prone UmuC/DinB or Y superfamily of DNA polymerases, affected the generation of revertants of the *hisC952* allele *via* the *B. subtilis* system (Sung *et al.*, 2003). In *E. coli*, *dinB* codes for DNA pol IV, which is known to be mutagenic by polymerizing from misaligned primer-template structures and/or to cause -1 frameshifts (Napolitano *et al.*, 2000; Wagner *et al.*, 1999). DNA pol V in *E. coli* is constituted by the postranlationally modified, a process mediated by the RecA-nucleoprotein complex, UmuD'$_2$C heterodimer complex (Opperman *et al.*, 1999) and bypasses abasic sites and thymine photoproducts (Tang *et al.*, 1999, 2000). Nonprocessed UmuD^{2}C delays replication and is proposed to provide the cell a DNA damage checkpoint (Sutton *et al.*, 2000). In the case of *B. subtilis*, homology analysis of the two *B. subtilis* Y DNA polymerases (YqjH and YqjW) studied by *Sung et al.* (2003) suggest that there are two DNA pol IV in *B. subtilis*. Gene expression and mutagenic experiments on *yqjH* and *yqjW* suggest that YqjH is constitutively expressed, functions as the *E. coli* DNA pol IV and slows growth as shown by the *E. coli* UmuD (Duigou *et al.*, 2004). In contrast, YqjW is expressed as part of the SOS response and its function resembles that of a catalytic DNA pol V. Further, it was postulated that the YqjW catalytic DNA pol V-like activity only requires one polypeptide, unlike its *E. coli* counterpart (Duigou *et al.*, 2004). Of note, there are other genes in *B. subtilis* showing homology to the *E. coli umuD*, however, these genes are part of the Spβ prophage (Kunst *et al.*, 1997) and their mutagenic function remains unclear (Duigou *et al.*, 2004).

Taken together, one possible explanation for the adaptive mutagenesis phenomenon of *B. subtilis* is that during periods of environmental stress sub-populations are differentiated. Within some of these subpopulation cells mutation frequencies can be increased (hypermutability) by the suppression of DNA repair systems

and/or the activation of mechanisms that would increase the introduction of DNA mutations into the genome (Sung *et al.*, 2003). Testing this hypothesis may be addressed by experiments involving strains carrying defective alleles that directly influence transcription of competence genes in combination with deficiencies in error-prone repair (Figure 2).

Adaptive Mutagenesis in B. subtilis—*Transcription Associated Mutagenesis Model*

The experiments examining the contribution of the mismatch repair and competence transcription factors also suggest that other molecular mechanisms are part of the adaptive mutation phenomena. This idea is supported by the following observations. First, adaptive mutation is not completely abolished by either overproduction of the mismatch repair or by deficiencies in competence and Y error-prone polymerases (Sung and Yasbin, 2002; Sung *et al.*, 2003) in *B. subtilis*. Second, the number and types of adaptive mutation vary depending on the allele tested and starvation conditions (Sung and Yasbin, 2000) in *B. subtilis*. Third, previous studies in *E. coli* and *B. subtilis* have associated aspects of transcription with the ability of cells to acquire mutations in conditions of stress (Doetsch, 2002; Rudner *et al.*, 1999; Wright, 2004). Finally, recent results involving strains differing only in the presence of a transcription elongation factor also support the transcription associated mutation concept. *B. subtilis* Mfd$^-$ strains showed a significant reduction or abolition of the ability of cells to acquire mutations under conditions of no growth in three chromosomal alleles (Ross *et al.*, 2006). What is more, in *E. coli* and Hela cells *mfd* or its human homologue, CSB, has also been associated with transcriptional mutagenesis, a process by which a transient phenotype is acquired via transcription in conditions of arrested growth (Bregeon *et al.*, 2003; Charlet-Berguerand *et al.*, 2006; Saxowsky and Doetsch, 2006).

Transcription-mediated mutation has the potential to couple cell physiology and genetic variation. It provides a potential feedback mechanism to increase mutation rates in genes that are highly transcribed due to selective pressure while supporting the randomness of the mutagenesis processes. By increasing rate of mutation as a function of transcription, the cell is also sparing the rest of the genome the acquisition of detrimental mutations and thus, potentially avoiding genetic load (Doetsch, 2002; Wright, 2004). The effect of transcription on mutagenic processes may be considered from two perspectives: consequences of derepression and consequences of transcriptional bypass.

Transcription/Derepression Predisposes DNA to Damage

Experiments that assay reversion to amino acid prototrophy and to catabolism of carbon sources supporting this hypothesis have been presented in diverse microorganisms and accrued over several decades (for a review, see Wright, 2000). Direct support comes from

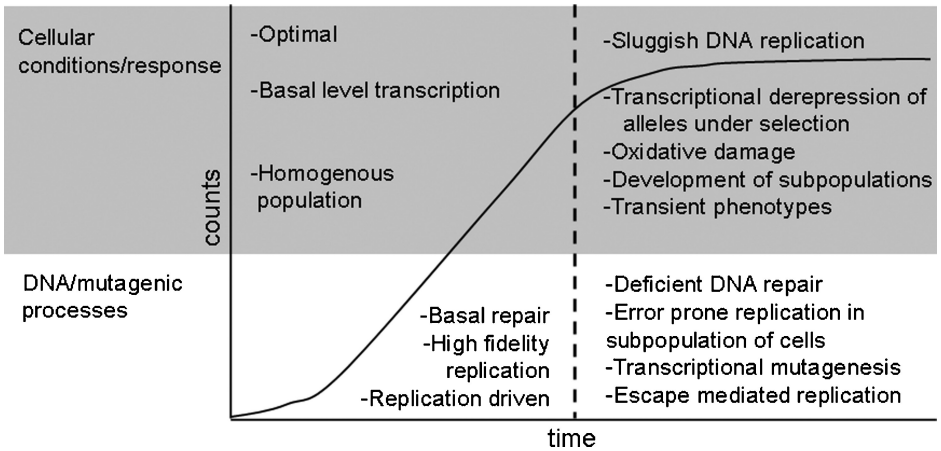

FIGURE 2 **Conditions, cellular responses, DNA and mutagenic processes taking place in stationary phase in *B. subtilis*. Exponential and stationary phase cellular conditions differ significantly in transcriptional activity of alleles under selection, DNA damage and repair as well as cell homogeneity. Stationary phase mutants may arise *via* error prone replication taking place in cell subpopulations undergoing competence. Mutants may also arise *via* escape driven replication mediated by transcriptional associated processes. RNAP may generate transient phenotypes *via* transcriptional bypass of premutagenic DNA lesions located to the template strand. Alternatively, transcription may prone DNA to the formation of premutagenic lesions on the coding strand. Both types of associated changes are locked into the DNA by replication if they provide escape from stationary phase.**

correlating rates of transcription and rate of reversion in *E. coli* strains carrying mutations in alleles that confer auxotrophy for different amino acids (Reimers *et al.*, 2004). One proposed molecular mechanism for this process poses that in the wake of transcription stresses DNA forms single-stranded structures that prime G and C residues for lesions (Reimers *et al.*, 2004; Wright, 2000). The likelihood of those residues to be mutated is associated with the formation and stability of stem-loop structures that result from transcription-generated supercoiling, their position in the aforementioned structure, and whether these residues are unpaired. The types of damage that these residues are more frequently subject to are deamination and oxidative stress (Wright, 2000). An interesting set of experiments would be to alter the formation/stability of a given stem and loop structure by introducing silent mutations at the codon level and determining whether the mutation rate is altered accordingly. Alterations to vulnerable residues would be expected to become heritable by lack of repair and subsequent replication. Nevertheless, it remains unclear how this happens in stationary phase conditions or whether it occurs in *B. subilis*. Transcription factors are likely to affect transcription levels and thus, rate of mutation, by dissociating stalled elongation complexes and permitting new transcription attempts.

Transcriptional Mutagenesis

Transcriptional mutagenesis is one less well understood route to generate mutagenic states in cells. This type of mutagenesis occurs in prokaryotic cells in stationary phase, and in nonreplicating cells of higher organisms (Doetsch, 2002; Holmquist, 2002; Rodin *et al.*, 2002). It is also based on the ease with which RNAP can bypass preformed DNA lesions (Holmquist and Maher, 2002; Larsen *et al.*, 2004; Saxowsky and Doetsch, 2006). At the level of mRNA synthesis, a mutant protein may be generated when RNAP encounters and bypasses nondistortive damage. Consequently, a nucleotide is misincorporated in the nascent elongating mRNA. The modified mRNA molecule results in an altered protein. The existence of most of these mutant proteins will be of no consequence to the cell and the originating damaged base would ultimately be repaired. However at the single cell level, it is possible that one or a few of these mutant proteins permit transient growth and allow DNA replication. DNA lesions that are not repaired before being reached by the advancing replication fork would then be locked into a daughter cell's DNA. This process has been denoted as transcriptional mutagenesis or retromutagenesis. Support for transcriptional bypass by RNA polymerase has recently been demonstrated in *E. coli in vivo* (Viswanathan *et al.*, 1999; You *et al.*, 2000). It is important to note that this in no way involves or necessitates a reverse transcriptase.

One could envision a scenario in which cells under highly selective, growth-inhibiting conditions, experiencing starvation, transcriptional derepression, and high DNA damage, generate mutant mRNAs, which bias the cell (based on stochastic processes) towards the generation of a heritable mutation in highly transcribed genes (Figure 2). There is some evidence supporting transcription associated mutagenesis as a universal phenomenon, experimental data for the two models presented above have been reported as an explanation for the generation of multiple mutations in *p53*, a tumor suppressor gene (Charlet-Berguerand *et al.*, 2006; Lloyd and Hanawalt, 2002; Rodin *et al.*, 2002).

Lastly, transcription mediated adaptive mutation offers the interesting prospect of cells escaping stationary phase via the dismissal of a checkpoint for DNA damage before replication. In *E. coli* this process is mediated by the unmodified UmuDC complex, which counters the effect of Fis, a DNA binding protein regulating cell division (Murli *et al.*, 2000). In addition to the effects of Y error-prone polymerases on adaptive mutagenesis in *B. subtilis* (Sung *et al.*, 2003), there are other proteins that associate with the cell cycle, chromosome structure and potential DNA repair checkpoints. Smc proteins affect chromosome structure, chromosome segregation and localizes differently depending on cell cycle (Dervyn *et al.*, 2004; Mascarenhas *et al.*, 2002). Also, Smc proteins are homologous to eukaryotic cohesins, which are associated with genetic instability checkpoints during mitosis (Dobles and Sorger, 2000; Wassmann and Benezra, 2001). One can speculate that a transient phenotype generated by the translation of a mutated message (transcriptional bypass) supplies cells with enough of a limiting nutrient or growth factor to either complete DNA repair or resume replication at the expense of generating a mutation. The "checkpoint" prospect and its link to stationary phase mutagenesis in *B. subtilis* may be examined by measuring the accumulation of mutations in cells deficient in Smc.

In *B. subtilis*, the concept of transcription associated mutation is supported by recent experiments that show how deficiencies in transcription termination and elongation factors influence the accumulation of adaptive

mutations (Ross *et al.*, 2006) and transcript levels in stationary phase conditions. Mfd was first described in *E. coli* by Witkin and coworkers (Witkin, 1966) and mediates transcription coupled repair by interacting with stalled transcription elongation complexes in bacteria, as does CSB in eukaryotes (Ayora *et al.*, 1996; Mellon *et al.*, 1986; Mellon and Champe, 1996; Park *et al.*, 2002; Selby and Sancar, 1993; Selby and Sancar, 1993; Selby and Sancar, 1997; Selby *et al.*, 1991; Svejstrup, 2002). Recruitment of Mfd or CSB to the stalled elongation complex results in either RNAP dissociation or rescue into active transcription (Charlet-Berguerand *et al.*, 2006; Park *et al.*, 2002; Roberts and Park, 2004; Svejstrup, 2002; Svejstrup, 2003). GreA, on the other hand, is also recruited to pause sites during transcription elongation and allows the ternary complex to resume transcription by processing the 3′ end of the nascent mRNA (Marr and Roberts, 2000; Toulme *et al.*, 2000). Unlike Mfd, GreA is not involved in DNA repair. Deficiencies in Mfd reduce or abolish the ability of cells to accumulate mutations in non-functional *hisC*, *metB*, and *leuC* alleles under conditions of starvation (Ross *et al.*, 2006). Similarly, a *greA*$^{-}$ strain shows a significant reduction in adaptive *leuC* mutation (in preparation). The response observed in the *greA*$^{-}$ strain was not as dramatic as the one reported for the *mfd*$^{-}$ strain (Ross *et al.*, 2006). Whether the observed reduction in the accumulation of adaptive mutations in Mfd and Gre deficient strains is due to a decrease in transcript accumulation or transcriptional bypass (transcriptional mutagenesis) remains to be elucidated. However, preliminary experiments measuring adaptive mutation and transcript accumulation in *B. subtilis* suggest that the lack of Mfd or GreA results in reduced transcript levels of the alleles under selection. Transcription of genes under neutral selection was not affected by Mfd or GreA deficiencies (in preparation).

The effect of Mfd on the adaptive mutagenesis process has been visited previously in *E. coli*, (Bridges, 1995). The results of these experiments led to the conclusion that Mfd was not involved in this process. However, results recently presented (Ross *et al.*, 2006) clearly indicate a role for Mfd in stationary phase induced mutagenesis in *B. subtilis.* The difference in results between *E. coli* and *B. subtilis* could be due to basic differences in the mechanisms involved or could be caused by some type of allele specificity (*i.e.*, more alleles need to be checked in the *E. coli* model system). In addition, Mfd, in *E. coli*, has also been hypothesized to influence transcriptional bypass (Saxowsky and Doetsch, 2006).

The effects of different transcription factors in transcriptional bypass in eukaryotes have been recently examined in a Hela cell-extract *in vitro* transcription system. This system relies in an immobilized template with engineered DNA damage residues, *e.g.*, 8-oxo-G or hydroxyuracil, serving as the substrate for transcriptional bypass. Transcriptional bypass of RNA pol II blocked by templated DNA damage was promoted to different levels depending on addition of different elongation factors. Addition of purified CSB and TFIIS (human homologs of Mfd and GreA, respectively) mediated transcriptional bypass of 8-oxo-G. More strikingly, addition of TFIIS to 8-oxo-G-blocked RNA pol II promoted less transcriptional bypass than addition of CSB (Charlet-Berguerand *et al.*, 2006). Thus, it is possible to speculate that the observed effects of Mfd and GreA deficiencies on adaptive mutagenesis in *B. subtilis* are mediated by their ability to promote transcriptional bypass similar to what is seen with their human homologs.

Transcription elongation factors promote transcriptional bypass in single and multicellular organisms. This raises the question of the fitness trade-off associated with this phenomenon. While in single-cell organisms transcriptional bypass may result in escape from stationary phase, the consequences of the same process in highly differentiated multicellular organisms may give rise to neoplasia and other detrimental effects (Doetsch, 2002). Why has the process of transcriptional bypass not been selected against in multicellular organisms since it promotes cell-selfish modes of replication (Holmquist, 2002)? Perhaps, the likelihood of genetic instability in cells unable to bypass certain types of DNA damage outweighs the likelihood of generating a transient phenotype that in most instances is of no consequence. This concept is supported by studies that show defects in elongation factors such as CSB and ELL2 are associated with disease states such as Cockayne's syndrome and Acute Myeloid Leukemia, respectively (reviewed by Conaway and Conaway, 1999).

In summary, there is a significant association between aspects of transcription and adaptive mutagenesis. Furthermore, factors that modulate transcription play key roles in transcription associated mutagenesis. Of the factors modulating transcription, Mfd appears very influential on the process of adaptive mutagenesis and provides a link between cell physiology (Mfd has been reported to mediate catabolite repression in *B. subtilis*

(Zalieckas *et al.*, 1998; Zeng *et al.*, 2000), DNA repair, transcription and mutagenic processes.

Methods to Study Adaptive Mutagenesis

It is important to mention that the current approaches used in the study of adaptive mutation in prokaryotes still rely heavily on testing all the cells in a bacterial culture, which assumes homogeneity of cellular responses to conditions of stationary culture. The pathways of differentiation taken by *B. subtilis*, the stoichiometry and protein interactions required for the activation of a specific regulon and the discussions on the two models presented above suggest that the onset of stationary culture generates heterogeneity in the population. As such, the current approaches examining adaptive mutation require the integration of techniques that allow the observation and manipulation at the subpopulation and single cell levels. Techniques for assaying transcriptional bypass, the observation of and manipulation of subpopulations are readily available in *B. subtilis* or may be adapted from *E. coli* (Bregeon and Doetsch, 2004; Charlet-Berguerand et al., 2006; Maamar and Dubnau, 2005; Viswanathan et al., 1999; You et al., 2000).

CONCLUSION

Studying adaptive mutagenesis in prokaryotic models, *E. coli* in particular, has incited a number of insights into the evolutionary process. Examination of adaptive mutagenesis in *B. subtilis* suggests two new models for the acquisition of mutations in conditions of stress. Much is yet to be learned about the effects of stress and the role of cell physiology and differentiation as it relates to mutagenesis. The use of better tools to examine bacterial culture at the subpopulation and single-cell levels, and to examine transcription in the absence of replication will provide clues to the mechanisms underlying the adaptive mutation phenomena in *B. subtilis*. Future experiments involving strains that combine defects in competence and different DNA repair systems; control of transcription of alleles under selection; and transcriptional bypass in *B. subtilis* and measurement of adaptive mutation will determine how critical the development of subpopulations of cells and transcription associated mutation is for increasing genetic diversity among the prokaryotes and for the development of neoplastic events in multicellular organisms.

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