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Mfd and transcriptional derepression cause genetic diversity in *Bacillus subtilis*

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1. ABSTRACT

Scientists have been aware for many years of genetic programs that get activated under stress and produce genetic variants in cells that escape non-proliferating conditions. These programs are well conserved in all organisms and expand our view of evolution. They mediate genome instability, create diversity in antibody formation, expand metabolism and increase fitness of pathogens within host environments. Error-prone DNA replication and repair are genetic variability-causing agents that get stimulated by the onset of cellular stresses. Embedded in these programs is the ability to limit mutagenesis to defined genomic regions and times, ensuring integrity of most of the genome. Recent evidence suggests that factors involved in RNA polymerase (RNAP) processivity or transcriptional derepression contribute to the generation of stress-induced mutations. In *Bacillus subtilis*, transcription-associated mutagenesis has been shown to be independent of recombination-dependent repair and, in some cases, of the Y DNA polymerases. Central to stationary-phase mutagenesis in *B. subtilis* is the requirement for Mfd, transcription coupling repair factor, which suggests a novel mechanism from those described in other model systems.

2. INTRODUCTION

Stationary phase mutagenesis is the process by which cells under stress, or non-proliferating conditions, activate programs that increase the frequency of mutations (1; 2; 3; 4). This includes beneficial mutations that permit cells to escape stressful conditions. Many studies have focused on how, or even whether, genes under selection are the target of programs that stochastically produce mutations. These studies have contributed greatly to our understanding of DNA repair and replication as well as broadened our view of the evolutionary process. Further, the factors affecting these programs are well conserved and generate beneficial and detrimental mutations in organisms spanning all three domains of life.

There are several reviews on stress-induced mutagenesis that use bacterial and eukaryotic systems which indicate that genetic variability is produced by different mechanisms (1; 2; 3; 4). The most studied model is that of the *E. coli* plasmid-borne FC40 *lac* frameshift system (5). In this system, Lac^- cells are placed under lactose starvation for prolonged times and measured for Lac^+ reversion. However here, we focus on the mechanisms of stress-induced transcriptional mutagenesis.

We examine the generation of mutations in *B. subtilis* cells under amino acid starvation stress and its dependence on Mfd, the transcription coupling repair factor.

First described in 1966 (6), Mfd is a known repair factor involved in promoting repair of DNA lesions that cause transcription elongation complexes to stall. Mfd has been shown to mediate this process by dissociating the RNAP and recruiting the nucleotide excision repair system to such sites (7; 8; 9; 10; 11). The nucleotide excision repair system functions by first generating nicks at the 5' and 3' of the lesion. A helicase (UvrD) then removes the DNA fragment and finally a DNA polymerase (PolI) refills the gap created by the helicase. This model suggests that the effect of Mfd is to prevent the formation of mutations; this has been shown amply in non-stressed cells. However, in nutritionally stressed *B. subtilis* cells, Mfd actually promotes the formation of mutations (12). In this short perspective, we provide brief descriptions of the *B. subtilis* system, factors influencing the generation of stress-induced mutations in this system, and a discussion of potential mechanism(s) by which Mfd mediates this mutagenic process. In the interest of space, we apologize to those whose work was not cited.

3. STATIONARY PHASE MUTAGENEIS IN *B. SUBTILIS*

The study of stress-induced mutagenesis in *B. subtilis* has relied on amino acid starvation as a model for stress (13; 14). In one system developed by Sung and Yasbin, chromosomal defective alleles for histidine (*hisC952* – nonsense), methionine (*metB5* – nonsense) and leucine (*leuC427* – missense) are used to interrogate cells for their ability to accumulate reversions to prototrophy over a ten-day period of starvation (14). In contrast to the FC40 *lac* system in *E. coli,* in which most reversions are acquired through frameshifts, reversion to prototrophy in the *B. subtilis* system relies on the generation of base substitutions and may be acquired by either suppression (His and Met markers) or true reversions (all three markers), which allows improved genetic analysis. What is observed in these assays is an increase in the number of revertant colonies as a function of the duration of incubation while the non-revertant cells remain in a non-growing state. Analysis of early (those that appear between 2 and 4 days after incubation) and late (those that appear after 5 days of incubation) appearing colonies indicate that most revertants are neither affected in rate of growth nor the ability to repair DNA (12; 14; 15).

3.1. Contributing factors of stationary phase mutagenesis in *B. subtilis*

Results from several studies that used the three marker-system described above indicate that *B. subtilis* manifests mutagenic processes during times of stress. Several unique factors that influence stationary phase mutagenesis have been determined. First, the accumulation of adaptive mutations in three chromosomal loci is recombination-independent (14). Second, activation of the regulon under the control of the stress sigma factor ($Sigma^B$ in *B. subtilis*) is not required for the generation of stationary phase mutants (14). Thirdly, the ability to generate stationary phase mutations appears to be dependent on the activation of a cell differentiation pathway (14). Lastly, the accumulation of mutations in genes under selection during stationary phase is mediated by Mfd and transcriptional derepression, or increases in the rate of transcription of the gene under selection (12,16).

In addition to the observations highlighted above, other factors influencing stationary phase mutagenesis in *Bacillus subtilis* include deficiencies in mismatch repair, oxidative damage prevention, and the PolYI Y-polymerase (15, 17, 18). PolY1 deficiency results in a decrease of almost half of the His^+ reversions and no change in the number of Leu^+ mutations in *B. subtilis* (15). Studies with agents that generate reactive oxygen species and strains that are defective in the ability to prevent oxidative (GO – 8 oxo-guanine) damage showed an increased occurrence of mutations in all three markers in exponentially growing cells, but in stationary phase cells only His^+ and Met^+ mutations were increased (19). Thus, it appears that activation of specific mutagenic DNA pathways (a more general one in conditions of growth – replication dependent) are affected by nutritional conditions in *B. subtilis*. The increased mutagenesis mediated by defects in the GO repair system mapped exclusively to ochre suppressor genes and could be complemented by overexpressing the components of the mismatch repair system (19). Further, the stationary-phase cells defective in GO repair did not accumulate reversions to leucine biosynthesis differently than the wild type, which suggests that adaptive Leu^+ mutations are not generated by oxidative damage. The role of repair of oxidative damage in stationary phase mutagenesis is further complicated by the results from experiments that included strains deficient in MutY activity (removes A from OG:A pairs). These studies showed that deficiencies in MutY nearly abolished the formation of Leu^+ mutations and suggest a mutagenic activity by this enzyme (20). Future research in the context of oxidative damage that examines how nutritional conditions activate mutagenic pathways will aid in elucidating the role of MutY in the generation of adaptive mutations.

4. MFD, TRANSCRIPTIONAL DEREPRESSIN AND STATIONARY PHASE MUTAGENESIS

The process of transcription is carried out by RNAP, a dynamic enzyme that is active in two conformations and interacts with many factors during gene expression. These interactions alter all aspects of the transcription process from initiation, elongation, and termination through the recycling of RNAP. During the process of transcription elongation RNAP often encounters pauses that are due to specific sequences, substrate availability, proteins occupying DNA, or to lesions which prevent synthesis of full messages or which decrease the rate of transcription. Mfd and other factors including Gre and Nus proteins significantly influence the processivity of RNAP (21). Mfd, the transcription repair coupling factor, was first described in experiments that investigated the repair of UV damage in *E. coli.* Auxotrophic strains were

UV-irradiated and inhibited for protein synthesis. These studies described a mutant strain that showed an increased mutation frequency compared to the parental strain (6). Later studies, in *E. coli*, indicated that this factor promoted repair in transcribed DNA regions and that it targeted the template DNA strand during transcription (11). More recent studies that examined protein structure and function indicate that Mfd is complex and has three different modules that allow this protein to interact with the upstream region of the transcription complex, recruit repair systems to transcribed regions and translocate RNAP (21). At sites in which the RNAP stalls, the translocate activity of Mfd may realign the active site of the backtracked/paused RNAP to the 3' terminus of the nascent mRNA. This translocase activity is ATP-dependent, is located within the C terminus and may promote the paused RNAP back into active transcription (22). Then, the interaction between Mfd and a stalled RNAP leads to one of two outcomes, RNAP and transcript release, or rescue into active transcription (23).

Recently, it has been shown that Mfd promotes the generation of mutations in stressed *B. subtilis* cells in experiments that measured base substitutions in the three marker-system described above (12). Because the system used in those experiments allows detection of revertants by tRNA suppressor mutations, the authors also concluded that most of the mutagenic promoting effect of Mfd was on the generation of mutations in the genes under direct selection. While the generation of true revertants was significantly decreased in Mfd deficiency, the generation of tRNA suppressors was not. These observations suggested that Mfd was affecting the generation of true revertants through a transcription-associated process. Gene expression measured in *leuC427* indicated that the absence of Mfd did not affect cell viability and only affected expression in starving cells (16). Taken together these reports lend strong support to the concept that stressed *B. subtilis* cells activate mutagenic processes in transcriptional derepresed genes that are under selection. In a broader context, linking mutagenic processes to DNA regions of high levels of transcription would be a good strategy for cells to escape non-proliferating states because it limits the generation of mutations in time and genome location.

The idea that transcriptional derepression promotes mutagenic events in growing cells is well established and several reports have demonstrated in different systems that in the presence of DNA damaging agents or deficiencies in DNA repair systems transcribed DNA is more prone to mutations than non-transcribed regions (24; 25; 26; 27; 28). The concept of transcriptional derepression mediating spontaneous mutagenic events has been tested in bacteria and eukaryotes, particularly in the context of conflicts with replication (29; 30; 31). Other experiments have studied the effect of transcriptional derepression on spontaneous rates of mutation (*argH*, *leuB* in *E.coli*; *lys-3* and *phe* in *B. subtilis*), as affected by the stringent response, and also suggest that replication-dependent mutations are significantly increased in conditions of active transcription (13; 32; 33; 34). Those experiments (in *E .coli* and *B. subtilis*) used growing cultures of strains carrying deficiencies in RelA, which synthesizes the alarmone effector molecule ppGpp/p and influences transcription levels of genes for amino acid biosynthesis when nutrients become limiting. The rates of reversion in auxotrophic mutants were higher in stringent cells than in relaxed cells and the increase in mutations was attributed to the increased levels of transcription observed in the stringent cells. Considering the reports on conflicts between replication and transcription, it is reasonable to speculate that the effect of transcriptional derepression on spontaneous mutation rates is a consequence of how conflicts between replication and transcription are processed, and that the presence of transcription elongation factors, such as Mfd, is to prevent the generation of mutations or genome instability. Evidence for how the presence of transcription elongation factors aids in restarting replication has been recently presented *in vitro* and *in vivo* in *E. coli* without activating the SOS response (35, 36). Since a majority of ORFs in the *B. subtilis* genome co-orient with replication, this suggests that co-orienting transcription of genes with replication is a measure to preserve genome integrity in conditions of active replication (31).

In the context of prolonged starvation, non-growing conditions, or when genome replication is halted or inhibited, what is the effect of the transcriptional derepression on the accumulation of mutations? There are a few studies that approach this question and they indicate that transcriptional derepression mediates mutagenic events in *B. subtilis* resting cells. The effect of transcriptional derepression on stress-induced mutagenesis in *B. subtilis* was demonstrated by an experiment that maintained cells in conditions of starvation even in the presence of mutations that revert to leucine prototrophy and as affected by transcriptional activation or repression. Experiments that used an IPTG promoter to control transcription of *leuC42*, showed that starving non-growing cells accumulated 10 times more leucine revertants in the presence of IPTG than in its absence (16). Because the cells tested in that study were subject to starvation for two amino acids and revertants were detected only after supplying one of the growth-limiting amino acids, it is possible to speculate that the effect of transcriptional derepression on the accumulation of mutations in *B. subtilis* cells do not require genome replication, collisions with replication forks or the formation replication intermediates.

5. STRESS-INDUCED MUTAGENEIS IN *E. COLI*

While the majority of this article focuses on stationary phase mutagenesis in *B. subtilis*, we would like to briefly discuss the well-studied *E. coli* FC40 model and contributing factors that influence the generation of stress-induced mutations in that system. In this plasmid-borne system, Lac^- cells are placed under lactose starvation for prolonged times and measured for Lac^+ reversion which can be generated through amplifications or frameshifts. One mechanism underlying the generation of Lac^+ stress-induced point mutations involves the formation of a subpopulation of cells and the repair of double stranded breaks in DNA. Cells that acquire a double stranded break

in their DNA repair such event by generating DNA replication intermediates that require recombination functions to resume DNA replication. This process faithfully repairs the double stranded break damage; however, in cells that enter a state of stress or starvation, the fidelity of the double stranded break repair is compromised or becomes mutagenic because DinB, an error prone DNA polymerase expressed under conditions of DNA damage or starvation, competes with the high fidelity polymerase PolIII at sites of repair (37, 17, 38, 39).

In addition to frameshifts and other point mutations, stress-induced amplifications or growth-dependent amplification have also been proposed to generate Lac^+ revertants (40, 41). The amplified *lac* variants are selected because expression of the *lacI'-'lacZ* fusion gene product confers a reduced ability to use lactose as a carbon source and so acquiring an amplified *lac* arrangement is beneficial to lactose-starving cells. The stress-induced *lac* amplifications may also be generated during acts of repair of DNA double stranded breaks; however, the formation and the components required for stress-induced *lac*-amplified variants differ somewhat from those required for the formation of point mutated Lac^+ revertants. While the increase of point mutated Lac^+ revertants occurs at a constant rate during stress, the majority of *lac*-amplified colonies appear late during starvation stress. The generation of stress-induced *lac*-amplified colonies is DinB-independent, but requires the activation of the stress response, controlled by $sigma^S$, and the 5-3 exonuclease activity of PolI. It is proposed that stressed cells may generate tandem arrangements of the *lac* region by template switching between the lagging strands of two replication forks (a recipient fork moving ahead of a donor one) mediating the repair of a double stranded break. In this model regions of microhomology (5-15 bases located at the junctions of the *lac* region) mediate the exchange of a newly synthesized 3'end from the donor fork to the recipient fork. This exchange results in a priming intermediate that is stabilized by the 5-3 exonuclease activity of PolI, which processes Okasaki fragments, and replicative extension. If the participating forks are at disparate points in replication and the template switching intermediate results in the formation of a Holliday junction, its resolution generates amplified *lac* arrangements (40). In summary the generation of Lac^+ variants is dependent on the manner in which repair of double stranded breaks in stressed cells proceeds. The models in the *E. coli* paradigm support the concept that cells activate mutagenic processes that are stochastic, limited to times of stress and to sites of double stranded breaks (2; 40).

The effect of transcription, as affected by Mfd, on the formation of adaptive mutations has been previously tested and those experiments, which tested tyrosine reversion in tyrosine-starved strains, showed no significant differences between Mfd-deficient and Mfd-proficient strains (42). Whereas Mfd does not affect starvation-associated mutations in *E. coli* at the *tyrA* locus, the NusA, an RNA polymerase (RNAP) processivity factor, significantly influences the generation of Lac^+ and Tc^r mutations in the *lacI* FC40 system (43). Specifically, a variation of transcription coupled repair, mediated by NusA, has been recently proposed as a mechanism in the formation of stress-induced Lac^+ mutations (43; 44). Two other striking observations from those NusA studies were the presence of a temperature effect on stress-induced mutagenesis and a DinB subordinate effect to NusA in the generation of mutations. Because NusA directly interacts with the error-prone polymerase DinB, it is hypothesized that the function of NusA is to potentiate DNA translesion synthesis at sites of stalled transcriptional elongation in stressed cells (45, 43). This transcription coupled-translesion synthesis was tested in the context of how cells survive to nitrofurazone or 4-nitroquinolone-1-oxide treatments and as affected by the partial loss-of-function phenotype of a temperature-sensitive *nusA* allele. Further experiments that involved transcription *in vitro* assays and strains with single and combined null mutations in *nusA* and *mfd* suggest that *i*) RNAP is paused at nitrofurazone-induced damage to or single stranded gaps on the template strand, *ii*) NusA interacts with the nucleotide excision repair system, previously shown to process nitrofurazone-induced damage, and acts additively with Mfd in the repair of UV- and alkylating damage, *iii*) NusA does not influence bypass of RNAP at such sites and *iv*) transcription pausing or stalling may not require nitrofurazone induced-damage (N^2-furfuryl –dG) to enter the active site of RNAP (46, 44). In the context of the models explaining the formation of Lac^+ mutations in the FC40 system, it remains to be elucidated how NusA-mediated transcription coupled repair is involved in the repair of double stranded breaks. In light of the role that transcription factors play during conflicts between transcription and replication, the observations in *E. coli* would then suggest that transcription elongation factors protect genome integrity in rapidly growing cells, but also potentiate the production of genetic diversity in times of stress or conditions that prevent genome replication.

6. PROPOSED MODELS ON HOW MFD PROMOTES MUTAGENESIS

How does Mfd influence the generation of mutations in *B. subtilis*? Considering the enzymatic analyses of Mfd in *E. coli* and eukaryotic systems and expanding on the idea that transcription elongation factors mediate the production of genetic diversity, it is tempting to speculate on two possible models that may not be mutually exclusive. A model in which Mfd recruits repair systems and error-prone polymerases to highly transcribed regions could explain the increase in the accumulation of mutations over time that has been previously reported (12). This Mfd-dependent mechanism is somewhat similar to the NusA-mediated mechanism proposed in *E. coli* in that it recruits translesion synthesis to sites of paused transcription elongation complexes. Another possibility is that Mfd promotes RNAP bypass of DNA lesions or restarts paused complexes leading to transient phenotypes through transcriptional mutagenesis. The transient phenotype would provide escape from the stress causing replication which may lock the lesion into a mutation in the absence of high fidelity repair (47; 48). Hence, by these two models Mfd could potentially generate variants subject to selection

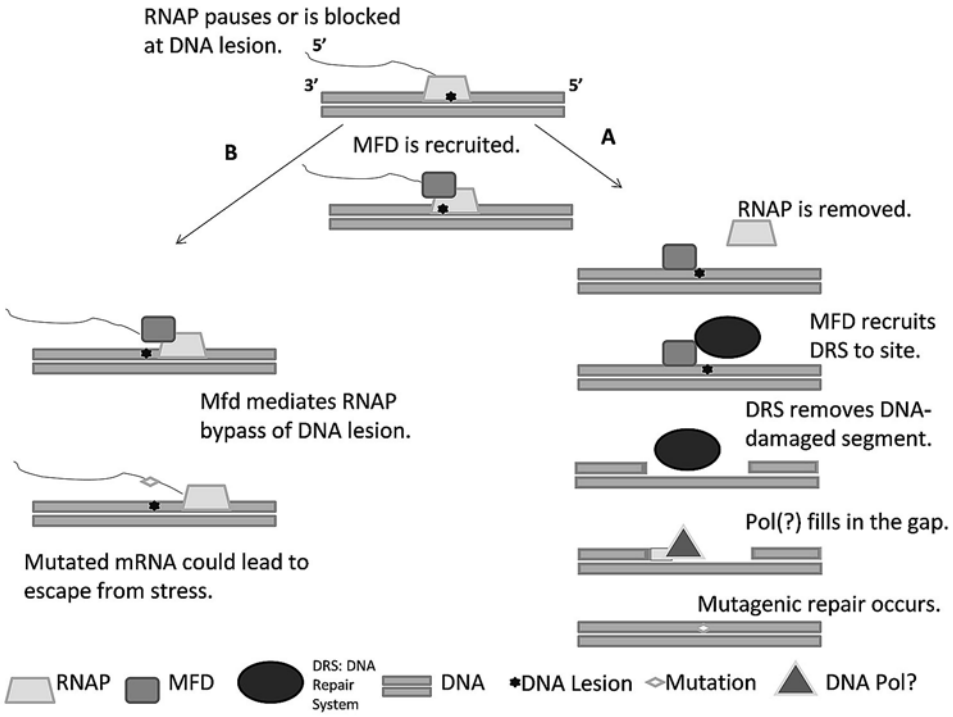

Figure 1. Proposed models for the role of Mfd in stationary phase mutagenesis. A) Mfd is recruited to highly transcribed regions in which RNAP stalls due to different DNA lesions, obstacles or sequence-specific pauses. Recruitment is followed by displacing of RNAP from the DNA template and enlisting of repair systems that generate gaps or nicks which are subsequently replicated by translesion synthesis polymerases or PolI. B) Mfd, alone or in combination with Gre factors, facilitates stalled transcription elongation complexes to go back into active transcription, by realigning backtracked RNAP and the DNA template strand. Such event may result in the formation of mutated mRNA that provides a transient phenotype that triggers a burst of growth/replication that introduces a mutation at a DNA site where the original lesion is located or any other lesion site that confers a growth advantage (not shown).

in times of stress at the DNA and RNA levels. These models are illustrated in Figure 1.

In the case of Mfd and *B. subtilis*, it is tentative to speculate that repair systems associated with transcription coupled repair and one or more polymerases are responsible for generating stress-induced mutations. Figure 1A illustrates such concept which is a variant of transcription coupled repair; at regions where transcription elongation stalls Mfd recruits a repair system in combination with low fidelity replication that engenders the generation of mutations. This scenario is supported by the following observations. First, cells in stationary phase accumulate mutagenic lesions, which would increase the potential for stalling or pausing at highly transcribed regions (see discussion on contributing factors), and the act of transcription itself has been shown to generate DNA structures that prone DNA residues to lesions (49). Second, Y-polymerases are expressed in conditions of stress in *B. subtilis* (50; 51). Thirdly, PolI (*polA*), in addition to processing Okasaki fragments, is part of the transcription-coupled repair pathway and, in the case of *B. subtilis*, lacks 3'-5' proofreading activity and has been postulated to participate in acts of translesion synthesis and/or spontaneous mutagenesis (50; 51). Lastly, Mfd, in *E. coli*, or its functional homologue, CSB, in mammalian cells have been shown to displace transcription complexes that are stalled at bulky and non-bulky lesions as well as single strand nicks or pauses and recruit different repair systems (caused by non-bulky lesions, protein barriers, specific sequences or low substrate) (8; 52; 53; 54; 55; 56). Hence, it is not unreasonable to entertain the hypothesis that Mfd, in *B. subtilis,* mediates two versions of transcription coupled repair, a high fidelity one and its mutagenic variant. No experiments on the influence of the interaction of Mfd, DNA repair factors or error-prone polymerases on stationary-phase have been presented in *B. subtilis*. Because Mfd does not influence the generation of stress-induced mutations in *E. coli*, comparative analysis between the *E. coli* and *B. subtilis* proteins is likely to shed some light on the mutagenic mechanism operating in *B. subtilis*.

Another possible model by which Mfd influences the generation of stationary-phase mutations is by promoting transcriptional bypass and the production of transient phenotypes (Figure 1B). Transcriptional bypass is the formation of altered mRNAs via the misincorporation of NTPs when the transcription elongation complex encounters a miscoding lesion in the template. It has been shown that RNAP bypasses different lesions with different

efficiencies (57). These erroneous messages generated by transcription stalling, DNA lesions or abasic sites, are efficiently translated and may give rise to altered protein functions that license a stressed or non-dividing cell back into replication. The lesion giving rise to the transient altered function gets locked into DNA if replication takes place before repair (47; 48). This process has been shown to influence bistable systems in bacteria and provide eukaryotic cells the potential for transient phenotypes and neoplasia (58, 59, 60). Whether Mfd affects transcriptional mutagenesis has not been directly addressed in *B. subtilis*, however, analyses in other models suggest that the effect of Mfd, or its functional homologues, on transcriptional bypass varies depending on the model used. The idea that Mfd influences the generation of altered mRNAs is supported by the observation that its translocase activity realigns the 3' end of the nascent mRNA with the active site of the backtracked RNAP and the base in the DNA template strand (23). In light of this, it has been speculated that Mfd influences the ability of RNAP to bypass different lesions (61). This speculation gathered support by experiments in which HeLa cells extracts were used to determine whether lesions on the template strand that stalled RNAPII could be bypassed upon addition of CSB, the functional homologue of Mfd in animal cells. Those experiments showed that CSB and other elongation factors homologous to Gre factors facilitated bypass of bulky (thymine glycol) and non-bulky (8-oxo-guanine) lesions at the expense of generating an altered transcript (62). However, in *E. coli* Mfd reduces transcriptional bypass by mediating transcription coupled repair and may or may not be recruited to non-bulky lesion sites (52; 53; 56). There are no experiments in *B. subtilis* that measure transcriptional bypass or the factors promoting it. However, the experiments that determined that transcriptional derepression increased stress-induced mutagenesis used conditions that eliminated any transient advantages, the trademark of transcriptional mutagenesis, by maintaining cells under double selection and suggest that such process is not involved in stress-induced mutagenesis in this Gram positive bacterial system (16).

7. CONCLUSIONS

Programs for the increase of genetic diversity in cells under stress are interesting because they provide mutagenic mechanisms that are limited in time and genomic space, which allows cells "to experiment" without compromising integrity of the whole genome or approaching genetic load. One interesting concept is that of stressed cells "experimenting" in regions that are transcriptionally derepressed. Here we hope to provide an account of stress-induced mutagenesis in *B. subtilis* and its dependence on Mfd as a potential link between transcription and mutagenesis. Interestingly, this factor does not affect starvation-induced mutations in *E. coli*, as examined by mutations in amino acid biosynthesis genes or the formation of mutated transcripts and their potential for transcriptional mutagenesis. However, the link between transcription and mutagenesis in *E. coli* stressed cells takes place through another transcription elongation factor, NusA, in the plasmid-borne FC40 system. Furthermore, Mfd and other transcription elongation factors resolve conflicts between transcription and replication, and maintain genome integrity in conditions of active growth. Perhaps in non-replicating conditions Mfd has an added function to mediating high fidelity repair of transcribed DNA in growing cells, that of increasing variability in genes under selection.

We propose two models by which Mfd affects the generation of stress-induced mutations in *B. subtili*, based on previous observations in either *E. coli, B. subtilis,* or other systems. While these Mfd-dependent models may not occur in *E. coli*, the models remain to be and should be tested in *B. subtilis* or other systems. Even more interesting is the subject of how or whether the Mfd-dependent increase in mutagenesis in non-proliferating conditions is selected and how it relates o the progression of the cell cycle in eukaryotes. In bacteria, Mfd mediates the formation of mutations that expand metabolism and resistance to antibiotics in cells under stress (12; 63). In humans its functional homologue, CSB, is associated with several degenerative diseases, Cockayne Syndrome most notably, and has been proposed as a therapeutic target in combination with the use of antiproliferative drugs, or as part of the pathway by which some drugs inhibit some cancers (8, 64, 65).

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