Contribution of the Mismatch DNA Repair System to the Generation of Stationary-Phase-Induced Mutants of *Bacillus subtilis*

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A reversion assay system previously implemented to demonstrate the existence of adaptive or stationary-phase-induced mutagenesis in *Bacillus subtilis* was utilized in this report to study the influence of the mismatch DNA repair (MMR) system on this type of mutagenesis. Results revealed that a strain deficient in MutSL showed a significant propensity to generate increased numbers of stationary-phase-induced revertants. These results suggest that absence or depression of MMR is an important factor in the mutagenesis of nongrowing *B. subtilis* cells because of the role of MMR in repairing DNA damage. In agreement with this suggestion, a significant decrease in the number of adaptive revertant colonies, for the three markers tested, occurred in *B. subtilis* cells which overexpressed a component of the MMR system. Interestingly, the single overexpression of mutS, but not of mutL, was sufficient to decrease the level of adaptive mutants in the reversion assay system of *B. subtilis*. The results presented in this work, as well as in our previous studies, appear to suggest that an MMR deficiency, putatively attributable to inactivation or saturation with DNA damage of MutS, may occur in a subset of *B. subtilis* cells that differentiate into the hypermutable state.

Adaptive or stationary-phase-induced mutagenesis occurs in nondividing cells during prolonged nonlethal selective pressure, e.g., starvation for an essential amino acid (25). While most of the research has involved *Escherichia coli* model systems, similar observations have been made in other prokaryotes (14, 30) as well as in eukaryotic organisms (8). The most widely studied system thus far has been the F′ lac frame-shift-reversion construct of *E. coli* (25). In this system it has been demonstrated that generation of Lac+ stationary-phase-associated revertants is dependent on (i) a functional Rec system (10), (ii) F′ transfer functions (6), and (iii) a component(s) of the SOS system (18, 19). In addition, both DNA polymerase III and the SOS-regulated DNA polymerase IV (19) have been shown to be responsible for the synthesis of errors that lead to these mutations (for review of the SOS regulon see reference 33). More recent evidence demonstrates that the mutations generated by this lac system during stationary phase are the result of actual cell growth and amplification of the plasmid-borne gene that is followed by SOS-induced mutagenesis and selection (11, 28).

The existence of stationary-phase-induced mutagenesis was recently demonstrated in *Bacillus subtilis* following the utilization of a reversion assay system (30). In contrast to the F′ lac system of *E. coli*, this type of mutagenesis in *B. subtilis* is not dependent upon a functional RecA protein (i.e., recombination or the activation of type 1 SOS functions [35] was not required). Moreover, it was also demonstrated that generation of *B. subtilis* adaptive mutants did not require a functional σB factor (RNA polymerase σB controls the general stress response in *B. subtilis* [34]). However, one of the most relevant outcomes derived from studying adaptive mutagenesis in *B. subtilis* was the observation that transcription factors such as ComA and ComK (21) did influence the eventual production of stationary-phase-induced mutants (30). More recent evidence has demonstrated that a null mutation in *ygiH*, which encodes a homolog of the UmuCDinB or Y superfamily of DNA polymerases, affected the generation of revertants of the *hisC952* allele in the *B. subtilis* system (32). Thus, for the adaptive mutagenesis phenomenon of *B. subtilis* a hypothesis was advanced that basically proposed that during periods of environmental stress subpopulations are differentiated within a culture in order to generate genetic diversity (30). Furthermore, it was proposed that within some of these subpopulations 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 damage into the genome (30).

The MutS and MutL proteins of the mismatch DNA repair (MMR) system have been conserved across the domains of life and play important roles in several DNA repair pathways (20). In addition to mutation avoidance, MMR has been linked to control of genome rearrangements (22), gene transfer among species (17), and the development of cancer (4, 23).

MMR was first linked to adaptive mutagenesis in the F′ reversion system of *E. coli*, following the observation that the type of mutants that gave the lac+ reversion genotype were almost all −1 deletions in a region of small mononucleotide repeats (5, 26). This was the category of growth-dependent revertants found in cells lacking a functional MMR system (16). These findings are apparently contradictory because production of adaptive lac+ mutants still occurs in wild-type cells where MMR is functional. Based on these collective observations, it was suggested that the function of the MMR system (namely, MutL) is in some way inhibited exclusively during the adaptive or stationary-phase-induced mutagenesis process (9).
Further evidence showed that overproduction of MutL (but not MutS) decreased the number of mutations generated by the adaptive process(es) without affecting growth-dependent mutagenesis (9). In addition to these findings in *E. coli*, a homolog of the MutS protein has also been found to be involved in the generation of stationary-phase-induced mutants in *Saccharomyces cerevisiae* (8).

In the present communication, we took advantage of the reversion system of *B. subtilis* (30) to study the effects of the MMR proteins, MutS and MutL, on the production of stationary-phase-induced mutants in this bacterium. Our results demonstrate that a deficiency of the MMR system increases the number of revertants during the stationary phase of *B. subtilis*. On the other hand overexpression of the entire mutSL operon or just the mutS gene product (but not the mutL product) significantly diminished the production of stationary-phase-associated revertants for the three markers tested. Thus, our results support the hypothesis that at least one mechanism for the generation of adaptive mutants in *B. subtilis* is the loss or decrease of MMR activity during stationary phase.

**MATERIALS AND METHODS**

The bacterial strains used are listed in Table 1. *B. subtilis* YB955 is a prophage “cured” strain that contains the hisC952, metB5, and leuC427 alleles (30, 36, 37). Procedures for transformation and isolation of chromosomal and plasmid DNA were as described previously (1, 2, 27). *B. subtilis* strains were maintained on tryptose blood agar base medium (PB medium; Difco Laboratories, Detroit, Mich.). Liquid cultures of *B. subtilis* strains were routinely grown in NOB medium (nutrient broth; Oxoid) supplemented with 2% yeast extract (NOB-YE). Stationary-phase mutagenesis assays. The bacterial strains used are listed in Table 1. *B. subtilis* YB955 is a prophage

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>YB955</td>
<td>hisC952 metB5 leuC427</td>
<td>37</td>
</tr>
<tr>
<td>MPRYB148</td>
<td>YB955 containing pMPRYB146</td>
<td>This study*</td>
</tr>
<tr>
<td>MPRYB149</td>
<td>YB955 containing pMPRYB147</td>
<td>This study</td>
</tr>
<tr>
<td>MPRYB150</td>
<td>YB955 containing pDG1048</td>
<td>This study</td>
</tr>
<tr>
<td>MPRYB151</td>
<td>YB955 carrying matSL neo</td>
<td>This study</td>
</tr>
<tr>
<td>MPRYB156</td>
<td>YB955 containing pMPRYB155</td>
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* See Materials and Methods for description of construction.

**RESULTS**

Generation of stationary-phase-induced mutants in an MMR-deficient *B. subtilis* strain. In *B. subtilis* the reversion frame of *mutS* and *mutL*, which encode the MMR proteins, are part of an operon (7). Therefore, we designed a plasmid termed pMPRYB140 to generate a *mutSL*-null mutant by

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eliminating part of the mutS and mutL ORFs and inserting a neomycin cassette in the middle of the operon (Fig. 1). This construction was transformed into B. subtilis YB955. A double crossover recombination event replaced the mutSL operon with the construction generating the Neo\(^r\) strain B. subtilis PERMYB151 (Fig. 1). Interruption of the mutSL operon containing the neomycin cassette of pBEST released with SmaI and the strain PERMYB150, which contained only the vector pDG148. For experiments 1 and 2, respectively. Thus, the \(\Delta\)mutSL strain had a mutation frequency that was at least 2 orders of magnitude greater than that of its parental repair-proficient strain.

As mentioned above, a reversion system to study stationary-phase-induced mutations in B. subtilis has recently been characterized (30, 32). We utilized this system to analyze the production of His\(^\ast\), Met\(^\ast\), and Leu\(^\ast\) revertant colonies during the stationary phase for the repair-proficient strain B. subtilis YB955 and its isogenic mutSL mutant derivatives. The results shown in Fig. 2A demonstrated that the number of his\(^\ast\) revertants accumulating during days 2 to 10 after plating was around three times greater in the MMR-deficient strain than with the parental strain YB955. As shown in Fig. 2B and C the \(\Delta\)mutSL strain produced between five and six times more met\(^\ast\) and leu\(^\ast\) revertant colonies during the stationary phase than did its parental strain. In general, Fig. 2 reveals that the number of adaptive mutants for the three markers in the mutSL operon or each of its components was overexpressed in strain YB955 harboring only the vector pDG148. For all three markers, the results demonstrated that overexpression of the mutSL or mutS genes significantly decreased the number of revertant colonies that arose as a result of stationary-phase-induced mutagenesis (Fig. 3). Interestingly, in contrast to the results obtained with mutS, the overexpression of mutL did not significantly affect the production of stationary-phase-induced mutants for the three markers tested (Fig. 3).

Effects of MMR overproduction in the generation of B. subtilis stationary-phase-induced mutants. As demonstrated above, B. subtilis cells lacking a functional MMR system had a significantly increased stationary-phase-induced mutation frequency compared to that of isogenic repair-proficient bacteria. This result suggested that the MMR proteins could be reduced or become nonfunctional in those cells responsible for generating stationary-phase-induced mutagenesis. Therefore, we next investigated this possibility. To this end, the entire mutSL operon or each of its components was overexpressed in strain YB955 and the numbers of His\(^\ast\), Met\(^\ast\), and Leu\(^\ast\) revertant colonies were scored during the stationary phase. The results were compared with those obtained with the B. subtilis strain YB955 harboring only the vector pDG148. For all three markers, the results demonstrated that overexpression of the mutSL or mutS genes significantly decreased the number of revertant colonies that arose as a result of stationary-phase-induced mutagenesis (Fig. 3). Interestingly, in contrast to the results obtained with mutS, the overexpression of mutL did not significantly affect the production of stationary-phase-induced mutants for the three markers tested (Fig. 3).

The results described in Fig. 4 revealed that the strains which overexpressed mutS, mutL, or both genes showed no differences in the rates of survival with respect to the parental strain PERMYB150, which contained only the vector pDG148. Therefore, the reduction in the number of His\(^\ast\), Met\(^\ast\), and
stationary-phase mutants generated by the strains PERMYB148 and PERMYB156, compared with those generated by the control strain PERMYB150, could not be attributed to a decreased survival of these strains on the selection medium.

The growth-dependent mutation rates of the strains PERMYB148, PERMYB149, and PERMYB156 for the generation of His\(^{+}\), Met\(^{+}\), and Leu\(^{+}\) colonies were also determined and compared with those of the control strain PERMYB150. The results shown in Fig. 5 revealed that the mutation rates of the strains which overexpressed either the individual genes or both genes of the mutSL operon were indistinguishable from those obtained for the strain PERMYB150. Thus, neither the independent nor the concomitant overexpression of the genes of the mutSL operon affected the spontaneous mutation rates of these strains during active growth stages.

Taken together, these results strongly support the conclusion that stationary-phase-induced mutagenesis occurs as a result of a significantly reduced amount of functional MMR proteins in B. subtilis.

**DISCUSSION**

The previously characterized reversion system of B. subtilis is being used to study stationary-phase-induced mutagenesis in order to yield insights into some of the mechanisms which control the production of adaptive mutants in this gram-positive microorganism (30, 32). Consequently, important differences have been found between the B. subtilis model and the F\(^{\prime}\) lac reversion system of E. coli, which has been the paradigm for adaptive or stationary-phase-induced mutagenesis. For instance, while the E. coli model requires functional recombination and SOS systems, such activities have been found to be dispensable in B. subtilis (30). Moreover, the production of stationary-phase-associated mutants has been shown to be influenced by the developmentally important transcription fac-
tors ComA and ComK (21) in B. subtilis (30), while no such interaction has been shown in the E. coli model.

Because of the involvement of these regulatory genes, it was suggested that, in B. subtilis, cell subpopulations with suppressed DNA repair systems (30) might be responsible for some or all of the adaptive or stationary-phase-induced mutagenesis. This hypothesis prompted us to investigate whether the MMR system is involved in stationary-phase-induced mutagenesis and whether this system might be inactivated or down-regulated in a hypermutable subpopulation of the culture. To this end we first constructed a strain of B. subtilis which lacked a functional mutSL operon. The results demonstrated that the rates of spontaneous mutation to rifampin resistance in this repair-deficient strain were around 100 times higher than those of its parental strain (see above). Compatible with this result, the growth-dependent mutation rates for the production of His\(^+\), Met\(^+\), and Leu\(^+\) colonies were also higher in the ΔmutSL strain than in the parental strain YB955 (Fig. 5).

In addition, the B. subtilis ΔmutSL strain was tested in the adaptive mutagenesis reversion assay. Results revealed that, in the absence of a functional MMR system, the mutant strain showed a clear propensity to increase the number of His\(^+\), Met\(^+\), and Leu\(^+\) revertants during stationary phase with respect to the number of revertants produced by the parental strain. In agreement with our results, the contribution of the MMR system in the adaptive mutation phenomenon was positively demonstrated in S. cerevisiae (8). Essentially, in that report the effects of each of the mutS and mutL homologs on the production of stationary-phase-associated mutants were independently tested and the strongest effect was obtained with an S. cerevisiae strain which lacked the MutS homolog MSH2 (8).

Although our results, utilizing the B. subtilis ΔmutSL strain, suggested that the MMR system is involved in adaptive mutagenesis, we wanted to determine the individual contribution of the MutS and MutL proteins to this phenomenon. Therefore, we implemented an approach similar to that utilized in E. coli (9). Specifically, the components of the MMR system were overexpressed in B. subtilis. With use of this approach, the overproduction of the proteins encoded by the mutSL operon resulted in a significant decrease in the number of mutations generated for all three markers via stationary-phase-induced mutagenesis (Fig. 3). A similar result was obtained following overexpression of the mutS gene. On the other hand, we did not observe a significant inhibitory effect in the production of adaptive revertants for any of the markers tested in the strain which exclusively overexpressed the mutL gene. Thus, our results strongly suggest that the MMR system, in particular the
MutS protein, is reduced in either amount or activity in those cells responsible for the mutation levels during the stationary phase of *B. subtilis*. However, overexpression of the mutSL operon did not completely suppress the production of stationary-phase-induced mutations. This result indicates either that we did not generate enough MMR proteins or that additional mechanisms are involved in the generation of stationary-phase mutations.

Our results also demonstrate that the complete elimination of the MMR system increases the production of stationary-phase-induced mutations. Thus, in *B. subtilis* either the overall functional level of MMR proteins is reduced in stationary-phase cells or there is a subpopulation that is lacking sufficient levels of active MMR proteins.

Although the MMR system has been shown to be involved in adaptive mutation in the F′ lac reversion system of *E. coli* (9), our results suggest that the mechanism of involvement could be different in *B. subtilis*. While the loss of function of the MutL protein was proposed to be responsible for an MMR deficiency during the stationary phase of *E. coli* (9), the evidence presented in this report strongly suggests that the MutS protein is the nonfunctional component of the MMR system for the production of stationary-phase-induced mutants in *B. subtilis*. In *S. cerevisiae*, the other microorganism where the MMR system has been shown to play a role in stationary-phase-induced mutagenesis, *MSH2*, a MutS homolog, has been implicated as the major contributor to this type of mutagenesis (8).

Taken collectively, the data tend to support the hypothesis that MMR is involved in at least a major share of the stationary-phase-induced mutagenesis process and that a completely functional MMR system might be absent or severely reduced in many if not all of those cells that mutate. Another possibility, and one that is not mutually exclusive, is that those cells responsible for producing stationary-phase mutations might have accumulated DNA damage that saturates the repair capacity of the MMR proteins. Experimental support for this latter hypothesis comes from results that demonstrate the involvement of error-prone polymerases in the generation of stationary-phase-induced mutations in the *E. coli* model system (6, 11, 18). In addition a recent report revealed that the generation of revertants of the hisC952 allele is significantly decreased in a *B. subtilis* strain that lacks the YqH protein, a homolog of the Y superfamily of *E. coli* DNA polymerases (32). This result suggests that error-prone replication occurs during the stationary phase of *B. subtilis*, as it does in *E. coli*. However, unlike the *E. coli* system, stationary-phase-induced mutagenesis in *B. subtilis* does not require RecA-controlled SOS functions (30).

The evidence presented in this report clearly indicates that a deficiency of functional MMR proteins exists in cells responsible for a significant portion of stationary-phase-induced mutagenesis. The question remains whether those cells that are responsible for this type of mutagenesis represent a distinct hypermutable subpopulation of the culture. With use of reporter gene technology, a previous report indicated that the level of transcription of the mutSL operon did increase in *B. subtilis* as the cells approached the end of exponential growth (7). These results would seem to be contrary to the ones that we have obtained (Fig. 3) unless a small subpopulation is envisioned as being responsible for the vast majority of the revertants generated via stationary-phase-induced mutagenesis. Such a differentiated hypermutable subpopulation is also suggested by the involvement of the *comA* and *comK* eubacterial differentiation transcription factors in the regulation of stationary-phase-induced mutagenesis (30). Interestingly, in the report by Ginetti et al. (7) mutations in *comA* or *comK* did not appear to affect the transcription of the *mutSL* operon. However, these results were based on the use of a reporter gene and could not measure actual translation levels or activity of MMR proteins.

Although the biochemical details of DNA repair through the MMR system have not been described for *B. subtilis*, it has been reported that the MutS homologs are very important in DNA repair, as they bind mismatches or small insertion-deletion events which can result from DNA polymerase errors (20). Therefore, mismatches generated during error-prone repair replication, in a subpopulation of non-growing *B. subtilis* cells, are possible targets which could contribute to saturating the DNA repair capacity of the MMR system. Equally plausible is the possibility that other DNA repair systems are reduced or inactivated in a subpopulation of the culture. Such activity would lead to the accumulation of mutations in these cells, especially if the MMR system had been reduced or eliminated. In this paper we believe that we have demonstrated the involvement of the MMR system in stationary-phase-induced mutagenesis of *B. subtilis*. Previously, we showed that one error-prone DNA polymerase was also involved in this mutagenesis process (32). We are continuing to study the genetic and developmental regulation of additional DNA repair systems in order to test the hypermutable subpopulation hypothesis. However, one problem that arises if a hypermutable subpopulation(s) actually exists is how do these cells generate a good mutation before they reach genetic load (3)? Basically, how does a hypermutable cell achieve a desired mutation (if mutations are random) before incurring a lethal mutation? Mathematical analysis of random mutagenesis in a subpopulation should argue against the existence of such a population. However, the data that we have obtained with the ComA and ComK proteins as well as with the MMR system strongly indicate that the cells responsible for stationary-phase-mutagenesis might have certain physiological differences from the general population. The answer to this conundrum may very well lie in the “retromutagenesis” hypothesis that has recently been advanced (12). Specifically, for the types of mutations that we are observing (revertants of amino acid requirements) one could imagine errors in mRNA being generated by the error-prone nature of the DNA polymerase. Such “mutant” RNA might very well yield an altered protein that would permit the transient growth of a bacterium. This transient growth could then allow for actual DNA mutations that might result in the generation of a true revertant. We are currently testing this hypothesis.

The existence of alternative explanations for the phenomenon described for the *E. coli lac* reversion system (28) points to the importance of examining multiple models of adaptive or stationary-phase mutagenesis. While the genetic characterizations of the *B. subtilis* reversion assay used in this study and the *E. coli lac* reversion system are basically different, it is clear that MMR proteins play important roles in both models (most
likely for different reasons). Characterizing the specific involvement of the MMR proteins in these two different systems should help in our understanding of the overall importance of mismatch repair mechanisms in mutagenesis, gene transfer, species isolation, and even cancer.

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