The Vsr Endonuclease of *Escherichia coli*: an Efficient DNA Repair Enzyme and a Potent Mutagen

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The Vsr endonuclease of *Escherichia coli* initiates the repair of T/G mismatches caused by deamination of 5-methylcytosine to thymine. In this paper, we examine the capacity of Vsr to prevent CG-to-TA mutations in cells with increased transcription of the cytosine methylase gene (*dcm*). We find that sufficient Vsr is produced by a single chromosomal copy of *vsr* to prevent mutagenesis. We also investigate the cause of the transition and frameshift mutations in cells overproducing Vsr. Neither the absence of the *dcm* methylase nor its overproduction affects Vsr-stimulated mutagenesis. However, addition of *mutS*, *mutL*, or *mutH* on multicopy plasmids has a significant effect: *mutL* or *mutH* decreases the number of mutations, while *mutS* stimulates mutagenesis. The *mut* containing plasmids have the same effect in cells treated with 2-aminopurine and in cells made defective in DNA proofreading, two experimental situations known to cause transition and frameshift mutations by saturating mismatch repair.

The Vsr endonuclease in *Escherichia coli* cleaves DNA at T/G mismatches in C(T/G)WG G and related sequences (W = A or T), producing a single-strand nick 5′ of the T. This is the first step in very short patch (VSP) repair, the end result of which is the replacement of the mismatch with a normal CG base pair (reviewed in reference 17). Since the *dcm* cytosine methylase of *E. coli* methylates the second C of the sequence CCWGG, the main function of VSP repair is probably the prevention of CG-to-TA mutations caused by deamination of 5-methylcytosine to thymine. Cells which contain *dcm* but not *vsr* have high frequencies of C-to-T mutations at 5-methylcytosine (16, 20).

The chromosomal arrangement of *dcm* and *vsr* is unusual: the 5′ end of *vsr* overlaps the 3′ end of *dcm* in a +1 reading frame (25). The two genes are probably transcribed as a single mRNA, with translation of *vsr* depending on translation of *dcm* (7). The arrangement of the genes suggests that coordinated production of the two proteins is important. For example, it could ensure that Vsr is present whenever the methylase is present, thereby minimizing mutations caused by 5-methylcytosine deamination.

Recently, we uncoupled the expression of *vsr* from *dcm* by cloning *vsr* into a multicopy plasmid under the control of the strong, constitutive *trc* promoter (8). We had hypothesized that elevated levels of Vsr in the cell would increase competition between VSP repair and postreplication mismatch repair (MMR) for the correction of T/G mismatches formed by DNA polymerase errors and that this would result in increased numbers of CTAGG-to-CCAGG mutations. As predicted, these mutations were elevated. Surprisingly, however, overproduction of Vsr also increases transition mutations in general and dramatically stimulates frameshifts. Thus, the unusual genetic arrangement of *dcm* and *vsr* may also serve to minimize mutations caused by Vsr. For example, the overlap between the coding regions of the two genes may decrease the efficiency of Vsr translation.

One purpose of the current study was to further investigate the biological reason for the cotranscription of *dcm* and *vsr*. Specifically, we wished to determine the extent of methylase-endonuclease-stimulated mutagenesis resulting from overexpression of *dcm* and *vsr* either alone or as part of the operon. We found that a single chromosomal copy of *vsr* produces sufficient Vsr to protect cells from the mutagenic effects of increased transcription of *dcm* but that increased production of the methylase does not protect cells from the majority of mutations caused by large amounts of Vsr. The second purpose was to investigate the mechanism by which increased production of Vsr causes widespread mutagenesis. Since the pattern of mutation is very similar to that produced in cells defective in MMR (8), we examined the effects of *mutS*, *mutL*, and *mutH* on Vsr-stimulated mutagenesis. The results support the hypothesis that Vsr-stimulated mutagenesis is caused indirectly by disruption of MMR.

MATERIALS AND METHODS

**Bacterial strains.** CC101 to -106 (4), CC107 to -111 (5), and CC113 (8) are all derivatives of CS142 [ara Δ(gpt-lac)3 thi], and contain an F′ lacZYA proAB episome. The strains differ only in the nature of the mutation in the *lac* gene. Isogenic mutant derivatives of these strains were constructed by using P1 transduction to introduce the mutant alleles into CS142, then adding the appropriate F′ lacZYA proAB episomes by conjugation with the original CC strains. The *dcm* allele was transduced from NR9605 (provided by R. M. Schaaper, National Institute of Environmental Health Sciences), and *mutS210::Tn5*, *mutH471::Tn5*, and *mutL211::Tn5* were transduced from GW3732, GW3733, and GW3734 (provided by G. C. Walker, Massachusetts Institute of Technology), respectively. Antibiotic-resistant transductants were tested for a mutator phenotype on Luria broth (LB) plates with rifampin. A *dcm*−*vsr* deletion [Δ(supD-dcm-fba) zees329::Tn10] was transduced from CC221 (8); the deletion was verified by Southern hybridization with a *dcm-vsr* probe.

**Cloning.** CC112 is *ara Δ(gpt-lac)3 thi gna argE(Asn) proB thi* and contains a plasmid with a synthetic tRNA gene (21). The tRNA inserts a glutamic acid at TAG (amber) codons. As a result of this nonsense suppression, a CAG-to-TAG mutation at codon 461 of *lacZ* produces a Lac− phenotype. A high-copy plasmid vector of CC112 was made by P1 transduction of the deletion from CC221 as described above. The SMB strains are *lacZ* derivatives of MC4100 (F araD139 rpsL150 relA1 thi-1 rbsR lacZ58), and contain the *lacZ* alleles from CC101 and CC112; SMB2065 and SMB206 contain the *lacZ* alleles from CC105 and CC109. *lacZ* is carried by the F episome in the CC strains and on the chromosome in the SMB strains. SMB strains were constructed by S. M. Benson (University of Maryland, College Park).

**Plasmids.** Overexpression of *dcm* and/or *vsr* was accomplished by cloning the gene(s) into ppK233-2 (Clontech), where they are transcribed from the synthetic *trc* promoter. pKK-K, (8), pKK-D, and pKK-DV contain *vsr*, *dcm*, and the *dcm-vsr* operon, respectively. The pKK plasmids could not be used in CC112, since the strain already contains a plasmid with a CoE1 origin of replication.

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Therefore, HindIII BamHI fragments containing the genes(s) and the tre promoter were subcloned from the pKK plasmids into pACYC184, a plasmid with a CoE1-compatible p15a origin (pdv series). pACYC184-based plasmids containing dcm (pdv101) and dcm-vsr (pdv102) expressed from the dcm promoter were constructed by subcloning from pdW and pdVW, respectively (20). pDCM28, a pACYC184-based plasmid containing vsr expressed from the dcm promoter, was obtained from A. S. Bhagwat (25).

Additional copies of mutL, mutS, and mutH were introduced into cells by transforming with pMQ339, pMQ341, and pMQ346, respectively (20). pMQ plasmids are derived from pACYC184, so they are compatible with pKK-V. Each pMQ plasmid was verified by its ability to complement the mutator phenotype of a strain with a Tn5 in the corresponding chromosomal mut gene.

Mutagenesis assays. The frequency of occurrence of specific base substitution and frameshift mutations in the CC and SMB strains was measured by using a Lac reversion assay (4, 5, 8, 21). For quantitative assays, 100-μl aliquots of saturated overnight cultures were spread on minimal lactose plates, and the number of colonies was counted after 36 h of incubation. For rapid assay, 100-μl aliquots of saturated overnight cultures were spotted onto minimal lactose plates and/or papillation plates and the number of colonies was counted after 36 h of incubation. For rapid assay (4, 5, 8). As shown in Table 2, cells transformed with pKK-DV show elevated frequencies of transition and frameshift mutations very similar to those seen in cells containing pKK-V. Only the CTAGG-to-CCAGG mutation (CC113) is stimulated more by the vsr plasmid than by the dcm-vsr plasmid. Overproduction of the methylase does not contribute to mutagenesis; cells containing pKK-D have a low level of mutation similar to that of control cells containing just the cloning vector (pKK233-2). The presence or absence of the dcm-vsr operon on the chromosome has no effect on the relative frequency of the various types of mutation with any of the plasmids (data not shown).

Effect of mutH, mutL, and mutS on Vsr-stimulated mutagenesis. The high numbers of transition and frameshift mutations in cells overexpressing Vsr (Table 2) are very similar to those seen in cells defective in MMR (5). Therefore, it is possible that excess Vsr causes mutation by interfering with MMR. If so, addition of pKK-V to cells with a defect in one of the mismatch repair genes should not increase mutagenesis above that caused by the loss of MMR. The data in Table 3 confirm this prediction: transition and frameshift mutations in mutS, mutL, or mutH strains are not increased when the cells are transformed with pKK-V.

If MMR is affected by overproduction of Vsr, then addition of plasmids containing the mutS, mutL, or mutH gene may influence mutation. We therefore transformed cells containing pKK-V with plasmids containing one of the three mut genes (pMQ341, pMQ339, and pMQ348) and measured the number of Lac− revertants per 108 viable cells due to a GC-to-AT transition mutation, a +1 frameshift mutation in a run of six As, or a CTAGG-to-CCAGG mutation. The data were normalized to the numbers of mutants produced by cells containing the control plasmid, pACYC184. Figure 1 shows that the mutS, mutH, and mutL plasmids have no statistically significant effect on the CTAGG-to-CCAGG mutation (Fig. 1, group IV).
TABLE 3. Overexpression of vsr does not increase mutation in cells defective in MMR

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>No. of Lac⁻ colonies/10⁸ viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F⁻ CC102</td>
<td>F⁻ CC107</td>
</tr>
<tr>
<td>mutH::Tn⁵</td>
<td>pKK233-2</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>pKK-V</td>
<td>215</td>
</tr>
<tr>
<td>mutL::Tn⁵</td>
<td>pKK233-2</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>pKK-V</td>
<td>173</td>
</tr>
<tr>
<td>mutS::Tn⁵</td>
<td>pKK233-2</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>pKK-V</td>
<td>255</td>
</tr>
</tbody>
</table>

However, the numbers of GC-to-AT transition (Fig. 1, group II) and +A frameshift (Fig. 1, group III) mutations are decreased substantially by the addition of the mutL or the mutH plasmid. In contrast, addition of the mutS plasmid increases the number of mutations. The mutS plasmid does not stimulate mutation in cells without pKK-V (data not shown).

The extremely high mutation rate characteristic of cells defective in DNA proofreading (mutD5) is due in large measure to saturation of MMR (22). Addition of a cloned mutH or mutL from Salmonella typhimurium was found to decrease mutation levels (as measured by rifampin or nalidixic acid resistance), while addition of the S. typhimurium mutS was either neutral or slightly mutagenic (23), results remarkably similar to those seen in Fig. 1 for cells overproducing Vsr. In order to make a direct comparison, we used the Lac reversion assay to assess mutagenesis in a dnaQ strain of E. coli containing a plasmid with the E. coli mutS, mutL, or mutH gene. As shown in Fig. 2, the mutH or mutL plasmid reduces the AT-to-GC transition (CC106) and the +A frameshift (CC110) in the proofreading mutant while the mutS plasmid increases both mutations. (The AT-to-GC transition is illustrated rather than GC to AT due to the difficulty in photographing dark-blue papillae on the pale-blue colony produced by CC102.)

It has been suggested that the large number of frameshift mutations caused by treatment of E. coli with base analogs is due to saturation of MMR (5), although this hypothesis has never been explicitly tested. For example, in 2-AP-treated cells, DNA polymerase replication errors are probably left unrepaired due to the presence of excess 2-AP-C base pairs (5), a lesion known to be subject to MMR (10). The results in Fig. 1 show that the +A frameshift mutation caused by treatment of cells with 2-AP is reduced by the addition of plasmid-born mutL, or mutH but increased by addition of cloned mutS (Fig. 1, group I). These results are consistent with those seen in cells defective in proofreading (Fig. 2) and in cells overproducing Vsr (Figure 1, group III).

Vsr-stimulated mutagenesis is not confined to lacZ alleles on an F episome. Some criticism has been levelled at studies which measure mutations occurring in target genes located on an F episome (9). Therefore, we determined the effect of overproduction of Vsr on the occurrence of mutations in lacZ on the chromosome; the lacZ alleles used to measure specific transition and frameshift mutations are identical to those used in the F⁻-based assay. Comparison of the data in Fig. 3A and B with the data in Table 2 shows that overexpression of Vsr stimulates the same types of mutation, to the same relative extent, in the chromosomal gene as in the episomal gene and that mutation is decreased by the addition of plasmids containing either mutL, or mutH and increased by the mutS plasmid. pKK-V also stimulates mutations in rpoB, the gene which codes for the β subunit of RNA polymerase, as shown by increased numbers of rifampin-resistant colonies (Fig. 3C). Mutation to Rif⁻ is influenced by the mut-containing plasmids in the same way as reversion to Lac⁺.

FIG. 1. Effect of cloned mut genes on 2-AP- and Vsr-stimulated mutations. Groups: I, CC110 grown in the presence of 700 μg of 2-AP/ml; II to IV, CC102, CC110, and CC113 transformed with pKK-V. Cells were transformed (I) or cotransformed (II to IV) with one of the following plasmids: pACYC184 (C), pMQ348 (H), pMQ339 (L), or pMQ341 (S). Data are normalized to the values obtained for cells containing the control plasmid (pACYC184): 480 (I), 19 (II), 928 (III), and 53 (IV) Lac⁻ colonies per 10⁸ viable cells.

FIG. 2. Effect of mut genes on base substitution and frameshift mutations in a dnaQ strain. The top half of the plate shows CC100 on papillation medium. The bottom half of the plate shows CC110 on minimal lactose medium. Cells are transformed with the following plasmids: pACYC184 (C), pMQ348 (H), pMQ339 (L), and pMQ341 (S).

DISCUSSION

The dcm cytosine methylase and the vsr endonuclease of E. coli both have the potential to cause mutation. It has been known for many years that methylation of cytosine in E. coli increases the frequency of CG-to-AT mutations (3) and that the mutation frequency is elevated in cells which lack vsr (16, 20). Strangely, although Vsr is required in order to avoid mutations at 5-methylcytosine, overexpression of Vsr causes transition and frameshift mutations throughout the genome (8). How does the cell exploit the DNA repair capacity of Vsr without suffering from its mutagenic effects? The solution may lie in the unusual genetic arrangement of dcm and vsr, in which the 5′ end of vsr overlaps the 3′ end of dcm (25). We therefore compared the frequency of occurrence of methylase-specific and Vsr-specific mutations in cells transformed with a multicopy plasmid containing the intact dcm-vsr operon and cells...
transformed with plasmids containing dcm or vsr alone. The cloned operon and the cloned individual genes are transcribed from the trc promoter. The combination of a high-copy-number plasmid and a strong promoter considerably augments production of the proteins (8, 14). Transformed cells were tested, with or without the dcm-vsr operon on the chromosome, by using an assay in which cells revert from Lac− to Lac+ via specific base substitution or frameshift mutations in lacZ.

**Methylase-stimulated mutagenesis.** Increased transcription of dcm stimulates only 5-methylcytosine-to-thymine mutations (Table 1); it has no effect on other types of base substitution and frameshift mutations (Table 2). The CCAGG-to-CTAGG mutagenesis depends on whether or not vsr is present. In cells with neither a chromosomal nor a plasmid-borne vsr gene, the presence of dcm stimulates the mutations. The frequency of mutation increases approximately sixfold when the gene is expressed from the trc promoter (Table 1). We have estimated from dcm::lacZ fusions that pDV104 produces about sixfold more methylase than pDV101 (14). This correspondence between levels of methylase and levels of mutagenesis suggests either that the target cytosine is normally poorly methylated or that the methylase actively contributes to the occurrence of mutations, as suggested by in vitro data (24).

Table 1 shows that a plasmid with both dcm and vsr (pDV102) does not stimulate mutation in cells lacking a chromosomal copy of vsr (CC112Δ). However, it should be noted that this result is at odds with data published by Bandaru and coworkers. They find that a dcm-vsr plasmid increases mutagenesis 26-fold in a strain which is Vsr− due to a nonsense mutation in dcm (the dcm-6 allele) (1). The discrepancy is not due to the different alleles used in the two studies: a dcm-6 version of CC112 transformed with pDV102 gives the same results (not shown) as CC112Δ (Table 1). The mutagenesis that the other group saw may have been due to mutations other than CCAGG to CTAGG resulting from increased production of Vsr. We have ruled out this possibility in our assay (pDV108).

A plasmid containing dcm alone does not stimulate mutation, even when overproduced, so long as there is a copy of vsr on the chromosome (e.g., Table 1, pDV101 and pDV104). This result, too, is at odds with data published by Bandaru and coworkers. They find that a dcm-containing plasmid, with the dcm promoter, produces a 47-fold increase in mutations in a vsr+ strain (2). There are several possible explanations for this discrepancy. The CCAGG target site in CC112 may be inefficiently methylated or it may be unusually resistant to deamination. Alternatively, the requirement for nonsense suppression in order to generate a Lac+ phenotype may reduce the sensitivity of our assay.

5-Methylcytosines are hotspots for mutation in E. coli (3). Several hypotheses have been put forward to explain these hotspots, including the possibility that repair of T/G mismatches caused by deamination of 5-methylcytosine to thymine is inefficient. Our results suggest that VSP repair is actually remarkably efficient.

**Vsr-stimulated mutagenesis.** Of the mutations we measured, only the CCAGG-to-CTAGG mutation was unaffected by overproduction of Vsr (Table 1). This lack of mutation is not due to a defect in the plasmid or to low copy number: pDV108 gives the same level of mutation as pKK-V when introduced into CC110 (data not shown). However, the reverse mutation, CTAGG to CCAGG, is stimulated, as is a broad spectrum of other base substitution and frameshift mutations in both episomal genes (Table 2) and chromosomal genes (Fig. 3).

Our data indicate that the mechanism by which overproduction of Vsr stimulates T-to-C mutations at CTAGG sites is distinct from that which stimulates other transition mutations and frameshifts. First, only the CTAGG-to-CCAGG mutation (CC113) is decreased by the presence of dcm with vsr on the plasmid (pKK-DV versus pKK-V); other types of mutation are unaffected by dcm on either the plasmid or the chromosome (Table 2). This protective effect of dcm may be due to binding of the methylase to C(T/G)AGG and exclusion of Vsr: it is known that some bacterial cytosine methylases bind tightly to recognition sites that contain mismatches (13, 27). Second, the CTAGG-to-CCAGG mutation is unaffected by the addition of plasmids containing mutH, mutL, or mutS (Fig. 1, group IV) while the other mutations are significantly affected (Fig. 1, groups II and III). We have suggested that the CTAGG-to-CCAGG mutations are caused by direct competition between VSP repair and MMR for T/G mismatches caused by misreplication of CTAGG (8). Although one might expect that adding extra Mut proteins would reduce mutation by stimulating MMR, it is possible that they stimulate VSP repair as much or more.

In our previous paper on Vsr-mediated mutagenesis (8), we noted that strains overproducing Vsr and strains defective in MMR show a remarkably similar mutagenic spectrum in the Lac reversion assay. This led us to suggest that the majority of Vsr-stimulated mutations are caused indirectly by disruption of MMR. The data in Table 3, which show that introduction of pKK-V into cells defective in mutS, mutL, or mutH does not increase base substitution or frameshift mutations beyond that
caused by inactivation of MMR, support this hypothesis. We also suggested that the disruption was due to a decrease in the availability of MMR proteins in cells overproducing Vsr (8). This hypothesis was based on genetic evidence that MutS and MutL contribute to VSP repair (12, 15, 28). Results presented in this paper strengthen this hypothesis.

It is known that inactivation of proofreading stimulates mutation by saturating MMR (23). There is good evidence that the saturation is due to exhaustion of one or more of the MMR proteins (6). We used a single assay (Lac reversion), and a set of isogenic strains, to directly compare mutagenesis in cells overproducing Vsr (Fig. 1, groups II and III) and cells defective in proofreading due to a mutation in daqQ (Fig. 2) following the addition of plasmids containing the E. coli mutH, mutL, and mutS genes. Since saturation of MMR has also been proposed as the mechanism for frameshift mutations produced by treatment of cells with base analogs (5), we performed the same experiment on cells treated with 2-AP (Fig. 1, group I). The striking similarities among the results of the three experiments suggest that MMR is inactivated through a common mechanism.

Our complementation experiments (this paper), and those of Schaaper and Radman with proofreading mutants (23), show that MutS is not limiting. The more likely cause of mutation is thus depletion of MutL, and/or MutH. The involvement of MutL, but not MutH, in VSP repair tends to point the finger of suspicion at MutL. It is also interesting that increased mutation in stationary phase is associated with a decrease in the level of functional MutL protein (11). Stimulation of mutation by mutS-containing plasmids may indicate that MutL is inactivated by interaction with MutS. MutH may decrease mutation by increasing the number of productive MutS-MutL interactions (i.e., those that result in MMR). However, the true nature of the interactions among the Mut proteins, and of their interaction with Vsr, will require further genetic and biochemical data.

The Dcm methylase poses less of a threat to the integrity of the E. coli genome than does the Vsr endonuclease, ostensibly a DNA repair enzyme. Our results indicate that increased transcription of the dcm-vsr operon would not increase Dcm-mediated mutations at 5-methylcytosines but would result in a Vsr-mediated, genome-wide increase in other transition and frameshift mutations. We suspect that Vsr production is probably tightly regulated in the normal cell to avoid mutagenesis. However, it is worth considering that Vsr could provide the cell with a mechanism for transiently increasing its mutation rate under adverse circumstances, including stationary phase.

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