JOURNAL OF BACTERIOLOGY, Feb. 2001, p. 921–927 Vol. 183, No. 3
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.3.921–927.2001
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Lowering *S*-Adenosylmethionine Levels in *Escherichia coli* Modulates C-to-T Transition Mutations

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Received 21 August 2000/Accepted 10 November 2000

Deoxycytosine methylase (Dcm) enzyme activity causes mutagenesis in vitro either directly by enzyme-induced deamination of cytosine to uracil in the absence of the methyl donor, *S*-adenosylmethionine (SAM), or indirectly through spontaneous deamination of [5-methyl]cytosine to thymine. Using a Lac reversion assay, we investigated the contribution of the first mechanism to Dcm mutagenesis in vivo by lowering the levels of SAM. *Escherichia coli* SAM levels were lowered by reducing SAM synthetase activity via the introduction of a *metK84* allele or by hydrolyzing SAM using the bacteriophage T3 SAM hydrolase. The *metK84* strains exhibited increased C-to-T mutagenesis. Expression of the T3 SAM hydrolase gene, under the control of the arabinose-inducible P_{BAD} promoter, effectively reduced Dcm-mediated genomic DNA methylation. However, increased mutagenesis was not observed until extremely high arabinose concentrations were used, and genome methylation at Dcm sites was negligible.

Methylated cytosines were first shown to be associated with an increase in the frequency of C-to-T transition mutations in the *Escherichia coli lacI* gene (6). In humans, methylation occurs predominantly at CpG dinucleotides and plays an essential role in many processes, including development, recombination, and X-chromosome inactivation (20, 39, 41). These methylated sites exhibit high rates of C-to-T transition mutations in both germ line and cancerous tissues (21, 23, 49). In prokaryotes, cytosine methyltransferases (CMTases) are generally associated with restriction-modification systems, and as a consequence of their action they play a role in bacterial genome evolution (3, 15, 31). Various CMTase recognition sites are mutational hot spots in *E. coli* (6, 24, 46).

CMTase function is well conserved in prokaryotes and eukaryotes. Upon binding to the target sequence, the protein flips the target cytosine into a catalytic enzyme pocket, where it becomes covalently linked to cytosine C6. This interaction activates cytosine C5, enabling transfer of a methyl group from the CMTase cofactor, *S*-adenosylmethionine (SAM), to produce DNA-[5-methyl]cytosine (5MeC) and *S*-adenosylhomocysteine (SAH)(22, 45).

Various mechanisms have been hypothesized to explain the increase in mutagenesis at CMTase target cytosines. While DNA replication errors can account for some of the observed mutations, spontaneous hydrolytic deamination of 5MeC to thymine was initially proposed as the most likely mechanism responsible (6). Indeed, 5MeC-to-T deamination events occur more frequently than C-to-U events (43), and the deamination frequency is enhanced on single-stranded DNA during transcription (2). However, CMTases may also directly cause 5MeC-to-T mutation (48). A number of other factors can affect the outcome of CMTase activity. The efficiency of DNA repair at sites of deamination or replication error, T/G or U/G, dictates the fixation rate of mutation. In bacteria, at least three separate DNA repair pathways are capable of dealing with the resultant mismatched bases, very short patch repair (VSP), uracil-DNA-glycosyl transferase (Ung), and mismatch repair (MMR) (25, 27, 33). The efficiency of each of these pathways varies from site to site and with growth phase (Fig. 1). It has also been shown that some CMTases bind with higher affinity to mismatched DNA, a characteristic which may impede repair of the lesion (47).

SAM is a universal methyl donor, produced from methionine and ATP by SAM synthetase; it is required for a host of cellular methylation reactions (34, 49), including CMTase methylation activity. It is now evident that reduced levels of this cofactor have a profound effect on the ability of various CMTases to induce deamination at target Cs in vitro. *Hpa*II methyltransferase (M.*Hpa*II) activity in a plasmid-based neomycin reversion assay increases the C-to-U reversion frequency about 10^4-fold. This reaction occurs only in the absence of SAM (42).

The deamination mechanism was first described for M.*Hha*I (45). At low SAM and SAH concentrations, the CMTase interaction with cytosine results in the production of a transient covalent CMTase-dihydrocytosine intermediate, rapidly followed by an enzyme-catalyzed exchange of the 5-H of cytosine for protons of water to produce uracil. This reaction is inhibited by SAM or SAH.

In *E. coli*, deoxycytosine methylase (Dcm)-mediated cytosine methylation occurs at the internal cytosine residues of CCWGG sequences. In the event of 5MeC/G conversion to T/G, the Vsr protein nicks the DNA 5′ to the mismatched T, and DNA polymerase I then replaces a short stretch of nucleotides in a process known as VSP repair. If repair does not take place, a C-to-5MeC-to-T transition occurs. If spontaneous or enzyme-mediated deamination causing C-to-U transition takes place, it has been assumed that the Ung enzyme will excise the U, followed by removal of the abasic site and resynthesis of the correct DNA. However, Vsr can also recognize and cleave at U/G mismatches; whether it is as efficient as Ung in this regard

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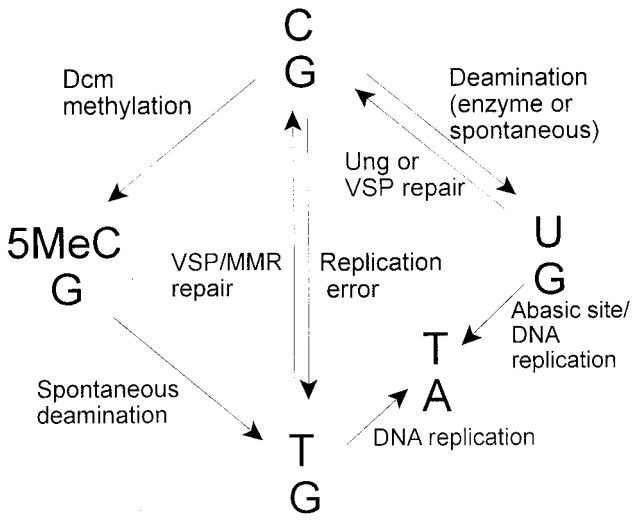

FIG. 1. Pathways to mutation at C · G.

remains unclear (12–14). It is interesting that the stability of 5MeC is similar to that of C in nondividing cells, implying that 5MeC-derived mutations occur during growth phase and are due to inefficient VSP repair (26), which may be reduced due to limited Vsr levels in exponentially growing cells (30).

The evidence supporting the C-to-U mutagenic potential of prokaryotic CMTases is mainly derived from in vitro studies using purified proteins and plasmid DNAs as substrates (1, 42, 45, 46, 50). In this study we have investigated the in vivo effects of Dcm in strains devoid of VSP and/or Ung repair activity. The mutagenic effect of Dcm activity is monitored using an episome which contains a Dcm recognition site within the *lacZ* gene sequence. C-to-T transition mutations at this site convert the strain to a Lac$^+$ phenotype (36). In an effort to detect enzyme-mediated deamination events, we also examined the effects of reduced SAM levels on Dcm-induced mutagenesis in vivo. Reduction of *E. coli* SAM levels was accomplished by reducing SAM synthesis using a *metK84* allele (17, 34), or by hydrolyzing cellular SAM using the bacteriophage T3 SAM hydrolase (T3SH) gene expressed from a plasmid. SAM synthetase is the product of the *metK* gene, and while mutants completely deficient in enzyme activity have not been isolated, *E. coli metK84* strains produce markedly reduced levels of SAM (17) and exhibit a complex phenotype if grown in minimal medium, including a cell division defect which produces an excessively filamentous phenotype (34). T3SH hydrolyzes SAM to methylthioadenosine and homoserine and has been used previously to deplete SAM pools in *E. coli* (37, 44).

MATERIALS AND METHODS

Bacterial strains, phage lysates, and plasmids. *E. coli* strains and plasmids are described in Table 1. BD2314 was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., and GM3888 *dcm-6 zed-501*::*Tn*10 is available from M. G. Marinus (http:/users.umassmed.edu/martin.marinus/dstrains.html). All mutant alleles were transferred to the test strains using standard P1 transduction protocols (32). Since the *metK84* allele is not marked or directly selectable in rich medium, a *serA*::*kan* derivative was produced to allow a subsequent cotransduction of the *metK84* mutation with *serA*$^+$. The *metK84* strains are notoriously unstable and accumulate suppressor mutations very rapidly (34). To ensure the integrity of our test strains, we prepared fresh sets of transductants for each experiment. Purified *metK84* transductants were tested for kanamycin sensitivity, filamentous growth in minimal glucose medium containing low concentrations of leucine (15 μg/ml), and growth on minimal glucose plates

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
Strains		
CSH142	Δ(*gpt-lac*)*5*	32
CC102	CSH142, F′ *lacZ*$^-$*YA, proAB*; G · C to A · T	8
CC106	CSH142, F′ *lacZ*$^-$*YA, proAB*; A · T to G · C	8
CC110	CSH142, F′ *lacZ*$^-$*YA, proAB*; +A frameshift	7
CC112	Δ(*gpt-lac*)*5 argE*(Am), F′*lacZ*$^-$*YA, proAB*; CCAGG to CTAGG	40
CC112Δ	CC112Δ(*supD-dcmvsr-fla*) *zee-3129*::*Tn*10	40
CC112*dcm*-6	CC112*dcm-6 zed-501*::*Tn*10	This work
BD2314	*ung-152*::*Tn*10	11
MEW30	*metK84*	34
MEW305	*ser*A::λ*plac*Mu9*kan*	34
CC500	CSH142, F′ *lacZ*$^-$*YA, proAB*; CCAGG to CTAGG	36
CC501	CSH142*ser*A::λ*plac*Mu9*kan*	This work
CC502	*metK84*	This work
CC503	*vsr*::*kan*	This work
CC504	*ung*::*Tn*10	This work
CC505	*vsr*::*kan metK84*	This work
CC506	*ung*::*Tn*10 *vsr*::*kan*	This work
CC507	*ung*::*Tn*10 *metK84*	This work
CC508	*ung*::*Tn*10 *vsr*::*kan metK84*	This work
LMG194	Δ*ara*	18
CC509	CC503 Δ*ara*	This work
CC510	CC506 Δ*ara*	This work
Plasmids		
pACYC184	Cm^r and Tet^r	5
pDV101	*E. coli dcm* gene in pACYC184 (Cm^r)	29
pDV102	*E. coli dcm* and *vsr* genes in pACYC184 (Cm^r)	29
pBAD24	Amp^r	18
pBAD-T3SH	Bacteriophage T3 SAM hydrolase gene in pBAD24	37

containing gamma glutamyl methyl ester (500 μg/ml) (34). To verify that the *ung* allele conferred the appropriate mutation spectrum, an increase in C-to-T transversions, it was introduced into *E. coli* strains containing different episomal *lacZ* mutations, including CC102 (GC to AT), CC106 (AT to GC), and CC110 (+A frameshift) (7, 8). As expected, an increased reversion frequency was produced only in CC102 *ung*.

The pBAD-T3SH plasmid contains the bacteriophage T3 SAM hydrolase gene under the control of the P_{BAD} promoter (18). Expression of this gene in *E. coli* grown in the presence of arabinose (Luria-Bertani [LB] broth plus ampicillin at 100 μg/ml and 13.3 mM arabinose) leads to a limited filamentation phenotype (37). This change in morphology was used to confirm the presence of the T3SH gene in our test strains.

Growth of cultures. The CC112 strain and its derivatives were transformed with various plasmids and grown overnight at 37°C in minimal medium containing 0.2% glucose, ampicillin (100 μg/ml), and chloramphenicol (20 μg/ml). Overnight cultures of *metK84* strains and their isogenic counterparts were diluted 10^3-fold into LB medium and grown for 15 h at 37°C. Analyses of mutation rates in *metK84* strains were carried out in LB medium to avoid any artifactual increases in mutation rate which may arise solely as a result of the very extensive cell filamentation associated with this strain. Previous studies have shown that decreasing leucine levels in minimal-glucose medium correlate with decreasing SAM synthetase levels in *metK84* strains and increasing filamentation (34). SAM synthetase levels are lowered by approximately 50% in *metK84* strains grown in LB medium (17). A comparison of mutation rates in the *metK84* strains grown under nonfilamenting conditions, either in LB medium or minimal-glucose medium containing leucine (100 μg/ml), indicated that the levels of mutagenesis were similar (data not shown).

Cells containing pBAD24 or pBADT3SH were grown overnight in LB medium containing ampicillin at 100 μg/ml (LBAmp), diluted to 10^{-6} in LBAmp with glucose (0.2%) or arabinose at various concentrations, and grown for 18 h at 37°C in a rotary shaker. Regardless of the method used to reduce SAM levels, the resultant cultures were used to produce samples for Lac reversion assays and for genomic DNA analysis to monitor the extent of DNA methylation.

Lac reversion assays. A papillation assay was performed as follows. Strain CC112, described previously (40), carries an episome which allows Lac reversion via C-to-T mutation at a Dcm site in the *lacZ* gene (aaC CAG Ggg). The strain carries a plasmid that contains a glutamic acid-inserting amber suppressor tRNA gene which allows the mutation of the CAG to TAG to be detected as a Lac^+ revertant. Five-microliter aliquots of each overnight culture were spotted onto papillation medium and incubated at 37°C until papillae were visible (35).

CC500 carries an episome, *pro462*, which contains a Dcm recognition site (CCA GGg) within the *lacZ* gene to monitor C-to-T transition mutations. In the event of a C-to-T mutation at the second cytosine, the proline residue (CCA) will be replaced by a leucine (CTA) which will change the phenotype from Lac^- to Lac^+ (36). Overnight cultures of all strains grown in LB medium were assayed for Lac reversion as previously described (32). All strains containing the *metK84* allele grew more slowly on the minimal lactose plates and were incubated for 40 h instead of 36 h.

DNA methylation assay. The extent of DNA methylation was assessed using the Bio-Rad CHEF-DRIII pulsed-field electrophoresis system and protocols. Approximately 5×10^8 cells were transferred to a microcentrifuge tube and centrifuged for 1 min at 13,000 × *g*. The cells were resuspended in 0.5 ml of cell suspension buffer (10 mM Tris [pH 7.2], 20 mM NaCl, and 50 mM EDTA) and equilibrated to 50°C in a water bath. The cell suspension was then combined with an equal volume of 2% agarose (pulsed-field gel electrophoresis grade), mixed, and transferred to a plug mold (100 μl of suspension per plug). The agarose was allowed to solidify at 4°C for 10 min. Each set of 10 plugs was then placed in a 50-ml conical centrifuge, followed by a series of incubations and washes at room temperature. The plugs were incubated in lysozyme buffer (10 mM Tris [pH 7.2], 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, and 1 mg of lysozyme per ml) for 1 h at 37°C. The plugs were rinsed with 25 ml of 1× wash buffer (20 mM Tris [pH 8.0] and 50 mM EDTA) followed by an incubation in 5 ml of proteinase K reaction buffer (100 mM EDTA [pH 8.0], 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, and 1 mg of proteinase K per ml) at 50°C overnight. Each sample was then washed four times in 50 ml of 1× wash buffer for 1 h with gentle agitation; phenylmethylsulfonyl fluoride (1 mM final concentration) was added to the third wash. Before digestion with restriction enzymes, the plugs were cut into quarters and washed in 0.1× wash buffer for 30 min with agitation. Each quarter plug was incubated in 250 μl of the appropriate 1× restriction enzyme buffer for 1 h with agitation. The quarter plug was then incubated in 75 μl of fresh 1× restriction enzyme buffer plus 10 U of restriction enzyme overnight at 37°C and then rinsed with 250 μl of 1× wash buffer. Each quarter plug was then inserted into a well in a 1.2% agarose–Tris-borate-EDTA gel and sealed into position using molten 1.2% agarose. Molecular weight markers were also loaded as plugs. Electrophoresis was carried out in 0.5× Tris-borate-EDTA at 14°C, 6 V/cm for 22 h with a switch time of 50 to 90 s.

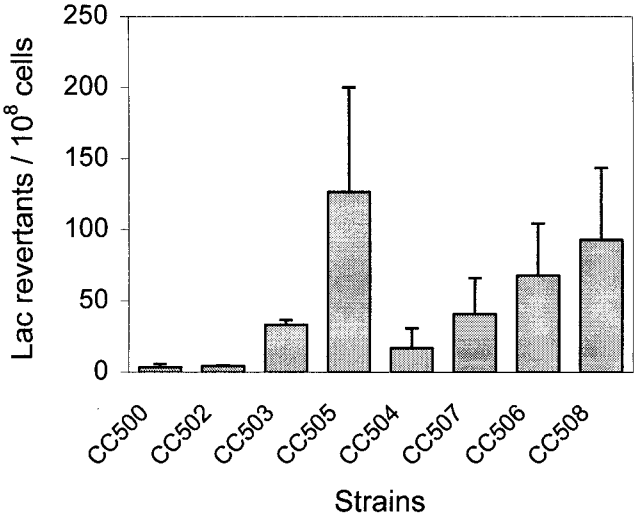

FIG. 2. Number of Lac revertants per 10^8 cells (plus standard error of the mean) resulting from C-to-T transition mutations at a Dcm recognition site, CCAGG, in an episomally located *lac* gene, in various genetic backgrounds. CC500 (wild type), CC502 (*metK84*), CC503 (*vsr*), CC505 (*vsr metK84*), CC504 (*ung*), CC507 (*ung metK84*), CC506 (*ung vsr*), CC508 (*ung vsr metK84*). Six replicate cultures for each strain were analyzed in triplicate.

RESULTS

The frequency of C-to-T mutations increased in a VSP-deficient strain. The wild-type strain CC500 exhibited a very low mutation frequency at the CCAGG site under study (Fig. 2). However, when the cells were VSP repair deficient, as in CC503, the mutation frequency was ninefold higher than that observed in a wild-type strain (Fig. 2), indicating the level of mutagenesis that results from Dcm activity. Therefore, using the *pro462* episome, we could easily measure the mutagenic outcome of wild-type levels of Dcm activity in the absence of VSP repair.

C-to-T transition mutations were enhanced in *metK* strains in vivo. The consequences of the *metK84* allele on Dcm function were assessed by comparing CC503 (*vsr*) with CC505, a *vsr metK84* strain (Fig. 2). In this instance, C-to-T transition mutations in the *vsr metK84* strain increased approximately fourfold over the isogenic *vsr* strain and were 35-fold higher than those in the CC500 strain. The fourfold difference in mutation frequency was consistently larger than that observed when the CC500 strain was compared to the *metK84* strain, CC502, which exhibited a 1.2-fold increase in C-to-T mutations over the CC500 strain (Fig. 2).

Uracil DNA glycosylase activity played a minor role in the repair of C-to-U mutations at the Dcm site under study. The C-to-T mutations detected in the *lac* reversion assay could potentially arise via 5mC-to-T or C-to-U-to-T events, and we have assumed that any pre mutagenic C-to-U lesions will be repaired by either the VSP or Ung repair pathways. To address the contribution of Ung to DNA repair, *ung* versions of the test strains were analyzed. In the *ung* strain CC504 and the *ung*

metK84 strain CC507, the mutation frequencies were approximately 5- and 10-fold higher, respectively, than those measured in the corresponding *ung*$^+$ isogenic strains CC500 and CC502 (Fig. 2). In CC506, a *vsr ung* strain, the mutation frequency showed a less dramatic twofold increase when compared to the isogenic *ung*$^+$ strain CC503 (Fig. 2). CC508, the *ung vsr met* strain, also exhibited little change in mutation rate when compared to CC505, its isogenic *ung*$^+$ counterpart (Fig. 2), as evidenced by a slight reduction in the Lac reversion frequency. While the additional *ung*::*tet* mutation revealed the underlying level of spontaneous deamination events normally corrected by Ung, it also suggested that Ung-mediated DNA repair is less important than VSP repair at the Dcm site used in this study, particularly in the VSP$^-$ strains. In contrast, a previous study found that the Ung repair system was responsible for the majority of DNA repair at a Dcm site (28). This study used a strain containing a *dcm-6* allele (9) to reduce VSP repair, which may have concealed the importance of this repair pathway.

CC112 *dcm-6* possesses VSP repair function. We measured the level of VSP activity in a strain containing the *dcm-6* allele, which contains two mutations in the *dcm* gene, a Gln to Arg change at codon 26 and a Trp to an opal stop at codon 45 (9). Strains that possess this allele do not produce Dcm as measured by a methylase assay (9) and by Western blot analysis (data not shown). The opal stop codon is thought to have a polar effect upon the expression of *vsr* (9). We compared the levels of VSP repair function in strains containing either wild-type *dcm* or the *dcm-6* allele with a strain deleted for *dcm* and *vsr*. The strains were transformed with various plasmids to restore Dcm and/or Vsr function as appropriate. CC112 *dcm*$^+$ *vsr*$^+$ exhibits a low level of Lac reversion in the presence of all plasmids tested, indicating that the chromosomal copy of *vsr* produces enough Vsr to repair the majority of lesions occurring at the Dcm test site (Fig. 3 column A, rows 1 through 3). When CC112Δ (*dcm vsr*) is examined in the presence of the same plasmids, the expression of plasmid-encoded *dcm* in the absence of *vsr* leads to a high level of mutagenesis (Fig. 3, column B, row 2), whereas the expression of *dcm* and *vsr* from a plasmid does not alter the mutation frequency, again due to efficient VSP repair (Fig. 3, column B, row 3). Analysis of CC112 *dcm*-6 indicates that in the absence or presence of both the *dcm* and *vsr* genes only background levels of mutagenesis are detectable (Fig. 3, column C, rows 1 and 3), as seen in the other strains. However, the expression of *dcm* in CC112 *dcm-6* (Fig. 3, column C, row 2) results in a much less dramatic increase in mutagenesis than that observed in CC112Δ overexpressing *dcm* (Fig. 3, column B, row 2). This suggests that moderate levels of VSP repair are present in the strain carrying the *dcm-6* allele.

Genomic DNA methylation in *metK* strains. To assess the effectiveness of our experimental approach at reducing methyl group availability to Dcm, we examined the levels of Dcm methylation of genomic DNA in *metK84* strains grown in LB using a clamped homogeneous electric field (CHEF) agarose gel analysis of genomic DNA. *Mva*I cuts DNA at Dcm sites irrespective of Dcm methylation of the DNA while *Eco*RII cleaves only unmethylated Dcm sites. Genomic DNA from all *metK84* strains tested was cleaved by *Mva*I but was insensitive to *Eco*RII hydrolysis under the growth conditions used for the experiment (data not shown). Therefore, the reduced SAM synthetase levels achieved in *metK84* strains grown in LB are not sufficient to alter Dcm methylation of DNA as detected by Dcm methylation-sensitive restriction enzyme analysis.

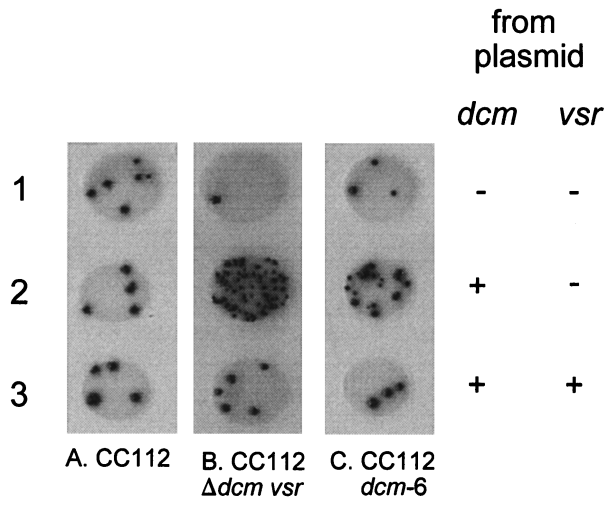

FIG. 3. Effect of the *dcm-6* allele on VSP repair. Five-microliter aliquots of saturated bacterial cultures were spotted onto papillation medium. Columns: A, CC112; B, CC112 Δ*dcm vsr*; C, CC112 *dcm-6*. Strains containing plasmids: row 1, pACYC184; row 2, pDV101; row 3, pDV102.

Induction of SAM hydrolase reduces Dcm methylation of genomic DNA. To reduce Dcm methylation levels more effectively without the risk of extensive filamentation, we used T3SH. The gene for T3SH is expressed from the plasmid pBAD24 (18), under the control of the P_{BAD} promoter, which allows extensive modulation of the levels of the protein using arabinose. Recent studies have shown that T3SH expression from this plasmid lowers SAM levels in *E. coli* and reduces methylation of adenines in chromosomal DNA (36). The CC509 *vsr* Δ*ara* strain transformed with pBAD24 or pBAD-T3SH was grown in LBAmp with increasing concentrations of arabinose (0 to 13.3 mM) and analyzed for Dcm methylation of chromosomal DNA. All DNA plugs were checked for cleavage at Dcm sites using the methylation-insensitive restriction enzyme *Mva*I. All genomic DNA samples were cleaved to completion with *Mva*I, indicating the purity of the DNA plug preparations (Fig. 4A).

The presence of pBAD24 had no effect on Dcm DNA methylation (Fig. 4B, lanes 1 to 6). The DNA appears to be cut to the same extent at all arabinose concentrations used, probably at naturally occurring unmethylated Dcm sites (38). CC509 transformed with pBAD-T3SH shows an increasing sensitivity to *Eco*RII as the levels of T3SH are increased using higher arabinose concentrations (Fig. 4B, lanes 7 to 12). Using this approach we were able to detect reduced Dcm methylation at arabinose concentrations of 13 and 130 μM which exhibited no enhanced cell filamentation (Fig. 4B, lanes 9 and 10, respectively). We also observed some EcoRII hydrolysis of the DNA prepared from the strain grown in LB containing 0.2% glucose, indicating that there is some limited expression of the T3 SAM hydrolase gene under repressing conditions (Fig. 4B, lane 7). At higher concentrations of the sugar (1.3 and 13.3 mM),

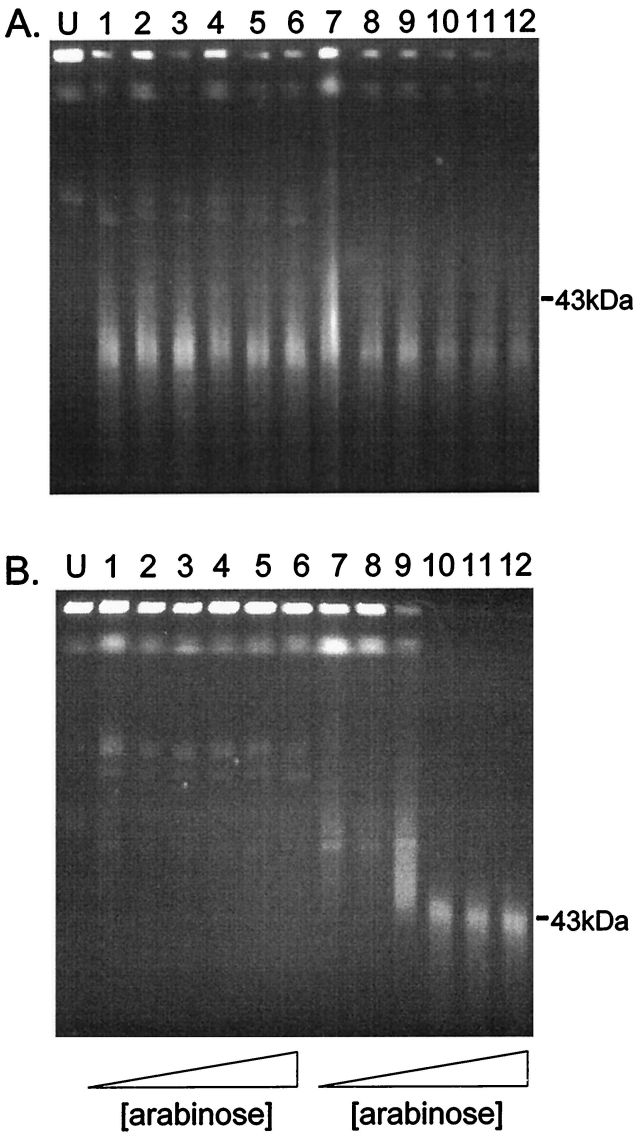

FIG. 4. Dcm methylation of genomic DNA is reduced upon induction of T3 SAM hydrolase. (A) *Mva*I, a methylation-insensitive enzyme, cleaves at Dcm sites in genomic DNA from CC509 containing pBAD24 (lanes 1 to 6) or pBADT3SH (lanes 7 to 12). (B) *Eco*RII, a methylation-sensitive enzyme, does not cut DNA from CC509 containing pBAD24 (lanes 1 to 6). EcoRII cleaves at unmethylated Dcm sites in CC509 overexpressing T3SH (lanes 7 to 12). Lane U, typical sample of uncut DNA. (A and B) Arabinose concentrations: Lanes 1 and 7, 0 mM (plus 0.2% glucose); lanes 2 and 8, 1.3 μM; lanes 3 and 9, 13 μM; lanes 4 and 10, 130 μM; lanes 5 and 11, 1.3 mM; lanes 6 and 12, 13.3 mM.

*Eco*RII sensitivity of the genomic DNA samples increased and cell filamentation became more obvious (Fig. 4B, lanes 11 and 12).

Overexpression of the SAM hydrolase gene alters the C-to-T mutation rate. C-to-T mutations were monitored in CC509 (*vsr* Δ*ara*) containing pBAD24 or pBAD-T3SH and grown in LBAmp containing glucose or a range of arabinose concentrations. The levels of mutagenesis were similar for the CC509 strain carrying either plasmid grown in LBAmp glucose (0.2%)

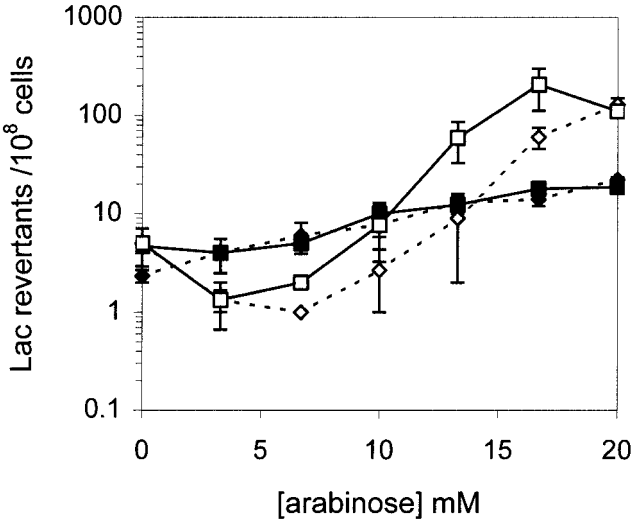

FIG. 5. The effect of reduced SAM levels on C-to-T transition mutations. A T3SH-containing plasmid was compared to a pBAD24 control plasmid under various induction conditions. Lac revertants were measured per 10^8 cells (± standard error of the mean) due to C-to-T transition mutations at a Dcm recognition site, CCAGG, in an episomally located *lac* gene in two strains, CC509 *vsr* Δ*ara* and CC510 *vsr ung* Δ*ara*. Four replicate cultures were assayed in triplicate. CC509/pBAD24 ◆; CC509/pBAD-T3SH ◇; CC510/pBAD24 ■; CC510/pBAD-T3SH □.

to repress expression from the P_{bad} promoter maximally (Fig. 5, 0 mM arabinose). At 1.3 μM arabinose, CC509/pBADT3SH exhibited a reduction in mutation frequency from five to one Lac revertants per 10^8 cells (not shown on graph); genomic DNA methylation appears normal at this concentration (Fig. 4B, lane 8). Arabinose (3.3 and 6.7 mM) also produced a modest reduction in mutation frequency in strains carrying pBAD-T3SH, indicative of reduced Dcm methyltransferase activity (Fig. 5). Figure 4B, lane 10, demonstrates that even at 0.13 mM arabinose, T3SH production was sufficient to reduce methylation at Dcm sites severely. At higher concentrations of arabinose (16.7 and 20 mM), the mutation frequency increased approximately 10-fold in the cultures producing T3SH. Mutagenesis was also enhanced in strains carrying the control plasmid, pBAD24, although the shape of the dose response curve was different; in this instance there was a slight but consistent increase in mutagenesis which correlated with increasing sugar concentrations (Fig. 5). Overexpressing the T3SH gene in CC510, an *ung vsr* Δara strain, produced a dose response curve that was similar in shape to the isogenic *vsr* Δ*ara* strain but exhibited slightly higher levels of mutagenesis (Fig. 5).

To ensure that the increasing SAM hydrolase levels do not have an adverse effect on MMR activity, due to decreased adenine methylation, the level of rifampin resistance (Rif^R) was also measured. Specific mutations in the *rpo*B gene confer Rif^r; these mutations do not occur at Dcm recognition sites (19). Since Dcm-mediated mutagenesis does not result in Rif^r, an increase in the frequency of Rif^r mutants is indicative of a defect in MMR. We did not detect an increase in Rif^r in any of the test strains (data not shown).

DISCUSSION

The purpose of this study was to ascertain whether the *E. coli* cytosine methyltransferase Dcm has the ability to mediate the deamination of C-to-U directly, leading to an increase in C-to-T transition mutations. Previous studies have suggested that this particular mutagenic event might be possible if SAM pools were to become limiting in vivo. This hypothesis is based mainly upon in vitro analyses including genetic and biochemical analyses of various bacterial methyltransferases, M.*Msp*I (4, 50), M.*Hpa*II (1, 42), and M.*Hha*I (45), that provide evidence that the enzymes are capable of mediating deamination of C leading to mutagenesis. A subsequent in vivo study did not support these findings (46).

In the present study we have assessed the effect of *E. coli* Dcm on C-to-T transition mutations at a Dcm site in an episomally located *lacZ* gene. To do this we used CC503, a *vsr*::*kan* strain, to eliminate VSP repair. In CC503 the frequency of C-to-T transitions is ninefold higher than in the wild-type strain, CC500 (Fig. 2), while the mutation frequency is increased only fivefold in CC504, an *ung* strain. In agreement with the previous plasmid-based studies, we show that chromosomally encoded Dcm significantly enhances the C-to-T mutation rate at target Cs. Our results suggest that Vsr plays a more significant role in DNA repair at this site than Ung. Previously, with a *dcm-6* strain in a Kan^r reversion assay, it was suggested that VSP repair was less efficient than Ung at Dcm sites (28). This discrepancy may be due to differences in sequence context around the Dcm site or, more likely, to VSP repair in strains carrying the *dcm-6* allele. The original characterization of the *dcm-6* allele, using a phage recombinogenic assay, indicated a reduced level of Vsp repair in strains carrying this gene (9). Subsequent studies using a Kan^r reversion assay have made the assumption that levels of repair are sufficiently low as to be insignificant (28). Our Lac reversion analysis indicates that strains carrying the *dcm-6* allele still have significant levels of VSP repair (Fig. 3), making it unsuitable for our study. Therefore, we have used a *vsr*::*kan* allele. This allows the study of wild-type levels of Dcm without the need to introduce a plasmid containing *dcm*, and more importantly, due to the insertion of the kanamycin cassette into the *vsr* gene, there is no VSP repair (10).

Using a SAM synthetase mutant allele, *metK84*, that possesses low SAM synthase activity (34) we have investigated the consequences of reduced SAM pools on CMTase-mediated deamination events in vivo. The introduction of the *metK84*, allele into the test strain to produce CC502 increases the reversion rate less than twofold (Fig. 2). However, C-to-T mutagenesis increases 35-fold over the wild type in CC505, a *vsr metK84* strain, and is fourfold higher than in the isogenic *vsr* strain CC503 (Fig. 2). The increase in mutation conferred by the additional *metK84* allele in a VSP-deficient strain suggests that Dcm-mediated mutagenesis is enhanced. However, genomic DNA methylation did not appear to be significantly reduced in the presence of the *metK84* allele. The substantial variability in mutation frequency observed using the double mutant, *vsr metK84*, is consistent with the occurrence of suppressor mutations that overcome the effects of the *metK84* mutation in the population.

We also determined the contribution of the Ung repair system to repair of C-to-U premutagenic lesions arising at the target C. Due to extensive variability in the number of Lac revertants produced by the *ung* strains, no statistically significant differences were found between isogenic pairs. However, the patterns of mutation seen in Fig. 2 are reproducible. Our results indicate that Ung contributes to repair at this site and that C-to-U mutations may be more prevalent in strains carrying the *metK84* allele. However, VSP repair makes a more significant contribution to the repair of premutagenic lesions than Ung, suggesting that VSP repair is primarily responsible for repair of C-to-U as well as 5MeC-to-T lesions at the Dcm site under investigation. The Ung enzyme may be unable to recognize U/G mismatches in the context of a Dcm site.

T3 SAM hydrolase has been used previously to investigate the role of SAM as an endogenous alkylating agent. Although adenine methylation by the Dam methyltransferase was inhibited, no significant changes in mutation frequency indicative of defective mismatch repair or alkylation damage were observed (37). In the present study we were able to correlate increasing induction of the T3SH gene with severe reductions in Dcm-mediated DNA methylation (Fig. 4B) and with changes in mutation frequency. A modest decline in the C-to-T mutation frequency was observed at a low arabinose concentration (1.3 μM) that did not induce a detectable reduction in genomic DNA methylation and at sugar concentrations (3.33 and 6.7 mM) that correlated with reduced Dcm-mediated DNA methylation (Fig. 5). This suggests that even subtle reductions in Dcm DNA methylation resulting in less 5MeC in the DNA reduce the potential for mutation via spontaneous deamination of 5MeC-to-T. However, at higher induction levels mutagenesis increased 10-fold (Fig. 5). It is unlikely that this increase was due to cell filamentation since the previous study using the T3 SAM hydrolase in a *lac* reversion assay did not detect increased mutagenesis under the same limited filamenting conditions (37). We also overexpressed the plasmids in the *vsr ung* Δ*ara* strain, CC510; although the outcome was very similar to that observed in the *vsr* Δ*ara* isogenic strain, the levels of mutagenesis were generally higher. Therefore, the increased mutagenicity may indicate a switch from 5MeC-to-T to C-to-U-to-T Dcm-mediated deamination. The lesions are not repaired in the absence of Vsr, indicating that the VSP repair constitutes the major DNA repair pathway at this site and that Ung has a more limited role, even when the genome is hypomethylated.

It had previously been suggested that the high mutation rates at CpG dinucleotides in human tumor cells may be the result of enhanced CMTase-mediated C-to-U deamination events induced by an altered SAM/SAH environment within the tumor cells. Subsequently, it has been shown that the SAM/SAH ratio is not significantly altered in these cells, reducing the likelihood that C-to-U mutations via this mechanism contribute to mutagenesis (16). Similarly, although we have observed an increase in C-to-T mutagenesis in VSP repair-deficient strains when SAM pools are reduced, it is doubtful that these changes in mutation frequency are physiologically relevant. Revertant or suppressor mutations which overcome the effect of the *metK84* allele give the cells a selective advantage, and they rapidly take over the population. Using the T3 SAM hydrolase, we have confirmed the predictions from previous in vitro studies and have demonstrated that Dcm can directly

mediate deamination in vivo when SAM levels are rapidly and drastically reduced. However, the massive reductions in SAM pools required to increase Dcm-mediated deamination of DNA significantly are unlikely to occur naturally in bacteria.

ACKNOWLEDGMENTS.

We thank Elaine B. Newman for supplying the *metK84* strain and for her advice on the use of this strain, Lan Jie for supplying various strains and lysates, and Leona Samson for supplying the pBAD-T3SH plasmid. Thanks also to Photini Pitsikas and Derek C. Bell for reading the manuscript. The CHEF-DRIII pulsed-field gel electrophoresis system was kindly made available to us by the Concordia University Center for Structural and Functional Genomics.

This research was funded by grants to C.G.C. from the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research.

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