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In Vivo DNA Protection by Relaxed-Specificity SinI DNA Methyltransferase Variants[▽]

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The SinI DNA methyltransferase, a component of the SinI restriction-modification system, recognizes the sequence GG(A/T)CC and methylates the inner cytosine to produce 5-methylcytosine. Previously isolated relaxed-specificity mutants of the enzyme also methylate, at a lower rate, GG(G/C)CC sites. In this work we tested the capacity of the mutant enzymes to function in vivo as the counterpart of a restriction endonuclease, which can cleave either site. The viability of *Escherichia coli* cells carrying recombinant plasmids with the mutant methyltransferase genes and expressing the GGNCC-specific Sau96I restriction endonuclease from a compatible plasmid was investigated. The *sau96IR* gene on the latter plasmid was transcribed from the *araBAD* promoter, allowing tightly controlled expression of the endonuclease. In the presence of low concentrations of the inducer arabinose, cells synthesizing the N172S or the V173L mutant enzyme displayed increased plating efficiency relative to cells producing the wild-type methyltransferase, indicating enhanced protection of the cell DNA against the Sau96I endonuclease. Nevertheless, this protection was not sufficient to support long-term survival in the presence of the inducer, which is consistent with incomplete methylation of GG(G/C)CC sites in plasmid DNA purified from the N172S and V173L mutants. Elevated DNA ligase activity was shown to further increase viability of cells producing the V173L variant and Sau96I endonuclease.

Type IIP restriction-modification (R-M) systems consist of a sequence-specific endonuclease and a sequence-specific DNA methyltransferase (MTase), which recognize the same DNA sequence (31). MTases transfer a methyl group from the methyl donor *S*-adenosylmethionine to a cytosine or adenine of the recognition sequence to produce 5-methylcytosine, *N*4-methylcytosine, or *N*6-methyladenine (14). The role of the modification MTase is to protect the host DNA from the cognate restriction endonuclease. The exquisite specificity and the availability of two enzymes recognizing the same DNA sequence but carrying out different chemical reactions make R-M systems uniquely interesting for studying sequence-specific DNA-protein interactions.

A wealth of sequence information (21, 27), mutational analysis (41), domain swap experiments (1, 20), biochemical studies (4, 42), and crystal structures for two enzymes (M.HhaI and M.HaeIII) (19, 29) support the view that prokaryotic DNA (cytosine-5) MTases (C5-MTases) share a common architecture and catalytic mechanism. C5-MTases contain 10 conserved amino acid sequence motifs and a variable region between conserved motifs VIII and IX. Prokaryotic C5-MTases consist of two domains; the large domain encompasses most of the conserved motifs, whereas the small domain contains the variable region and conserved motif IX. The large domain contains the catalytic site and the cofactor binding site, and the variable region is predominantly responsible for sequence-specific DNA recognition (see also below). The two domains form a cleft, which holds the DNA with the major groove facing the small domain and the minor groove facing the large domain (19, 21, 29).

A crucial element of the catalytic mechanism of C5-MTases is the formation of a transient covalent bond between the C6 carbon of the substrate cytosine and the sulfur of the active-site cysteine (4, 42). C5-MTases employ a base extrusion mechanism (base flipping) to make the target base accessible for the chemical reaction (19, 29).

Mutational analysis can be very useful to test structural models (11, 22) or, in cases when a structure is not available, to probe amino acids for a possible role in the function of the protein. Changing the recognition specificity of MTases by mutagenesis, as a means to identify amino acid residues playing a role in sequence-specific DNA recognition, has proven to be a difficult task. There are only a few documented cases when the recognition specificity of a MTase was changed. Miner et al. isolated T4 Dam MTase mutants which showed activity on Dam-modified DNA, indicating methylation of noncanonical sites, but the exact change in specificity was not investigated (25). Rational protein design was used to change the target base preference of the N6-adenine MTase M.EcoRV from adenine to cytosine (32). A relaxed-specificity mutant of the Eco57I adenine MTase was isolated by random mutagenesis and in vitro biochemical selection. The mutant enzyme, which is part of the type IIG bifunctional restriction endonuclease/modification MTase, methylated, in addition to the canonical CTGA<u>A</u>G, CTGG<u>A</u>G sites (30). Other authors employed a directed in vitro evolution technique to achieve a change in the specificity of the HaeIII MTase (5).

The SinI MTase, a component of the SinI R-M system of *Salmonella enterica* serovar Infantis, recognizes the sequence GGW<u>C</u>C (W = A or T) and methylates the internal C to produce 5-methylcytosine (15, 16). We wanted to study the molecular mechanism used by M.SinI to recognize A · T or

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TABLE 1. Plasmids used in this study

Plasmid	Vector/resistance	Relevant encoded protein(s)	Reference
pSI4	pUC19[a]/Ap	M.SinI, R.SinI	15
pSI4-106	pUC19/Ap	M.SinI^{V173L}, R.SinI	This work
pSin5	pUC19/Ap	M.SinI	18
pSin10-19	pUC19/Ap	M.SinI^{N172S}	18
pSin10-106	pUC19/Ap	M.SinI^{V173L}	18
pSin10-5mut	pUC19/Ap	M.SinI$^{A34V+K44Q+M66T+L214S+Y229H}$	This work
pSau1	pBR322[a]/Ap	M.Sau96I	37
pSau2	pBR322/Ap	M.Sau96I	This work
pSau21	pOK12[b]/Kn	M.Sau96I, R.Sau96I	This work
pOB-RSau96I	pOK12/Kn	R.Sau96I[c]	This work
pSTC-MSau96I	pSC101^{ts}/Cm	M.Sau96I	This work
pMSin-RSau	pUC19/Ap	M.SinI, R.Sau96I	This work
pMSin(V173L)-RSau	pUC19/Ap	M.SinI^{V173L}, R.Sau96I	This work
pOK-ligA	pOK12/Kn	*E. coli* DNA ligase	This work
pJAT13*araE*[d]	pJN105/Gm Em	Arabinose transporter	17

[a] Carries the ColE1 origin of replication.
[b] Carries the p15A origin of replication.
[c] Controlled expression from the P_{BAD} promoter.
[d] Carries the pBBR-1 origin of replication.

T · A but exclude G · C and C · G (= S) in the middle of the recognition sequence. Because of a lack of structural information, in vitro random mutagenesis and selection for GGNCC (N = A, C, G, or T) specificity was used to obtain enzyme mutants which, while preserving the canonical GGWCC specificity, also methylated GGSCC sites (18, 38). Three variants displaying highly relaxed specificity were isolated: two mutants with single substitutions (N172S and V173L) and a third mutant (5mut) containing five amino acid replacements (A34V, K44Q, M66T, L214S, and Y229H). Surprisingly, none of these substitutions were in the variable region, which was previously thought to be the sole determinant of sequence specificity. Obtaining substitutions which are located far from the variable region yet lead to altered sequence specificity shed light on the function of large-domain–minor-groove interactions in determining sequence specificity. This role, i.e., exclusion of the G · C and C · G base pairs, presumably by steric clash with the 2-amino group of the guanine, was confirmed by in vitro studies using an oligonucleotide substrate containing a hypoxanthine-cytosine base pair in the central position of the recognition sequence (18). The L214S Y229H double mutant MTase, a derivative of the phenotypically similar parental 5mut enzyme, was subjected to steady-state kinetic analysis. This analysis revealed that the catalytic activity (k_{cat}/K_m) of the mutant MTase was ~5-fold lower for the canonical GGWCC site and 20-fold higher for the GGSCC site than that of the wild-type (WT) enzyme. The increase of noncanonical activity was due to the enhanced k_{cat} for GGSCC (38). Methylation kinetics of the N172S and V173L variants were not analyzed in detail, but preliminary data suggest that they too have increased k_{cat}s for the GGSCC site (our unpublished observations).

The natural function of modification MTases is to protect the host DNA against the cognate restriction endonuclease. In this work we tested the level of protection provided by the M.SinI mutants against a restriction enzyme cleaving both GGWCC and GGSCC sites. We describe experiments using an in vivo system in which the mutant MTases were coexpressed with the GGNCC-specific Sau96I endonuclease, whose expression could be tightly controlled, and viability of the cells was quantitatively estimated. To test the hypothesis that in *E. coli* a limited number of DNA double-strand scissions made by restriction endonucleases producing cohesive ends can be repaired in vivo by DNA ligase, viability assays were also performed with host bacteria containing elevated levels of DNA ligase.

MATERIALS AND METHODS

Strains and media. The *E. coli* strains DH10B F$^-$ *endA1 recA1 galU galK deoR nupG rpsL* Δ*lacX74* ϕ80*lacZ*Δ*M15 araD139* Δ(*ara leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$ (9), ER1398 F$^-$ *endA1 thi1 hsdR2 supE44 mcr1* λ$^-$ (28), and N2604 *lig ts7* (8) were used as cloning hosts. Bacteria were grown in LB medium (34). Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Kn), 50 μg/ml; chloramphenicol (Cm), 25 μg/ml; gentamicin (Gm), 10 μg/ml; and erythromycin (Em), 15 μg/ml. Glucose was used at 0.2% and L-arabinose (Sigma) as indicated for the various experiments.

Plasmids. The plasmids used in this work are listed in Table 1, and restriction maps of some of the plasmids are shown in Fig. 1. pSI4 carries the genes of the SinI R-M system cloned in pUC19 (15). pSin5, pSin10-19, pSin10-106 (18), and pSin10-5mut are similar plasmids; they carry the WT or a mutant SinI MTase (*sinIM*) allele in pUC19. pSin10-5mut was made by replacing the 1,245-bp BseRI-NdeI fragment encompassing most of the M.SinI coding sequence in pSin5 with the corresponding fragment of pTZSmut (38), which codes for an M.SinI variant with five substitutions (A34V, K44Q, M66T, L214S, and Y229H). pSI4-106 was constructed by reinserting the previously deleted (18) HindIII fragment of pSI4 into pSin10-106 to reconstitute the *sinIR* gene. pSau1 (37) and its deletion derivative pSau2 carry the Sau96I MTase gene. pSau21, which contains the complete Sau96I R-M system, was constructed by transferring the 3.1-kb SalI-BamHI fragment from pSau3 (37) into pOK12, a plasmid compatible with pUC vectors (40). Plasmid pSTC-MSau96I contains the Sau96I MTase gene cloned in pST76-C, a pSC101^{ts}-based plasmid vector (26) with temperature-sensitive replication; it can be maintained in the cell at 30°C but is lost at 42°C. pSTC-MSau96I was constructed by transferring the PstI-BstYI fragment, carrying the *sau96IM* gene, from pSau21 into pST76-C. Plasmid pOK-BAD, a derivative of pBAD24 (10) was constructed by ligating the 1,830-bp ClaI-PaqI fragment of pBAD24 to the 1,838-bp StuI-FspI fragment of pOK12. pOK-BAD carries the replication origin and the Kn^r gene of pOK12 and the *araC* regulator gene, the *araBAD* promoter, the polycloning site, and the *rrnB* terminator sequence of pBAD24. pOB-RSau96I contains the PCR-amplified coding sequence of the Sau96I endonuclease gene inserted between the NcoI and XbaI sites of pOK-BAD. pOB-RSau96I can be maintained in cells containing pSTC-MSau96I at 30°C. pMSin-RSau, a derivative of pSI4, carries a hybrid R-M system: the gene encoding the SinI MTase and the gene for the Sau96I endonuclease. It was constructed by replacing the NruI-SphI fragment of pSI4 with the ApaLI-SphI fragment of pSau21. pMSinI(V173L)-RSau is identical to pMSin-RSau except

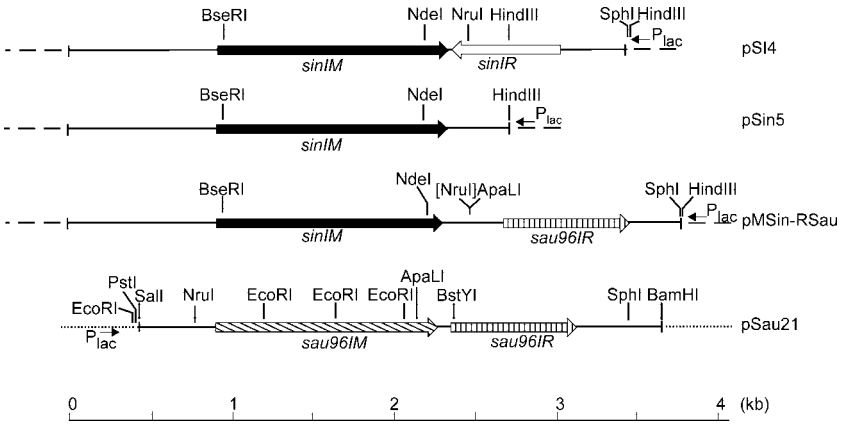

FIG. 1. Schematic map of the inserts of some of the plasmids used. The map of pSI4 also represents pSI4-106; the map of pSin5 also represents pSin10-19, pSin10-106, and pSin10-5mut; and the map of pMSin-RSau also represents pMSin(V173L)-RSau. Only restriction sites used to make the different constructs are shown. The NruI site shown in square brackets was lost during construction of pMSin-RSau. Continuous line, insert; broken line, pUC19 vector; dotted line, pOK12 vector.

that it carries the *sinIM* allele encoding the V173L variant. pOK-ligA contains the *E. coli* DNA ligase gene. It was constructed by transferring the ~2.7-kb NheI-BamHI fragment of pLG2520 (13) into the EcoRV site of pOK12. In pOK-ligA the orientation of the insert is such that the *lig* gene can be transcribed from the *lac* promoter on the vector. Expression of DNA ligase from pOK-ligA was verified by testing its capacity to restore viability of the ligase mutant N2604 *lig ts7* strain at the nonpermissive temperature of 42°C. Plasmid pJAT13*araE* (Gm^r Em^r), which constitutively expresses an arabinose transporter protein and is compatible with both ColE1 and p15A replicons, was used to ensure uniform expression of R.Sau96I in all cells of the culture (17).

DNA techniques. The recombinant DNA work used standard methods (34), and enzymes were purchased from Fermentas and New England Biolabs.

Viability assay. DH10B cells harboring pJAT13*araE* and a plasmid carrying either the WT or one of the mutant *sinIM* alleles was transformed with an HpaI-digested plasmid preparation containing pOB-RSau96I and pSTC-MSau96I. The role of HpaI digestion was to select against pSTC-MSau96I, which has a HpaI site, whereas pOB-RSau96I is not cut by HpaI. Gm^r Ap^r Kn^r transformants were selected on glucose-containing plates at 42°C. The absence of pSTC-MSau96I was verified by testing for the Cm^s phenotype at 30°C. Samples of the Gm^r Ap^r Kn^r Cm^s clones were stored at −80°C, and used to inoculate cultures for testing plating efficiency or growth rate.

For determination of plating efficiency, overnight cultures grown in LB-Gm-Ap-Kn-glucose medium were diluted 1:20 into fresh medium and grown at 37°C to an optical density at 550 nm of 0.3 to 0.6. The cultures were adjusted to equal optical density and serially diluted. To determine the viable cell counts, 0.1-ml aliquots of the different dilutions were plated on LB-Gm-Ap-Kn plates containing either glucose or 0.01% arabinose. The plates were incubated at 37°C overnight.

Sau96I endonuclease assay of crude extract. *E. coli* DH10B harboring pJAT13*araE*, pSau2, and pOB-RSau96I was grown in 50 ml LB-Em-Ap-Kn-glucose to an optical density at 550 nm of ~0.4. Cells were sedimented by centrifugation and then resuspended in 50 ml LB-Em-Ap-Kn–0.005% arabinose. After growing for 3 h at 37°C, cells were harvested by centrifugation, resuspended in 3 ml extraction buffer (50 mM Tris-HCl [pH 8.0], 10 mM 2-mercaptoethanol, 1 mM EDTA), and disrupted by sonication. Cell debris was removed by centrifugation, and dilutions of the supernatant prepared with extraction buffer were used to digest 0.6 μg lambda phage DNA in 50 mM K-acetate–20 mM Tris-acetate–10 mM Mg-acetate–1 mM dithiothreitol (pH 7.9) for 1 h at 37°C.

RESULTS AND DISCUSSION

Viability as an indication of protection of GGWCC and GGSCC sites by the mutant SinI MTases in vivo. Digestion of the plasmids carrying the mutant *sinIM* alleles (pSin10-19, pSin10-106, and pSin10-5mut) with methylation-sensitive restriction enzymes showed that GGWCC sites in the mutant plasmids were modified, whereas modification of GGSCC sites was not complete (Fig. 2). pSin10-19 and pSin10-106 were better protected against digestion by the GGNCC-specific Cfr13I, an isoschizomer of Sau96I, than pSin10-5mut, suggesting that the N172S and V173L single mutants were more active toward GGSCC sites than the 5mut variant (carrying A34V, K44Q, M66T, L214S, and Y229H).

The function of the MTase of an R-M system is to provide protection against the cognate endonuclease. As a first test of the ability of the mutant enzymes to function as the counterpart of a GGNCC-specific restriction endonuclease, a plasmid which is compatible with ColE1 replicons, which carries the complete Sau96I system, and from which the *sau96IM* gene can be easily deleted was constructed. This plasmid (pSau21) contains four EcoRI sites, and the positions of these sites are such that EcoRI digestion and subsequent circularization of the plasmid backbone by ligation delete most of the *sau96IM* gene,

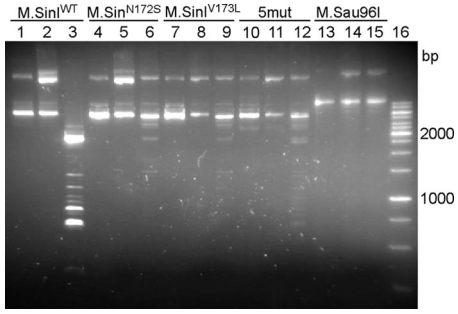

FIG. 2. Methylation status of plasmids tested by digestion with methylation-sensitive restriction enzymes and electrophoresis in a 1% agarose gel. Lanes 1 to 3, pSin5(WT); lanes 4 to 6, pSin10-19(N172S); lanes 7 to 9, pSin10-106(V173L); lanes 10 to 12, pSin10-5mut(A34V+K44Q+M66T+L214S+Y229H); lanes 13 to 15, pSau2; lane 16, DNA fragment size marker (Fermentas). Lanes 1, 4, 7, 10, and 13, undigested; lanes 2, 5, 8, 11, and 14, digested with Eco47I (GGWCC); lanes 3, 6, 9, 12, and 15, digested with Cfr13I (GGNCC). The plasmids pSin5, pSin10-19, pSin10-106, and pSin10-5mut contain two GGWCC and eight GGSCC sites. GGW<u>C</u>C and GGN<u>C</u>C sites with C5-methylated internal cytosines (underlined) are resistant to Eco47I and Cfr13I, respectively.

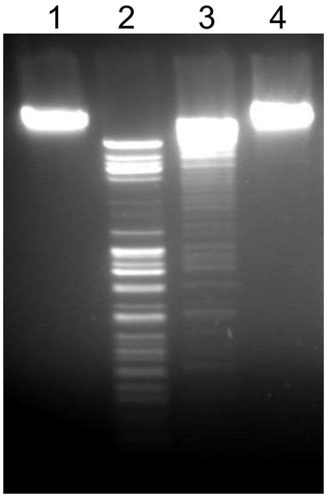

FIG. 3. Sau96I endonuclease activity in the crude extract of *E. coli* DH10B(pJAT13*araE*, pSau2, pOB-RSau96I) grown and induced with 0.005% arabinose as described in Materials and Methods. Agarose gel electrophoresis of lambda phage DNA digested with different dilutions of the cell extract is shown. Lanes: 1, undigested; 2, 10× diluted extract; 3, 100× diluted; 4, 1,000× diluted.

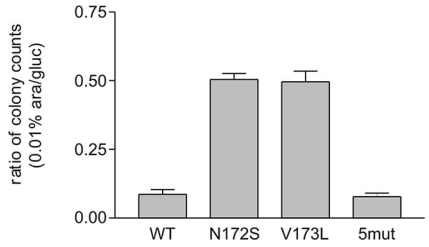

FIG. 4. Plating efficiency of *E. coli* DH10B cells expressing Sau96I endonuclease and relaxed-specificity mutants of the SinI MTase. Cells contained pJAT13*araE*, pOB-RSau96I, and a plasmid encoding the WT SinI MTase (pSin5) or a mutant SinI MTase: pSin10-19(N172S), pSin10-106(V173L), or pSin10-5mut(A34V+K44Q+M66T+L214S+Y229H). Aliquots of serially diluted cultures were pipetted onto LB-Gm-Kn-Ap agar plates containing either 0.2% glucose or 0.01% arabinose. Glucose served to repress and arabinose to induce Sau96I endonuclease expression. The data shown are the averages and standard errors of the means from five to eight experiments.

leaving the *sau96IR* gene and the vector intact (Fig. 1). To test if the N172S and the V173L mutant SinI MTases can protect the host DNA against the Sau96I endonuclease in vivo, pSau21 was cleaved with EcoRI and ligated, and the ligated DNA was used to transform *E. coli* ER1398 cells harboring one of the Ap^r plasmids pSin10-19, pSin10-106, or pSau1. pSau1 is a ColE1-based plasmid carrying the gene for the Sau96I MTase (Table 1). Transformation with the ligated pSau21 DNA yielded several thousand Ap^r Kn^r double-resistant clones in host cells containing pSau1, whereas no double-resistant transformants were obtained with host cells harboring pSin10-19 or pSin10-106. Similar transformation efficiencies of the three kinds of competent cells were verified with a transformation control using intact pSau21, which yielded several ten thousands of transformants in all three types of cells. These results showed that the mutant SinI MTases could not protect the cell DNA from Sau96I endonuclease expressed from its native promoter and/or from the vector *lac* promoter (Fig. 1) in vivo.

GGWCC-specific methylation of the mutant plasmids seemed to be complete (38) (Fig. 2), suggesting that the mutant enzymes can protect the host DNA against the SinI endonuclease in vivo. To test this assumption, a plasmid (pSI4-106) which contains the *sinIR* and *sinIM* genes in their native arrangement but in which the MTase gene has the V173L mutation was constructed. DH10B cells harboring this plasmid had a normal growth rate. These data indicated that M.SinI(V173L), and most probably also the N172S and the 5mut variants, preserved the capacity to function as a genuine GGWCC-specific modification MTase, but their acquired GGSCC-specific activity was too low to protect the cell against a GGNCC-specific restriction endonuclease.

To test the GGNCC-specific activity of the mutant SinI MTases in vivo in a more controlled manner, a system in which expression of the Sau96I endonuclease can be tightly regulated was designed. A plasmid (pOB-RSau96I) expressing R.Sau96I under the control of the *araBAD* promoter was constructed as described in Materials and Methods. Transcription of the *sau96IR* gene in pOB-RSau96I can be repressed by adding glucose or induced by adding arabinose to the medium. pOB-RSau96I is stable in cells containing pSTC-MSau96I, which expresses the Sau96I MTase, but is lethal in m^- cells even in the presence of glucose. It can be propagated under glucose repression for shorter periods in cells also containing pSin5, which encodes the WT SinI MTase, but such cells tend to accumulate mutations inactivating the Sau96I endonuclease upon extended culturing. To estimate the intracellular level of Sau96I endonuclease activity under inducing conditions, an extract was prepared from a culture of *E. coli* DH10B(pJAT13*araE*, pSau2, pOB-RSau96I) induced with 0.005% arabinose, and Sau96I endonuclease activity was assayed by digesting λ phage DNA. The digestion pattern obtained with a 10-fold-diluted extract indicated that the cells contained a substantial amount of Sau96I endonuclease (Fig. 3).

To assess the viability of cells coexpressing the mutant MTases and Sau96I endonuclease, *E. coli* DH10B cells were transformed with three plasmids: pJAT13*araE*, pSin5 or its mutant derivatives, and pOB-RSau96I. The methylation status of plasmids isolated from the triple transformants was similar to that of plasmids isolated from the single transformants shown in Fig. 2. Viability was tested by plating aliquots of cultures grown in the presence of glucose onto agar plates containing glucose or different concentrations of arabinose. Preliminary experiments showed that even at an arabinose concentration of as low as 0.0001%, the cells producing the N172 or the V173L mutant MTases formed colonies with higher efficiency than cells containing the WT MTase (not shown). Viability was quantitatively assessed by determining plating efficiency at 0.01% arabinose as described in Materials and Methods. At this arabinose concentration, the plating efficiency of cells producing the N172S or V173L mutant MTases was approximately fivefold higher than that of cells producing the WT or the 5mut enzyme (Fig. 4), indicating that the N172S and V173L mutants of M.SinI can provide significant protection against Sau96I cleavage in vivo.

To exclude the possibility that the increased viability of the N172S and the V173L mutants observed in the plating exper-

iments was the result of inactivation of the Sau96I endonuclease by a secondary mutation, plasmid DNA was prepared from the triple transformants investigated in the viability assays and used to transform cells containing pSTC-MSau96I. Cm^r Kn^r double transformants were selected at 30°C and then screened for the Ap^s phenotype, which indicated the absence of pSin5 or its mutant derivatives. Such Cm^r Kn^r Ap^s clones were subsequently tested for the ability to grow on Kn-Cm plates containing 0.1% arabinose at 30 or 42°C. All clones tested grew at 30°C but died at 42°C, a temperature nonpermissive for pSTC-MSau96I, proving that the Sau96I endonuclease in the triple transformants was functional.

The experiments described above demonstrated that the N172S and V173L mutant SinI MTases provide significant protection in vivo against a restriction endonuclease with GGNCC specificity. However, when the colonies obtained on the arabinose-containing plates were transferred onto fresh arabinose-containing plates, they usually did not grow, indicating that the level of methylation at GGSCC sites was insufficient to support long-term survival. To further test the viability of cells in colonies appearing on arabinose-containing plates, colonies taken from arabinose-containing as well as glucose-containing plates were suspended and replated on glucose-containing plates. These tests revealed that for both N172S and V173L, colonies that appeared on arabinose-containing plates contained approximately 10- to 100-fold fewer viable cells than colonies taken from glucose-containing plates.

Interestingly, although the methylation level of GGSCC sites in pSin10-5mut was not dramatically lower than that in the two single mutants (Fig. 2), the clone producing the variant with five substitutions displayed no increase in plating efficiency relative to the WT (Fig. 4). This suggests that there is a minimum level of methylation which is essential to have a detectable effect on viability under the conditions of the plating assay.

Effect of elevated level of DNA ligase. At first glance, even the limited protection provided by the N172S and V173L mutants seems surprising, as methylation of the GGSCC sites in the plasmid DNA, and presumably in the genomic DNA, was not complete (Fig. 2), probably leaving many of the 4,218 GGSCC sites in the *E. coli* genome (J. Pósfai, personal communication) unprotected. We assumed that viability under these conditions was due to DNA ligase-mediated repair of Sau96I endonuclease-inflicted DNA scissions. This model was suggested by two previous observations. It was shown that *E. coli* DNA ligase can repair a moderate level of double-strand scissions caused by the EcoRI endonuclease in cells whose DNA is not protected by EcoRI-specific methylation (12). The role of DNA ligase was also demonstrated in rescuing *E. coli* cells carrying the PvuI restriction endonuclease gene without the cognate MTase gene (36).

To test the hypothesis that DNA ligase can repair DNA scissions occurring at incompletely modified GGSCC sites, two plasmids encoding SinI-Sau96I hybrid R-M systems [pMSin-RSau and pMSin(V173L)-RSau] and a compatible plasmid (pOK-ligA) encoding *E. coli* DNA ligase were constructed. pMSin-RSau carries the WT *sinIM* and the *sau96IR* genes, whereas pMSin(V173L)-RSau carries the V173L mutant allele of the *sinIM* gene and the *sau96IR* gene (Fig. 1; Table 1). Because of unprotected GGSCC sites, these plasmids can be maintained only in cells containing the *sau96IM* gene on a compatible plasmid, such as pSTC-MSau96I. When plasmid preparations containing pMSin-RSau and pSTC-MSau96I were used to transform ER1398(pOK-ligA) cells at 42°C, a temperature nonpermissive for pSTC-MSau96I, Ap^r Kn^r colonies of various sizes were obtained. Of 1,000 Ap^r Kn^r Cm^s transformants, only two grew when transferred onto fresh Ap-Kn plates or inoculated into LB-Ap-Kn liquid medium. Analysis of these two clones revealed that they did not contain Sau96I endonuclease. Similarly, transformation of ER1398(pOK12) cells with a plasmid sample containing pMSin(V173L)-RSau and pSTC-MSau96I did not yield stable Ap^r Kn^r Cm^s transformants. In contrast, when ER1398(pOK-ligA) cells were transformed with the same plasmid preparation [pMSin(V173L)-RSau/pSTC-MSau96I], approximately 10% of the 200 Ap^r Kn^r Cm^s clones tested were stable enough to grow up to high density in a liquid culture. Digestion of plasmid DNA purified from these clones showed the characteristic partial fragmentation pattern with Cfr13I, and cell extracts prepared from the clones contained Sau96I endonuclease, proving that the increased viability was not due to the presence of the Sau96I MTase or the absence of the Sau96I endonuclease.

These results and previous observations (12, 36) suggest the generalization that the same mechanism (ligase-mediated repair) saved *E. coli* cells from self-destruction in all cases where the puzzling phenomenon of viable r^+ m^- clones was observed. In this context, it is worth to call attention to the so-far-perhaps-unrecognized fact that, to our knowledge, in all R-M systems where viable r^+ m^- clones have been reported, the restriction enzyme produces cohesive ends, which (unlike blunt ends) are substrates for *E. coli* DNA ligase: PaeR7I (C/TCGAG) (7), TaqI (T/CGA) (2, 35), HaeII (RGCGC/Y), HgiAI (GWGCW/C), HinfI (G/ANTC), PstI (CTGCA/G), XbaI (T/CTAGA) (reference 3 and unpublished observations reported in reference 35), MwoI (GCNNNNN/NNGC) (23), BanIII (AT/CGAT) (24), BstVI (C/TCGAG) (39), and PvuI (CGAT/CG) (36). Moreover, the restriction enzymes cloned by the "endo-blue method," which is based on the expression of the restriction endonuclease in cells whose DNA is unprotected or is only partially protected by methylation, belong to the type making overhanging ends: TaqI, Tth111I (GACN/NNGTC), and BsoBI (C/YCGRG) (6, 33).

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