JOURNAL OF BACTERIOLOGY, June 1988, p. 2533–2536
0021-9193/88/062533-04$02.00/0

Vol. 170, No. 6

# Isolation and Characterization of the Modification Methylase M · *Sin*I

CHRISTIAAN KARREMAN* AND ADRIAN DE WAARD

*Department of Medical Biochemistry, Sylvius Laboratories, University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands*

Received 17 November 1987/Accepted 9 March 1988

**A sequence-specific modification methylase (M · *Sin*I) was isolated and purified from *Escherichia coli* harboring a derivative of recombinant plasmid pSI4 (see accompanying manuscript: C. Karreman and A. de Waard, J. Bacteriol. 170:2527–2532, 1988), which contains a *Salmonella infantis* DNA insert. The enzyme uniquely methylates the internal deoxycytidylate residue in the nucleotide sequence GG(A/T)$^{Me}$CC, thereby protecting DNA completely against cleavage by restriction endonuclease R · *Sin*I or R · *Ava*II [GG(A/T)CC], and in part against cleavage by R · *Sau*96I (GGNCC).**

In the bacterial cell, modification methylases protect the DNA against restriction enzymes. They can be isolated and exploited in vitro to render DNA resistant to the action of particular restriction enzymes.

The endonuclease R · *Sin*I (7) recognizes a degenerate sequence, i.e., it cleaves the sequence G^G(A/T)CC, whereas the same sequence is expected to be protected against this nucleolytic attack by appropriate methylation, presumably of cytosine residues. Another endonuclease, R · *Sau*96I (11), cleaves the more degenerate sequence G^GNCC. By treating DNA with M · *Sin*I before incubation with R · *Sau*96I, one expects to change the net specificity of R · *Sau*96I into G^G(G/C)CC. This possibility prompted us to purify the modification methylase of *Salmonella infantis* and also to study the specificity of this enzyme.

## MATERIALS AND METHODS

**Culture conditions.** *Escherichia coli* MC1061 (2) harboring the recombinant plasmid pΔSPHH1 (see below) was grown to the early stationary phase in brain heart infusion medium (Oxoid Ltd. Basinstoke, United Kingdom) at 37°C. Cells were harvested by centrifugation and stored at −30°C.

**Chemicals and enzymes.** *S*-Adenosyl-methionine was purchased from Boehringer (Mannheim, Federal Republic of Germany). Restriction endonucleases were obtained from Promega-Biotec (Leiden, The Netherlands), New England Biolabs (Beverly, Mass.), or Pharmacia (Uppsala, Sweden) and used as indicated by their manufacturers. Hydrazine (pure) was from Serva (Heidelberg, Federal Republic of Germany), and piperidine was from E. Merck AG (Darmstadt, Federal Republic of Germany). The oligonucleotide 5′-CCGGACCTCGAGGT-3′ was synthesized by J. H. van Boom and co-workers (University of Leiden).

**Determination of sequences.** Plasmids were cut with R · *Eco*RI, terminally labeled with [α$^{32}$P]dATP, and then digested with R · *Hha*I. Fragments were isolated from a polyacrylamide gel and subjected to nucleotide sequence determination (9).

**Isolation of M · *Sin*I.** Frozen cells (about 10 g) were thawed and incubated for 10 min with lysozyme (Boehringer) in the presence of EDTA at room temperature and subsequently sonicated (sonifier from Branson Sonic Power Co., Danbury, Conn.) at 4°C with 100-W bursts totaling 3 min. All further steps were carried out at 4°C.

The crude lysate was treated with polyethyleneimine (1%, final concentration) at pH 7.9 in the presence of 0.4 M NaCl to precipitate nucleic acids. After their removal by centrifugation (10,000 × *g* for 1 h), the supernatant was dialyzed twice for 2 h against buffer A (20 mM Tris hydrochloride, 0.1 mM EDTA, 2 mM β-mercaptoethanol [pH 7.4]). The enzyme solution was clarified by brief centrifugation and chromatographed on a 20- by 2-cm column of Whatman P11 phosphocellulose with buffer A to which 10% glycerol had been added. A 400-ml gradient of 0 to 0.65 M KCl was run with a gradient programmer (P250; Pharmacia). The methylase eluted at 0.5 M KCl.

**Assay of M · *Sin*I.** The enzyme was assayed on the basis of its capacity to protect bacteriophage lambda DNA (35 potential sites) against cleavage by endonuclease R · *Sin*I. Bacteriophage lambda DNA (0.75 μg) was incubated with a 5-μl enzyme sample in the presence of 1 mM *S*-adenosylmethionine in a total volume of 20 μl (50 mM Tris, 10 mM EDTA, 125 mM KCl) for 120 min at 37°C. After 2 h, $MgCl_2$ was added (16 mM, final concentration), and incubation was continued with 10 U of R · *Sin*I for an additional hour. The samples were then subjected to agarose gel electrophoresis. One unit of M · *Sin*I was defined as the amount of enzyme required to render 1 μg of lambda DNA resistant to cleavage with R · *Sin*I after 1 h of incubation.

**Construction of plasmids.** All plasmids mentioned below are derivatives of plasmid pSI4 (5), which codes for both R · *Sin*I and M · *Sin*I. (Fig. 1). The first descendant (pΔHH2) was obtained by deleting the small R · *Hin*dIII-R · *Hin*dIII fragment (step 1); this plasmid is M · *Sin*I$^+$ and R · *Sin*I$^-$. To minimize the size of the plasmid an R · *Sma*I-R · *Pst*I fragment was also deleted (step 2), yielding plasmid pΔSPHH1.

Two plasmids were constructed for the determination of the target base for methylation by M · *Sin*I. Both are based on pΔHH2; for the first (pS→XΔHH3), this plasmid was opened at its unique *Sma*I site with R · *Ava*I, and the partly palindromic oligonucleotide 5′-CCGGACCTCGAGGT-3′ was ligated into it (step 3). The second plasmid (pS→

* Corresponding author.

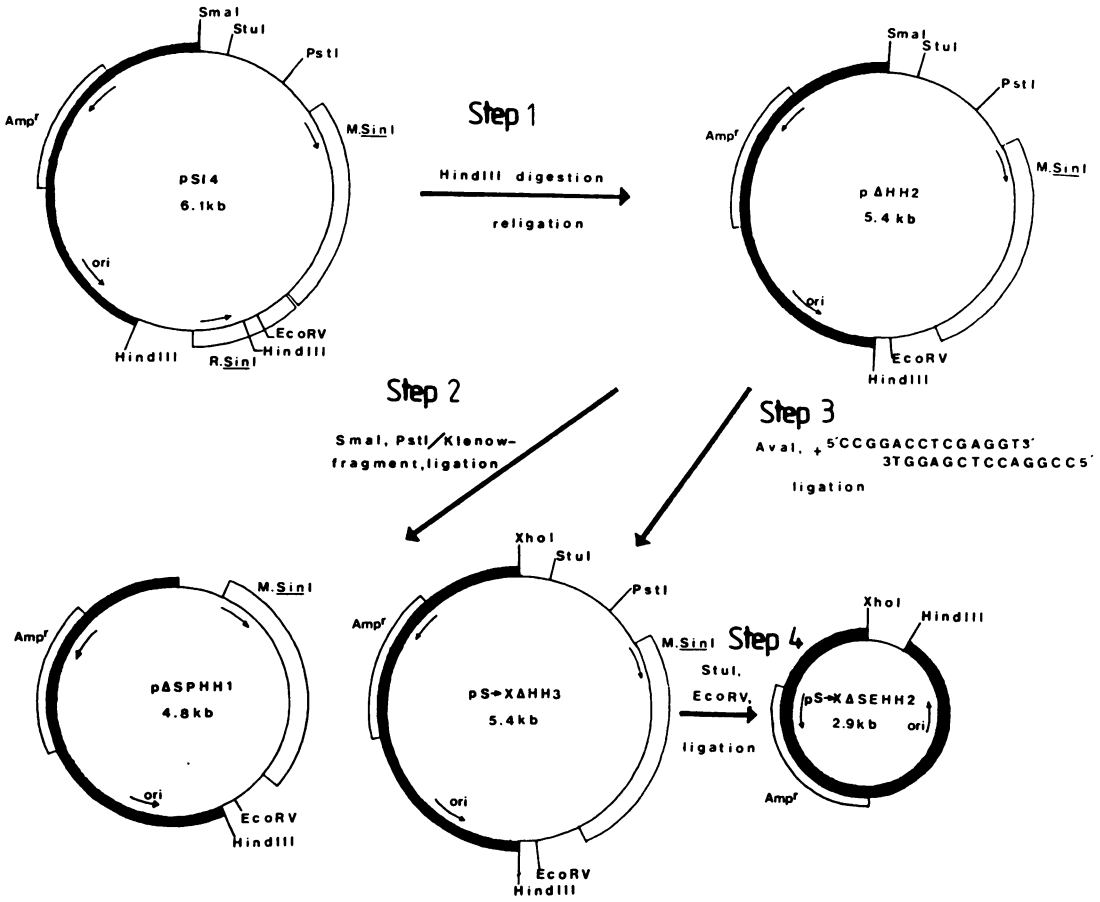


FIG. 1. Plasmids described in the text. On the upper left-hand corner the original clone pSI4 is shown with vector DNA by thick lines, and the *S. infantis* insert DNA is shown by thin lines. The genes are indicated by boxes.

XΔSEHH2) that is phenotypically M · *Sin*I$^-$, was made by deleting a R · *Stu*I-R · *Eco*RV fragment (step 4).

## RESULTS AND DISCUSSION

Approximately 10 g of cells harboring plasmid pΔSPHH1 was sonicated and, after fractionation on a Whatman P11 column, yielded a total of 10,000 U of exonuclease-free M · *Sin*I. The enzyme was found to have an absolute requirement for *S*-adenosyl-methionine as a methyl donor. It protected DNA uniquely against R · *Sin*I and R · *Ava*II (Fig. 2), which both recognize the sequence GG(A/T)CC. It did not protect against enzymes with other recognition sequences (R · *Stu*I, R · *Eco*RV, R · *Hin*dIII, R · *Pst*I, R · *Xba*I).

We were able to prove that the inner C of the recognition site of M · *Sin*I is methylated. Since the recognition sequence of M · *Sin*I-R · *Sin*I contains two deoxycytidylate residues, we had to consider three alternative methylation patterns: methylation of the internal cytosine, the external cytosine, or less probably both. Two different methods were used to clarify this point; both made use of the synthetic oligonucleotide linker described in Materials and Methods. The first method used plasmid pS→XΔSEHH2 after it had been methylated in vitro by M · *Sin*I; in this recombinant, the introduction of the synthetic linker (step 3) had resulted in the sequence:

*Sin*I *Sin*I

5'-CCGGACCTCGAGGTCCGG-3'
3'-GGCCTGGAGCTCCAGGCC-5'

*Msp*I *Xho*I *Msp*I

in which the potential recognition-cleavage sites are indicated. Endonucleases R · *Msp*I and R · *Xho*I are sensitive to

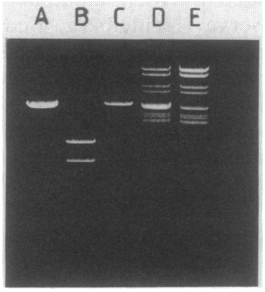


FIG. 2. Resistance to R · *Ava*II of M · *Sin*I-modified DNA. Lanes: A, unmethylated, linearized plasmid pUC19; B, unmethylated plasmid incubated with R · *Ava*II; C, methylated plasmid with R · *Ava*II; D, methylated plasmid mixed with phage lambda DNA and R · *Ava*II; E, phage lambda DNA with R · *Ava*II.

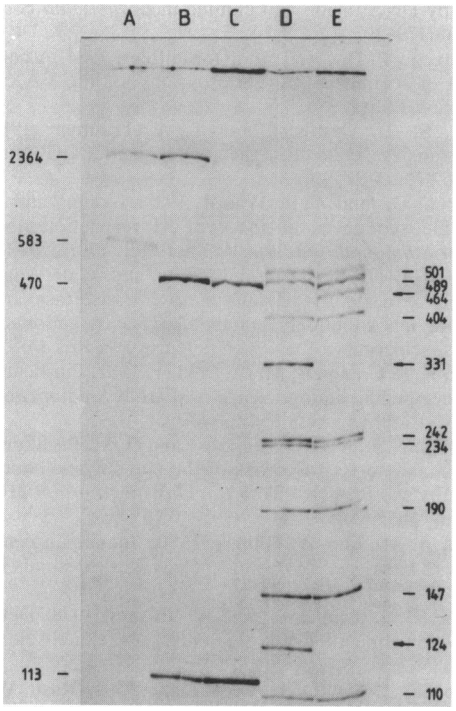


FIG. 3. Protection of M · *Sin*I modificated DNA against the action of R · *Msp*I and R · *Xho*I with overlapping sites. Lanes A, unmethylated plasmid pS→XΔSEHH2 with R · *Pvu*II; B, unmethylated plasmid with R · *Pvu*II and R · *Xho*I; C, methylated plasmid with R · *Pvu*II and R · *Xho*I; D, unmethylated plasmid with R · *Msp*I; E, methylated plasmid with R · *Msp*I. The lengths of the bands are given in base pairs.

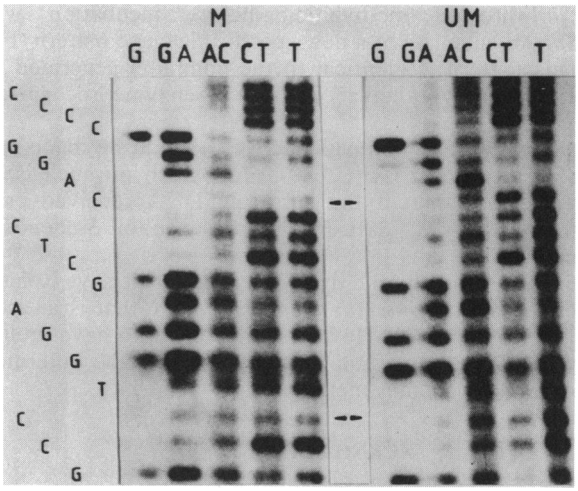


FIG. 4. Comparison of sequence ladders generated with in vivo-methylated DNA. The nucleotide sequence of analogous stretches of two plasmids was determined with the chemical cleavage method. Plasmid pS→XΔHH3 (M) was methylated in vivo; this plasmid codes for the M · *Sin*I protein. The other plasmid, pS→XΔSEHH2 (UM), has had this gene deleted. The cytosines that are methylated are indicated.

methylation at specific bases in their recognition sites (4, 6); R · *Msp*I cleavage is blocked in the case of methylation of the internal cytosine of the *Sin*I site, and R · *Xho*I cleavage is blocked in the case of methylation of the external cytosine. This experiment is illustrated in Fig. 3; pS→XΔSEHH2 was incubated with M · *Sin*I and subsequently with either a combination of R · *Pvu*II and R · *Xho*I or separately with R · *Msp*I. As a control, pS→XΔSEHH2 was also treated with the same endonucleases but without prior methylation with M · *Sin*I.

Lanes B and C of Fig. 3 show the resulting patterns of unmethylated and methylated pS→XΔSEHH2 incubated with R · *Xho*I: both are clearly sensitive to this enzyme, which implies that the external cytosine residue is not methylated. Lanes D and E contain R · *Msp*I-treated unmethylated and methylated plasmids, respectively; lane E (M · *Sin*I-methylated DNA) contains one large band representing the sum of the two smaller bands figuring in lane D (unmethylated DNA). This signifies that only the internal cytosine in the two recognition sites in this plasmid is methylated. This conclusion was confirmed by the second experiment.

In the second experiment, the linker oligonucleotide present in the plasmid pS→XΔHH3 and its M · *Sin*I$^-$ derivative pS→XΔSEHH2 was analyzed for its content of methyl groups by subjecting it to the chemical sequencing procedure of Maxam and Gilbert (9). Here we made use of the fact that 5-$^{Me}$C is resistant to the action of hydrazine (10), which is used to introduce specific cleavage at pyrimidine sites. So methylation leads to the loss of the corresponding band in a sequence ladder. On the other hand, methylation of a cytosine residue at the 4N position does not lead to hydrazine resistance (1); therefore, this experiment gave information as to which position in the cytosine ring is methylated, i.e., whether the product is 4N-$^{Me}$C or 5-$^{Me}$C.

In Fig. 4 the Maxam-Gilbert analyses of the oligonucleotides of pS→XΔHH3 and pS→XΔSEHH2 are compared. In the analysis of the plasmid that was methylated in vivo the bands corresponding to the internal C residues of the two recognition sites are reduced to background level, indicating their resistance toward hydrazine treatment. However, the equivalent bands are present in the unmodified control. This not only confirmed the conclusion of the first experiment but also showed that the cytidylate residue is converted to 5-$^{Me}$C and not 4N-$^{Me}$C.

The M · *Sin*I modification methylase reported here may find use in restriction enzyme analysis of DNA, not only for the protection of DNA against R · *Sin*I and R · *Ava*II but also in combination with R · *Sau*96I. This enzyme recognizes the sequence GGNCC (11), which is less specific than that of R · *Sin*I [GG(A/T)CC]. The protection of DNA by

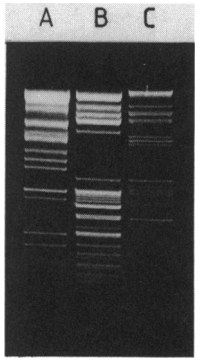


FIG. 5. New specificity for the M · *Sin*I-R · *Sau*96I combination. Lanes: A, phage lambda DNA with R · *Sin*I; B, phage lambda DNA with M · *Sin*I and R · *Sau*96I; C, phage lambda DNA with R · *Sau*96I.

M · *Sin*I-directed methylation before incubation with R · *Sau*96I gave rise to a new specific cleavage pattern (Fig. 5). This pattern is identical to the computer-generated (3) pattern of a hypothetical restriction enzyme recognizing GG(G/C)CC.

Endonuclease R · *Sau*96I (GGNCC) could be made specific for the subset GG(G/C)CC, for which no nuclease is currently available, if the substrate DNA received prior treatment with M · *Sin*I in the presence of *S*-adenosylmethionine.

It is interesting to note that Matvienko et al. have isolated a methylase from *Bacillus megaterium* 216 that is an isoschizomer of M · *Sin*I. However, they claim to have preliminary data suggesting that in their case it is the outermost cytosine that becomes methylated (8).

## ACKNOWLEDGMENTS

We are indebted to H. van Ormondt for critical reading of the manuscript.

Financial support was obtained from the Foundation for the Technical Sciences in The Netherlands.

## LITERATURE CITED

1. **Butkus, V., S. Klimasauskas, D. Kersulyté, D. Vaitkevicius, A. Lebionka, and A. Janulaitis.** 1985. Investigation of restriction-modification enzymes from *M. varians* RFL19 with a new type of specificity toward modification of substrate. Nucleic Acids Res. **13**:5727–5746.
2. **Casadaban, M., and S. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
3. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
4. **Jentsch, S., U. Günthert, and T. A. Trautner.** 1981. DNA methyltransferase affecting the sequence 5'CCGG. Nucleic Acids Res. **9**:2753–2759.
5. **Karreman, C., and A. de Waard.** 1988. Cloning and complete nucleotide sequences of the type II restriction-modification genes of *Salmonella infantis*. J. Bacteriol. **170**:2527–2532.
6. **Karreman, C., N. Tandeau de Marsac, and A. de Waard.** 1986. Isolation of a deoxycydylate methyl transferase capable of protecting DNA uniquely against cleavage by endonuclease *R · AquI* (isoschizomer of *AvaI*). Nucleic Acids Res **14**:5199–5205.
7. **Lupker, H. S. C., and B. M. M. Dekker.** 1981. Purification of the sequence-specific endonuclease *Sin*I from *Salmonella infantis*. Biochim. Biophys. Acta **654**:297–299.
8. **Matvienko, N. I., V. M. Kramarov, and D. M. Pachkunov.** 1987. Isolation and some properties of the site-specific endonuclease and methylase *Bme*216I from *Bacillus megaterium* 216. Eur. J. Biochem. **165**:565–570.
9. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
10. **Ohmori, H., J. Tomizawa, and A. Maxam.** 1978. Detection of 5-methylcytosine in DNA sequences. Nucleic Acids Res. **5**: 1479–1485.
11. **Sussenbach, J. S., P. H. Steenbergh, J. A. Rost, W. J. van Leeuwen, and J. D. A. van Embden.** 1978. A second site-specific restriction endonuclease from *Staphylococcus aureus*. Nucleic Acids Res. **5**:1153–1163.