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Isolation of a deoxycytidylate methyl transferase capable of protecting DNA uniquely against cleavage by endonuclease R.*Aqu* I (isoschizomer of *Ava* I)

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ABSTRACT

A sequence-specific modification methylase (M.AquI) was isolated and purified from Agmenellum quadruplicatum (Synechococcus PCC 7002). This enzyme uniquely methylates the deoxycytidylate residue in the sequence *CYCGRG indicated by the asterisk. It was shown to protect DNA against cleavage by restriction endonucleases AvaI, SmaI and XhoI, which recognize the sequences CYCGRG, CCCGGG, and CTCGAG, respectively.

INTRODUCTION

Sequence-specific DNA modification methylases (the counterparts of type II restriction endonucleases) are beginning to find use in molecular-biological research, e.g. for alterating of restriction enzyme cleavage patterns and for modifying DNA to be used in transfection experiments.

Restriction endonucleases are found in many genera and species of cyanobacteria (for a review see Tandeau de Marsac and Houmard (1)). Agmenellum quadruplicatum contains such an endonuclease, R.AquI (2) which is an isoschizomer of endonuclease R.AvaI (C↓YCGRG). We report here the first sequence-specific modification methylase M.Aqu I to be isolated from a cyanobacterium.

MATERIALS AND METHODS

Culture conditions

Agmenellum quadruplicatum PR-6 (strain 7002 of the Pasteur Culture Collection) was grown in medium ASN III (3) buffered with 2 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) buffer, pH 7.8, and supplemented with 100 µg vitamin B12/litre. The culture was illuminated with cool-white fluorescent light at 1000 lux and aerated with a stream of sterile air (95%)/CO_2 (5%) bubling through. The cells were harvested in late log phase and stored at -30°C.

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Chemicals and enzymes

S-adenosyl-methionine (SAM) was purchased from Boehringer Mannheim. Restriction endonucleases were obtained from Anglian Biotechnology Ltd or Promega-Biotec.

Isolation of modification methylase M.AquI

Frozen cells of Agmenellum quadruplicatum (10 g) were processed as described earlier (4). The crude extract was treated with polyethylene imine at pH 7.9 (1% final concentration) in the presence of 0.6 M NaCl to precipitate nucleic acids. After removal of these by centrifugation (10.000 x g, 10 min), the supernatant was saturated to 70% with ammonium sulphate. The precipitated proteins were dissolved in 20 mM potassium phosphate (pH 7.4) - 1 mM Na_2EDTA, 2mM 2-mercaptoethanol and dialysed against 2x2 l of the same buffer. The enzyme solution was clarified by brief centrifugation and chromatographed on a 20x2.5 cm column of Whatman P11 cellulose phosphate equilibrated with the same buffer. Two consecutive gradients were applied, first 40 ml from 0 to 0.2 M NaCl in the above buffer (also containing 10% glycerol) and secondly, 200 ml from 0.2 to 0.4 M NaCl in the same buffer. The methyl transferase eluted in the latter gradient, ahead of the restriction endonuclease R.AquI.

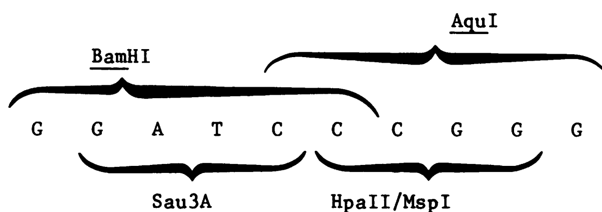
Assay of methylase M.AquI

The method of McClelland (5) was used which is based on the capacity of a modification methylase to protect DNA against a restriction endonuclease with identical sequence recognition properties. Bacteriophage lambda DNA (0.6 µg) was incubated for 120 min with 56 mM Tris-Cl (pH 7.4), 10 mM Na_2EDTA, 6 mM 2-mercaptoethanol in the presence of 1 mM SAM, in a total volume of 20 µl. The reaction was terminated by heating the mixture at 65°C for 10 min. $MgCl_2$ and NaCl were then added to 16 mM and 100 mM, respectively, and 3 units of purified endonuclease R.AquI were added (reaction volume now 35 µl); incubation was continued at 37°C for 60 min. Agarose gel-electrophoretic examination showed which fractions were capable of protecting λ DNA against cleavage by endonuclease R.AquI.

Strategy used to identify the base methylated by M.AquI

All known DNA methyltransferases are specific for either adenine or cytosine. The nature of the AquI recognition sequence excludes the former possibility. In order to determine which of the possible cytosine residues in this sequence is methylated by the M.AquI enzyme, the following strategy was developed. Plasmid pUC9 was converted to a new construct, pKW2, by

cutting pUC9 at the unique sites for R.*Aqu*I and R.*Bam*HI, filling in the "sticky" ends using the large fragment (Klenow) of polymeraseI and religating the so formed "blunt" ends. In this way the second C of the original sequence: 5'-GGATCCCCGGG-3'was deleted. The essential feature of this new DNA molecule is the presence of four overlapping sites for the endonucleases *Sau*3A, *Bam*HI, *Aqu*I and *Hpa*II/*Msp*I, as shown below.



It is known that the sequence GAT^{me}C is resistant to cleavage by endonuclease *Sau*3A (6). Incubation of the above plasmid with M.*Aqu*I in the presence of SAM and subsequent treatment of the methylated pKW2 with *Sau*3A should therefore tell whether the leftmost cytosine of the *Aqu*I sequence is the target base for the *Aqu*I methylase.

It is also known that the sequence ^{me}CCGG is resistant to both endonucleases *Hpa*II and *Msp*I, whereas C^{me}CGG is susceptible to cleavage by *Msp*I and resistant to *Hpa*II (7). A similar methylation experiment as described above and subsequent digestion of the product with either *Msp*I or *Hpa*II would establish whether the third cytosine has become methylated. The second cytosine of the sequence is not expected to be a substrate for the methylase *in vivo* in view of the degeneracy (T/C) of this position. If, however, this base could accept a methyl group *in vitro* (as an artefact) this would be detected by the failure of both *Msp*I and *Hpa*II to digest such a sequence.

RESULTS AND DISCUSSION

The methylating enzyme M.*Aqu*I had an absolute requirement for SAM as a donor of methyl groups. It had the unique property of protecting various DNA molecules against the endonucleolytic action of *Aqu*I and *Ava*I (which both specifically cleave the nucleotide sequence CYCGRG) and also that of *Xho*I (CTCGAG) and *Sma*I (CCCGGG) (Fig.1). It did not protect DNA molecules against cleavage by restriction endonucleases with different recognition

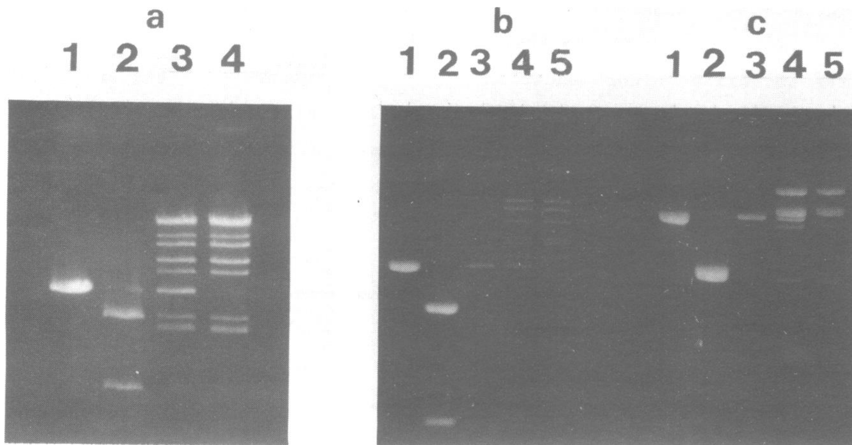


Figure 1. Protection of various DNA molecules against cleavage by restriction endonucleases R.AvaI, R.SmaI and R.XhoI.
a) AvaI. Lane 1, linearized plasmid pUC19. Lane 2, (unmethylated) plasmid pUC19 incubated with R.AvaI. Lane 3, plasmid pUC19 methylated by M.AquI and subsequently mixed with bacteriophage λ DNA and digested with R.AvaI Lane 4, λ DNA digested with R.AvaI.
b) SmaI. Experimental set up as in a. Lane 3, plasmid pUC19 methylated by M.AquI and subsequently incubated with R.SmaI. Adenovirus type 5 DNA was used as control.
c) XhoI. Experimental set up as in b, except that bacteriophage ØX174 RF DNA was used as a substrate. Adenovirus type 2 DNA was used as a control.

properties such as PstI (CTGCAG), NcoI (CCATGG), EcoRV (GATATC) and Asp700 ($GAAN_4TTC$).

We were able to prove that the leftmost cytidylate residue in the CYCGRG sequences is the receptor of the methyl group from the donor molecule SAM. The evidence for this came to a large extent from the experiment with Sau3A, HpaII and MspI endonucleases as discussed in the Methods section. This experiment is documented in Fig.2. The 2448-bp SinI-A fragment of pKW2 was first methylated in vitro by M.AquI and then incubated in separate incubations with Sau3A, HpaII and MspI. Lanes 4-7 give the results with HpaII and MspI, which bear on the innermost cytosine present in the AquI recognition sequence. In this figure, the smallest 101-bp fragment in lane 7 is slightly underrepresented in lane 5, suggesting that the innermost C might be methylated. However, other experiments did not substantiate this conclusion. Firm proof that, indeed, the outermost C is methylated by M.AquI is given in lanes 1-3. The two Sau3A fragments of the SinI-A fragment of pKW2 which border the AquI site (lane

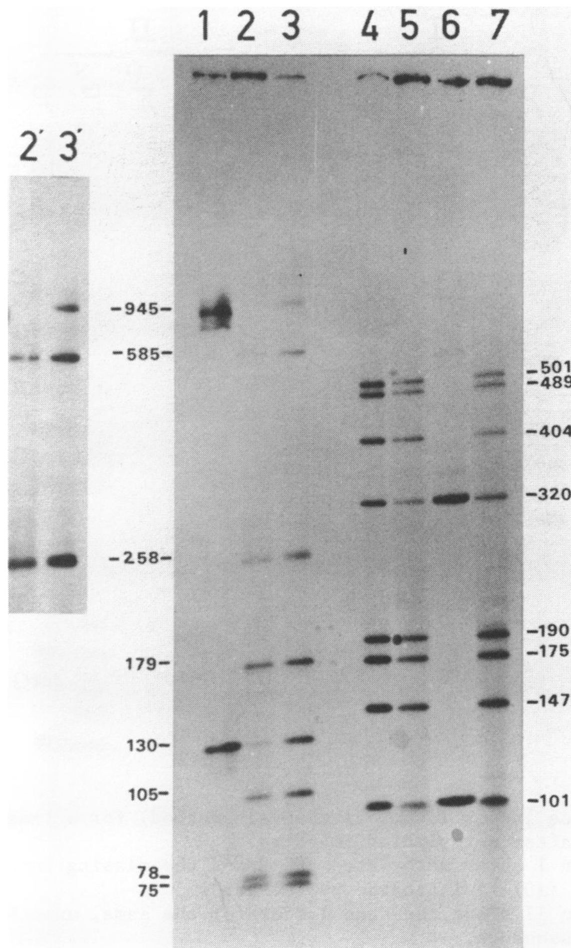


Figure 2. Localization of ^{5me}C in the AquI recognition sequence. Sizes of bands are given in base-pairs.

Lane 1. The two Sau3A fragments which border the AquI site.

Lane 2. Fragment SinI-A of pKW2 methylated *in vitro* by M.AquI and treated with R.Sau3A.

Lane 3. Fragment SinI-A of pKW2 unmethylated, and incubated with R.Sau3A.

Lane 4 Fragment SinI-A of pKW2 methylated *in vitro* x R.MspI.

Lane 5. Fragment SinI-A of pKW2 methylated *in vitro* by M.AquI and subsequently incubated with R.HpaII.

Lane 6. The two MspI fragments which border the AquI site.

Lane 7. Fragment SinI-A fragment of unmethylated pKW2, incubated with R.MspI.

Lane 2' and 3' are identical to lane 2 and 3, only the autoradiogram has been exposed for a longer time.

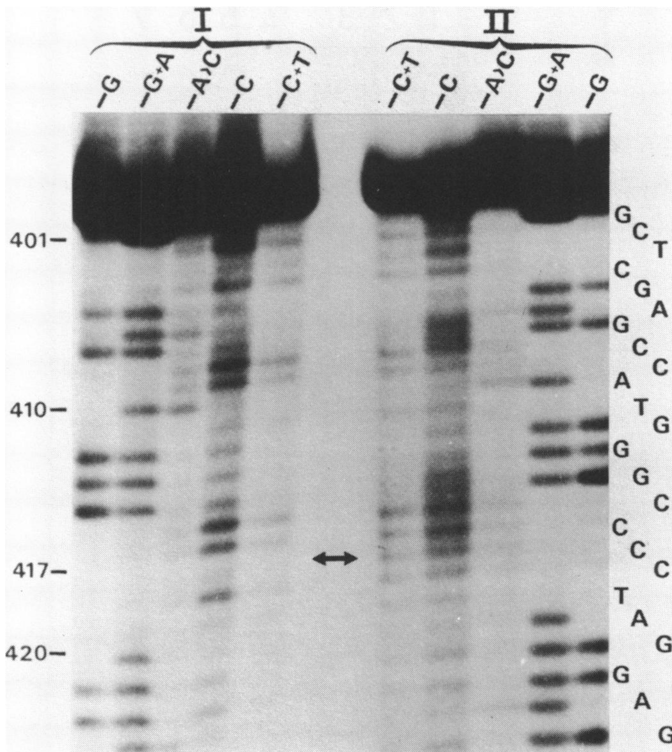


Figure 3. Sequence ladder (chemical cleavage method) for a fragment of pUC19 after methylation _in vitro_.
Section I shows methylated pUC19 and the missing band of residue no.417 (10) is indicated by the arrow.
Section II shows the same ladder for the same, unmethylated, pUC19 sequence.

1) are present when unmethylated pKW2 is digested with _Sau_3A (lane 3), but absent when this DNA molecule had been methylated previously by M._Aqu_I (lane 2). This is the predicted result when the _Aqu_I sequence has been modified to meCYCGRG.

We confirmed this conclusion by performing a sequence analysis of the methylated DNA. It is known that 5-methylcytidylate residues are rather resistant to attack by hydrazine, the base-specific reagent used in the sequencing procedure of Maxam and Gilbert (8,9). Fig.3 shows a sequence ladder of a fragment of pUC19 DNA harbouring an _Aqu_I-recognition site which has been methylated _in vitro_ by methylase M._Aqu_I. It is seen that nucleotide 417 (numbering according to (10)) is absent from the sequence

ladder (see legend). This in contrast to all other cytidylate residues - present in other than AquI recognition sites - in this or any other sequence ladder that was made with DNA preincubated with M.AquI.

This result proves both the nature of the methylated C (^{5me}C) as well as its position in the recognition sequence. The alternative product N^4-methyl C (recently found in a number of bacterial species (11)) cannot be a product of the enzyme modification reaction as this nucleotide behaves like unmodified C in the cleavage reaction with hydrazine (12).

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Abbreviations: bp=base-pair; R=purine; SAM=S-adenosyl-methionine; Y=pyrimidine;

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