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Construction of a *Neisseria gonorrhoeae* MS11 Derivative Deficient in *Ngo*MI Restriction and Modification

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We have cloned from *Neisseria gonorrhoeae* MS11 the gene encoding a methylase that modifies the sequence GCCGGC. The corresponding restriction enzyme was also encoded by this clone. Sequence analysis demonstrated that the methylase shares sequence similarities with other cytosine methylases, but the sequence organization of M.*Ngo*MI is different from that seen for other cytosine methylases. A deletion was introduced into the chromosome of *N. gonorrhoeae* MS11 to produce strain MUG701, a strain that is inactivated in both the methylase and the restriction genes. Although this strain no longer methylated its DNA at the *Ngo*MI recognition sequence, cells were viable and had no other significant phenotypic changes. Transformation data indicated that MS11 does not produce enough restriction activity to block plasmid transformation in the gonococcus, even though restriction activity could be demonstrated in *E. coli* containing the cloned gene.

DNA restriction endonucleases and methyltransferases have been identified in most procaryotes (21). The presence of restriction enzymes in a bacterium can prevent genetic transfer by way of conjugation, transformation, or transduction (1, 7, 11). However, the existence of restriction enzymes that cut infrequently (such as the 8-bp recognition sequences) suggests that some restriction enzymes may exist for reasons other than protecting bacteria from parasitic DNA.

Restriction enzymes with a variety of specificities have been detected in different strains of *Neisseria gonorrhoeae* as follows: *Ngo*I, recognizing PuGCGCPy (21a); *Ngo*II, recognizing GGCC (4); *Ngo*III, recognizing CCGCGG (17); *Ngo*MI, recognizing GCCGGC (3); and *Ngo*BI, recognizing GGTGA (19). Each gonococcal strain usually expresses one or two of these restriction activities at detectable levels. The DNA of *N. gonorrhoeae* is highly methylated at numerous sites, with cytosine modification occurring in all of the sequences recognized by known restriction enzymes (9, 10). However, several DNA sequences are methylated in the gonococcus at sequences unrelated to known gonococcal restriction systems. In addition to these cytosine methylases, an adenine methylase that recognizes the same sequence as the *dam* methylase of *Escherichia coli*, is expressed in some strains of *N. gonorrhoeae* (6).

The reason all the methylation activities are expressed in the absence of detectable corresponding restriction activity is a mystery. The *Ngo*II restriction enzyme restricts DNA during transformation (28). The methylation of plasmid RSF1010 in *E. coli* drastically increases conjugation frequencies with *Neisseria* species (2). We have been able to isolate at least one restriction enzyme from all strains of *N. gonorrhoeae* that we have tested (28, 29); however, the amount of enzyme isolated and its relationship to the amount of DNA methylase produced varies widely (19). The presence of one or two restriction enzymes would be sufficient to prevent the introduction of foreign DNA into the gonococcus. However, the reason for every strain needing to express all of the cytosine methylation activities is unknown. One possibility is that each gonococcal strain methylates its DNA in order for the DNA to retain its ability to act as a donor in transformation. This would presume that mixed infections are common in vivo and that these genes are maintained by selection. An alternative possibility is that some of the restriction and/or modification enzymes may be involved in other processes besides host-mediated restriction.

In the process of cloning a DNA methylase that recognizes the sequence GCCGGC, we also isolated a neighboring gene that encoded a restriction enzyme that corresponded to this methylase. We characterized this gene pair in *E. coli* and generated a mutation in the chromosome of *N. gonorrhoeae*. We propose that many, if not all, of the modified sites in the gonococcal genome are the target for restriction systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are shown in Table 1. All *E. coli* strains were tested for methylcytosine-dependent restriction by using plasmid pHaeIII (encoding M.*Hae*III) and pHpaII (encoding M.*Hpa*II). Plasmids used in this study are shown in Table 1, and plasmids constructed in this study are shown in Table 2. *E. coli* strains were grown on Luria-Bertani agar or in L broth (13). Antibiotics were added to the cultures as needed. The growth medium for *N. gonorrhoeae* was GCK, and the liquid medium was GCP (30). Broth cultures were supplemented with growth supplements (8) and sodium bicarbonate to a final concentration of 0.042% before inoculation.

Chemicals, reagents, and enzymes. All chemicals used were of analytical grade or better and were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Chemicals used for DNA isolation were of molecular biology grade and were also purchased from Sigma. Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or Promega (Madison, Wis.) and were used according to the manufacturers' instructions.

Genetic transformations. *E. coli* strains were transformed

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant phenotype[a]	Source or reference[b]
Strains		
N. gonorrhoeae		
MS11	NgoMI M.NgoMI	M. So
MUG701	R.NgoMI$^-$ M.*Ngo*MI$^-$	This study
E. coli		
HB101	McrA$^+$ McrB$^-$ Mrr$^+$	H. Boyer
DH5αMCR	LacZΔM15 McrA$^-$ McrB$^-$ Mrr$^-$	BRL
JM101	LacZΔM15 McrA$^-$ McrB$^-$ Mrr$^-$	E. Raleigh
MM294	McrA$^+$ McrB$^+$	E. Raleigh
ER1562	Derived from MM294, McrA$^-$ McrB$^-$	E. Raleigh
ER1563	Derived from MM294, McrA$^-$ McrB$^+$	E. Raleigh
NM554	McrA$^-$ McrB$^-$	E. Raleigh
GC6	McrA$^-$ McrB$^-$	Laboratory strain
Plasmids		
pHSS6	Km^r	24
pHSS7	Km^r	24
pBR322	Ap^r Tc^r	13
pUC19	Ap^r	13
pUC8	Ap^r	13
pKAN18	Km^r	20
pHaeIII	Ap^r	NEB
pHpaII	Ap^r	NEB
pFT180	Ap^r Pc^r (*N. gonorrhoeae*)	30
pNa16	Ap^r Nal^r (*N. gonorrhoeae*)	27

[a] Km^r, resistance to 30 μg of kanamycin per ml; Ap^r, resistance to 30 μg of ampicillin per ml; Tc^r, resistance to 5 μg of tetracycline per ml; Pc^r, resistance to 2 μg of penicillin per ml; Nal^r, resistance to 1 μg of nalidixic acid. Except where noted otherwise, *E. coli* DH5αMCR was the plasmid host.

[b] BRL, Bethesda Research Laboratories; NEB, New England Biolabs.

by the standard $CaCl_2$ procedure (13). *N. gonorrhoeae* was transformed by resuspending piliated cells to ~1 × 10^8 cells per ml in GCP broth containing 10 mM $MgCl_2$ and 0.042% $NaHCO_3$ (30). After the addition of DNA, cells were incubated with agitation for 6 h at 37°C, prior to plating on GCK agar containing 2 μg of penicillin per ml. Transformations were also performed by using a cotransformation assay. In this assay, DNA encoding a nonselectable marker was added with the cloned gonococcal gene that confers nalidixic acid resistance (26). The nalidixic acid-resistant marker was added at a limiting concentration 10^6-fold less than the experimental DNA. Cells were plated on GCK with 1 μg of nalidixic acid per ml, and the resulting transformants were screened for the acquisition of the desired marker.

Cloning DNA methylase enzymes. Chromosomal DNA was isolated from gonococcal strain MS11, partially digested with *Hpa*II and *Mbo*I, and inserted between the *Cla*I and *Bam*HI sites of the plasmid vector pHSS7. After ligation, the DNA was used to transform *E. coli* GC6 (32). Plasmid DNA from 3,200 transformants (pCBB) was isolated, digested with *Nae*I, and used to transform *E. coli* GC6. About 5,000 colonies were pooled, and plasmid DNA was isolated, digested with *Nae*I, and used to transform *E. coli* GC6. All of the colonies that arose after the second round of screening contained one of two plasmids, pCBB49 or pCBB20.

Detection of endonuclease and DNA methylase activities. For the detection of restriction endonucleases, cell extracts were made as previously described (19). After passage through a phosphocellulose P11 column, 2 μl of each fraction was mixed with 30 μl of digestion buffer (25 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 2 mM β-mercaptoethanol) containing 1 μg of lambda DNA. The mixtures were incubated at 37°C for 1 h before gel electrophoresis. Restriction enzyme activity was detected by the ability of the extract to digest lambda DNA. For the detection of DNA methylase activity, 2 μl of each fraction was mixed with 30 μl of methylation buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 5 mM β-mercaptoethanol, 4 μM *S*-adenosyl-L[^{3}H]methyl-methionine [AdoMet]) containing 100 μg of lambda DNA per ml and incubated at 37°C for 2 h. The reaction mixture was deproteinized by extraction with phenol-chloroform (1:1) three times, and the aqueous phase was loaded on a minicolumn (0.5 by 4 cm) of Sephadex G-50 to separate the DNA from free AdoMet. The incorporation of ^{3}H-methyl groups from ^{3}H-AdoMet into DNA was measured by using a scintillation counter.

DNA sequence analysis. DNA sequencing was done on templates derived from two sets of experiments. In one set, small restriction fragments of the 3-kb fragment containing the RM.*Ngo*MI genes were prepared and inserted into M13mp18 or M13mp19 (14). In the other set, sequential deletions of the DNA were prepared by using an Erase a Base kit obtained from Promega. These fragments were used as templates in the chain termination procedure for DNA sequencing (22). Computer analysis of the DNA sequences was performed by using the program NUCLEIC ACID ALIGNMENT. Homology searches and translational analysis were performed by using the program GENEPRO (Riverside Scientific, Seattle, Wash.). An amino acid sequence alignment of the cytosine-specific DNA methylases was carried out by hand.

Construction of restriction and modification mutants. For the construction of the RM.*Ngo*MI-negative gonococcal mutant, pCBB49ΔCSUP was used to transform *N. gonorrhoeae* MS11. Transformants were plated on GCK agar and incubated for 36 h at 37°C, and replicate plates were made and screened for their inability to react with the 423-bp *Ssp*I-*Ssp*I sequence from pCBB49.1 in a colony hybridization experiment. Incorporation of [α-^{32}P]dCTP (Dupont, NEN Research Products, Boston, Mass.) into the *Ssp*I fragment was performed by nick translation using a commercial kit (Bethesda Research Laboratories, Gaithersburg, Md.). The transformants were blotted from one set of plates onto nitrocellulose membrane BA85 (Schleicher & Schuell, Keene, N.H.) and lysed with alkaline solution by the method of Moseley et. al. (15). Hybridization with α-^{32}P-labeled probe was carried out at 65°C. Washes using a salt concentration of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C were performed. Autoradiography was carried out overnight at −70°C by using Kodak X-OMAT AR diagnostic X-ray film. Colonies that had lost the RM.*Ngo*MI genes were identified, and their DNAs were now sensitive to cleavage with *Nae*I.

Growth curves. Growth curves for MUG701 and MS11 were determined by inoculating T4 colonies into 20 ml of supplemented GCP broth. Cells were incubated at 37°C with shaking, and the turbidity was monitored with a Klett-Summerson Colorimeter with a green filter.

Nucleotide sequence accession number. The DNA sequence described above has been submitted to GenBank and has been assigned accession no. M86915.

TABLE 2. Plasmids constructed in this study

Plasmid	Size (kb)	Vector	Site of insertion	Fragment cloned	R/M system[a]	Construction scheme[b]
pCBB20	8.2	pHSS7	*Cla*I-*Bam*HI	*Hpa*I-*Mbo*I	RM.*Ngo*MI	1
pCBB49	6.8	pHSS7	*Cla*I-*Bam*HI	*Hpa*I-*Mbo*I	RM.*Ngo*MI	1
pCBB49.1	5.3	pHSS7			RM.*Ngo*MI	2
pCBB49.12	4.4	pHSS7			M.*Ngo*MI	3
pCBB49.13	3.9	pHSS7			M.*Ngo*MI	4
pCBB49.15	3.7	pHSS7				5
pCBB49.17	5.1	pHSS7				6
pBS1	4.3	pUC19	*Sma*I	*Bgl*I-*Sma*I	R.*Ngo*MI	7
pBS2	4.8	pUC19	*Sma*I	*Bst*NI-*Sma*I	R.*Ngo*MI	8
pSS0.5	3.1	pKAN18	*Sma*I	*Ssp*I-*Ssp*I		9
pCBB49ΔCS	5.6	pHSS7				10
pCBB49ΔCSUP	5.6	pHSS7				11

[a] R, restriction; M, methylation.

[b] Construction schemes were as follows: 1, pHSS7 with a chromosomal insert from the gene bank (*Mbo*I-*Hpa*I); 2, derived from the deletion of the 1.5-kb *Eco*RI-*Cla*I internal fragment of pCBB49; 3, derived from the deletion of the 0.9-kb *Ssp*I-*Sma*I internal fragment of pCBB49.1; 4, derived from the deletion of the 1.4-kb *Ssp*I-*Ssp*I-*Sma*I internal fragment of pCBB49.1; 5, derived from the deletion of the 1.6-kb *Bgl*I-*Ssp*I-*Ssp*I-*Sma*I internal fragment of pCBB49.1; 6, derived from the deletion of the 0.2-kb *Bgl*I-*Ssp*I internal fragment of pCBB49.1; 7, 1.6-kb *Bgl*I-*Ssp*I-*Ssp*I-*Sma*I internal fragment of pCBB49.1 cloned into the *Sma*I site of pUC19; 8, 2.1-kb *Bst*NI-*Bgl*I-*Ssp*I-*Ssp*I-*Sma*I internal fragment of pCBB49.1 into the *Sma*I site of pUC19; 9, 0.5-kb *Ssp*I-*Ssp*I internal fragment of pCBB49.1 into the *Sma*I site of pKAN18; 10, deletion of the 2.0-kb *Cla*I-*Ssp*I internal fragment of pCBB49; 11, addition of the 800-bp *Sph*I-*Cla*I internal fragment (containing uptake sequences) of pLV155 to the *Cla*I-*Sph*I site of pCBB49CS.

RESULTS

Detection of restriction endonucleases and DNA methylases present in *N. gonorrhoeae* strains. From the DNA sequence of the cryptic plasmid pJD1, Korch et. al. (9) predicted that *N. gonorrhoeae* should produce five cytosine-specific DNA methylases. Subsequent to this work, three additional DNA methylases, M.*Ngo*III, M.*Ngo*VI (an adenine-specific DNA methylase), and M.*Ngo*VIII, were postulated to exist (10). In an effort to determine the distribution of the various DNA methylases in *N. gonorrhoeae*, we isolated chromosomal DNA from three gonococcal strains and challenged them with endonucleases that are isoschizomers of the predicted gonococcal DNA methylases. All three strains produced six of the seven methylation activities. WR220 and MS11 were missing the *dam* homolog M.*Ngo*VI, which is only present in some gonococcal strains (6). MUG116 did not express the *Hae*III homolog, M.*Ngo*II. The same strains were assayed for their ability to produce restriction enzymes by screening cell extracts for the presence of each restriction activity. Each of the strains produced one or two detectable restriction activities. These data are summarized in Table 3.

Cloning the M.*Ngo*MI gene. All sequenced pilin gene copies contain the DNA sequence GCCGGC near the junction of the constant and semivariable portions of the gene, and there are data suggesting that differential methylation could occur at this sequence (23). In order to investigate the role of DNA methylation at this sequence, we cloned the DNA that encoded this methylase. Chimeric plasmids containing *N. gonorrhoeae* MS11 DNA were constructed, and a pool of plasmids representing the entire chromosome was isolated in *E. coli* GC6. In order to identify a plasmid expressing GCCGGC methylating activity, pooled plasmid DNA was digested with *Nae*I, a commercially available isoschizomer that recognizes the same DNA sequence. Since this plasmid has two recognition sites in the vector, any plasmid isolated from a cell expressing the methylase should be resistant to *Nae*I digestion. After two rounds of digestion and transformation, 40 transformants were screened and >90% contained an 8.2- or 6.8-kb plasmid. DNA was isolated from several of these clones, and their resistance to cleavage with *Nae*I was confirmed. Two plasmids, representing the two size classes, were chosen for further study. pCBB20 carried a 5.9-kb insert, while pCBB49 contained a 4.5-kb insert. Restriction analysis showed that

TABLE 3. Restriction endonucleases and DNA methylases present in *N. gonorrhoeae* strains

Protected DNA sequence	System	Presence or absence of activity[a]					
		Predicted enzyme for (M):			Enzyme isolated from (R/M):		
		WR220	MUG116	MS11	WR220	MUG116	MS11
5′-PuG^{m}CGCPy-3′	S.NgoI	+	+	+	+/−	−/−	+/−
5′-GG^{m}CC-3′	S.NgoII	+	−	+	−/+[b]	−/−	−/−
5′-CCGCGG-3′	S.NgoIII	+	+	+	+/−	−/−	+/−
5′-G^{m}CCGGC-3′	S.NgoIV	+	+	+	−/−	−/−	−/−
5′-GGNN^{m}CC-3′	S.NgoV	+	+	+	−/−	−/−	−/−
5′-G^{m}ATC-3′	S.NgoVI	−	+	−	−/−	−/−	−/−
5′-G^{m}C(C/G)GC-3′	S.NgoVII	NT	NT	NT	−/−	−/−	−/−
5′-T^{m}CACC-3′	S.NgoVIII	+	+	+	−/−	+[b]/+[b]	−/−
5′-GTAN5^{m}CTC-3′	S.NgoIX	NT	NT	NT	−/−	−/+[b]	−/−

[a] +, presence of methylase activity (M) as indicated by the resistance of chromosomal DNA to digestion with specific restriction enzymes or the presence of restriction enzyme activity (R) as measured by the ability of cell extracts to cleave λ DNA. −, absence of enzyme activity. NT, not tested.

[b] Enzymes isolated by Piekarowicz et. al. (19).

TABLE 4. Detection of restriction and modification by using transformation[a]

Strain transformed	No. of transformants/CFU with pFT180 from:		
	MUG701	MS11	HB101
E. coli HB101(pHSS7)		1.2×10^{-3}	1.1×10^{-3}
E. coli HB101(pCBB49.1)		1.1×10^{-3}	$<7.5 \times 10^{-8}$
N. gonorrhoeae MUG701	4.1×10^{-6}	4.0×10^{-5}	$<3.0 \times 10^{-8}$
N. gonorrhoeae MS11	7.1×10^{-5}	6.3×10^{-5}	$<3.1 \times 10^{-8}$
E. coli HB101	3.6×10^{-4}	3.3×10^{-4}	5.2×10^{-4}

[a] Plasmid DNA was isolated from the strain indicated at the top of each column, and 0.1 μg was used to transform ~2×10^8 cells as indicated at the left.

both clones were identical at one end of the insert but that pCBB20 carried an additional 1.4-kb insert at the other end. A 1.5-kb *Eco*RI-*Cla*I deletion of pCBB49 (pCBB49.1) retained DNA methylase activity and was used in all subsequent experiments.

Detection of an associated restriction activity on pCBB49.1. When we attempted to introduce the compatible plasmid pFT180 into *E. coli* HB101(pCBB49.1) by transformation, we never recovered any transformants. However, pCBB49.1 was efficiently introduced into *E. coli* HB101(pFT180). We postulated that an activity was expressed by pCBB49.1 that was restricting the incoming pFT180 DNA. To investigate this phenomenon further, plasmid pFT180 was isolated from *N. gonorrhoeae* MS11, where it was fully methylated at the *Nae*I sites, and from *E. coli* HB101, where it was unmethylated at these sites. Only the methylated plasmid DNA isolated from the gonococcus could transform *E. coli* HB101(pCBB49.1) (Table 4). Thus, the insert in pCBB49.1 expressed both methylation and restriction activities. The existence of the restriction activity was later confirmed biochemically (see below).

Location of the restriction and modification genes on pCBB49.1. The exact location of the DNA methyltransferase gene on pCBB49.1 was determined by further deletion analysis (Fig. 1). One clone, pCBB49.13, contained a 1.7-kb insert that expressed the DNA methylase activity. Subclone pCBB49.17 (deletion of the internal *Bgl*I-*Ssp*I fragment) showed neither DNA methylation nor restriction activity. Attempts to delete the *Cla*I-*Bgl*I fragment or the *Cla*I-*Bst*NI fragment did not result in any viable clones. Since we were unable to delete the *Cla*I-*Bst*NI and *Cla*I-*Bgl*I fragments, we felt that the restriction enzyme gene must be located on the *Bgl*I-*Sma*I fragment. Therefore, HB101(pCBB49.13) was used as a methylation-expressing host to isolate subclones that only express the restriction endonuclease gene. One subclone, pBS1, containing the 1.6-kb *Bgl*I-*Sma*I fragment cloned into the *Sma*I site of pUC19, possessed restriction activity when tested in both in vivo and in vitro assays. An identical result was obtained for the subclone, pBS2. Attempts to transform pBS1 and pBS2 directly into *E. coli* HB101 in the absence of a plasmid encoding the DNA methylase gene were unsuccessful. These results indicated that the restriction enzyme gene is located on the *Bgl*I-*Sma*I fragment.

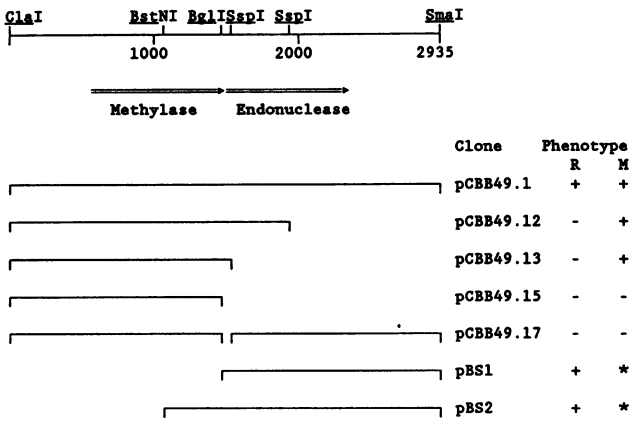

FIG. 1. Deletion analysis of plasmid pCBB49.1. Thin horizontal lines indicate the insert DNA sequences remaining in the various deletion derivatives. Phenotypes were scored by in vivo and in vitro analyses as described in Materials and Methods. R, restriction activity; M, DNA methylation activity; +, presence of enzyme activity; −, absence of enzyme activity; *, DNA methylase gene carried by a compatible plasmid, pCBB49.13, present in the cell. The direction of transcription as deduced by the computer reading frame analysis is shown.

Properties of the restriction enzyme *Ngo*MI. The restriction endonuclease encoded by pCBB49.1 was purified from crude lysates of HB101(pCBB49.1) by column chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified *Ngo*MI indicated that the molecular mass of the restriction enzyme was 33,000 Da (data not shown). The recognition sequence for this enzyme was verified by digesting several different DNAs of known sequence and comparing the digestion patterns generated with those that could be predicted from the DNA sequences. The restriction enzyme isolated from HB101(pCBB49.1) has a recognition sequence that could not be distinguished from *Nae*I (data not shown). The nature of the ends of DNA molecules generated after cleavage with *Ngo*MI was determined. DNA sequencing reactions were performed on plasmid DNA containing an *Nae*I site ~50 bases from a site complementary to a synthetic oligonucleotide primer. DNAs were digested with *Nae*I or *Ngo*MI, and the DNA sequence was determined. The data indicate that DNA cleaved with *Ngo*MI contains a 4-base 5′ extension (data not shown). Repeated attempts to detect the restriction activity in MS11 cell lysates were unsuccessful.

Nucleotide sequence of the S.*Ngo*MI restriction and methylation system. In order to begin studying the genetic regulation of RM.*Ngo*MI, we determined the DNA sequence of these genes. A detailed restriction map of the insert and the sequencing strategy is shown in Fig. 2, and the sequence is shown in Fig. 3. Three significant open reading frames were present in the sequence, although one of them lacked an initiation codon in the insert and extends from the adjacent vector sequences. On the basis of the deletion mapping data (Fig. 1), the DNA methylase gene and the restriction endonuclease gene were assigned to the other two open reading frames. The DNA methylase gene is encoded by an open reading frame that extends from base 79 to base 1014 (Fig. 3), while the restriction endonuclease gene is in the same reading frame, begins at base 1018, and extends through base 1875. Assuming that translation begins at the first ATG in each of these open reading frames, then the DNA methylase will contain 313 amino acids with a predicted molecular weight of 35,225. The restriction endonuclease would be a 286-amino-acid polypeptide with a molecular weight of 31,759. In the case of the restriction endonuclease gene, SDS-PAGE of the purified restriction endonuclease agrees

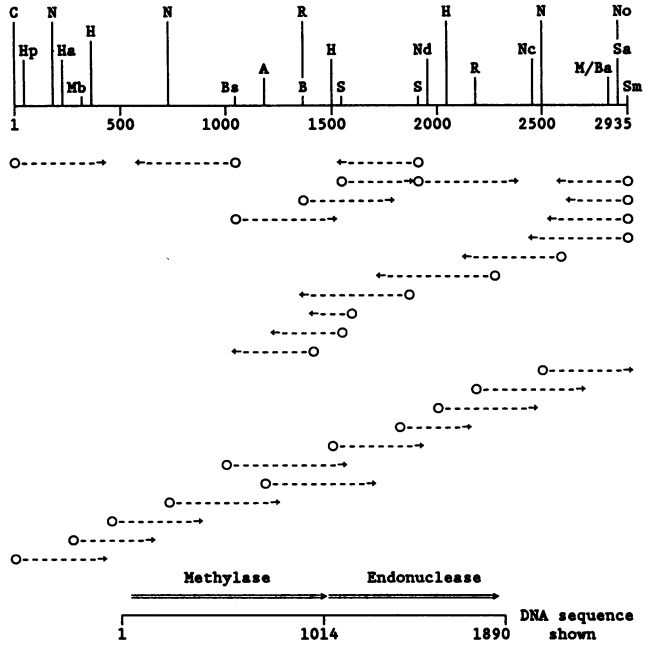

FIG. 2. Restriction map of insert and sequencing strategy used to determine the DNA sequence of the insert in pCBB49.1. Circles and dashed lines indicate the extent of individual sequencing readings. Restriction enzyme sites: A, *Ava*II; H, *Hinf*I; N, *Nae*I; R, *Rsa*I; B, *Bgl*I; Ha, *Hae*II; Nc, *Nco*I; S, *Ssp*I; Ba, *Bam*HI; Hp, *Hph*I; Nd, *Nde*I; Sa, *Sac*II; Bs, *Bst*NI; M, *Mbo*I; No, *Not*I; Sm, *Sma*I; C, *Cla*I; Mb, *Mbo*II.

with the predicted molecular weight of the restriction endonuclease (33,000 versus 31,759).

Comparison with other restriction and modification enzymes. We compared the DNA sequence of the S.*Ngo*MI restriction and DNA methylase genes with other restriction and DNA methylase genes. In addition, the deduced amino acid sequences were used to search for homologies with the complete PIR or GenBank data bases (November 1989 release). No significant homologies at either the DNA or protein level for the restriction enzyme were detected. However, significant and extensive homologies with other DNA methylases known to catalyze the formation of 5-methylcytosine were detected. The alignment of the amino acid sequences of these DNA methylases is shown in Fig. 4; the presence of four conserved domains (core elements [CE]) described by Lauster (12) is also shown. Although the domains of M.*Ngo*MI are present in the same order, the size and spacing between these domains are significantly different from those seen in other cytosine-specific DNA methylases. All of the other cytosine-specific DNA methylases aligned in this manner contain about 50 amino acids between CE I and II. In M.*Ngo*MI, CE I and CE II are immediately adjacent. Also, M.*Ngo*MI is missing about 40% of CE I.

Construction and characterization of RM.NgoMI-negative gonococcal mutant. In order to begin our studies on the role of restriction and methylation in *N. gonorrhoeae*, a deletion that mutated both the restriction and methylation genes was constructed. In order to efficiently introduce DNA into the gonococcus by transformation, the transforming DNA must contain an uptake sequence (25). DNA sequence analysis of pCBB49.1 indicated that this plasmid lacked the DNA uptake sequence. A deletion derivative of pCBB49 (pCBB49ΔCS) that was defective for both the restriction and modification genes was constructed. Two transformation uptake sequences were introduced into the *Sma*I site of the plasmid pCBB49ΔCS to make pCBB49ΔCSUP (Table 2). Plasmid pCBB49ΔCSUP was used to transform *N. gonorrhoeae* MS11 by congression with a nalidixic acid resistance-encoding plasmid as previously described (26). Nalidixic acid-resistant transformants were tested for the incorporation of the desired deletion by testing for their failure to produce a signal in a DNA colony hybridization experiment with the probe of α-^{32}P-labeled pSS0.5 (Fig. 1). One transformant, MUG701, was isolated and further characterized. DNA isolated from MUG701 was susceptible to cleavage with *Nae*I, demonstrating the inactivation of the methylase gene (data not shown). The presence of the deletion was confirmed by Southern hybridization experiments (data not shown).

Role of RM.NgoMI system in cell growth. Since we were able to isolate transformants that lacked the RM.*Ngo*MI system, we knew that this enzyme system was not required for cell growth. In order to determine the possible effect of this mutation on cell function, growth curves for the parent and the mutant were performed. The data indicated that MUG701, lacking the RM.*Ngo*MI system, grows at approximately the same growth rate as its parent, MS11 (data not shown).

Since RM.*Ngo*MI enzymes were not required for cell growth, we tested the mutant to see whether any other properties were altered. We analyzed the SDS-PAGE profile of LOS, isolated from MS11, and MUG701. Although the LOS profiles were significantly different, subsequent tests showed that this difference was unrelated to the acquisition of the RM defect (data not shown). We analyzed the rates of pilin phase variation in the two strains, but no significant differences were detected (data not shown).

Genetic test reveals that R.NgoMI is not expressed in MS11. The mutated MS11 derivative, MUG701, allowed us to determine whether the restriction activity was expressed at a level too low to be detected biochemically. Plasmid pFT180 was isolated from MUG701 and MS11, in order to compare the transformation frequencies of plasmids that differed only in methylation at the S.*Ngo*MI recognition sequences. The data in Table 4 indicate that strain MS11 fails to produce enough *Ngo*MI restriction enzyme in vivo to alter its transformation with unmethylated pFT180. Since pFT180 isolated from *E. coli* HB101 could not transform MUG701, this indicates that MUG701 produces other restriction enzymes. We have been able to detect restriction enzymes with a specificity of S.*Ngo*II and S.*Ngo*III from both of these strains but have never detected the *Ngo*MI activity in gonococcal cell lysates (data not shown).

DISCUSSION

Our studies show that, although strain MS11 expresses at least six methylase activities, only two corresponding restriction activities can be detected in lysates. We cloned the DNA encoding the M.*Ngo*MI methylation activity in *E. coli* as a first step in our studies to determine whether methylation by this enzyme could influence gene expression in the gonococcus. To our surprise, we detected an accompanying restriction activity expressed at high levels in *E. coli*. Genetic experiments failed to detect any enzyme expression in the gonococcus.

Korch et al. (9, 10) initially predicted that *N. gonorrhoeae* produces six different cytosine-specific DNA methylases.

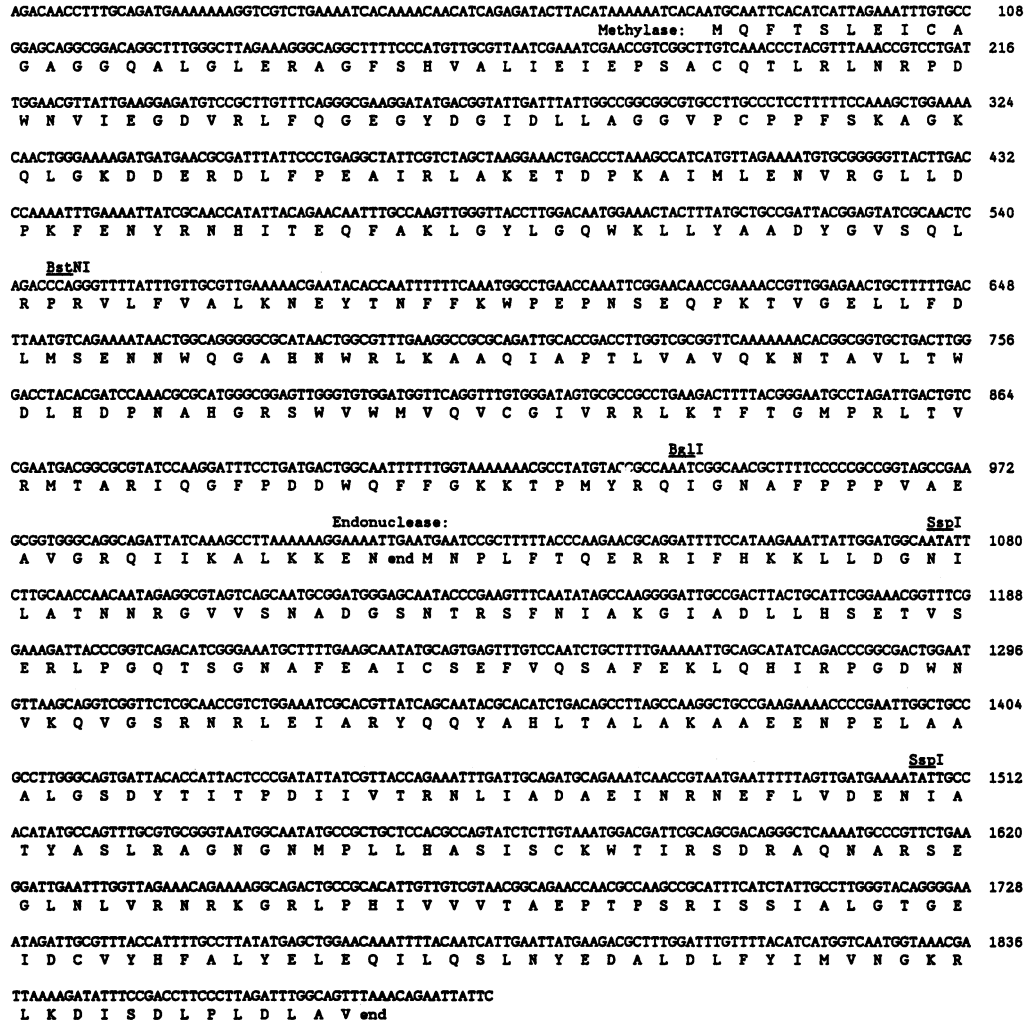

FIG. 3. DNA sequence of the *Ngo*MI restriction and modification genes and the predicted amino acid sequences.

Two additional enzyme specificities were subsequently identified (6, 17). The recognition sequences for these enzymes are PuGCGCPy (S.*Ngo*I), GGCC (S.*Ngo*II), CCGCGG (S.*Ngo*III), GCCGGC (S.*Ngo*IV), GGNNCC (S.*Ngo*V), GATC (S.*Ngo*VI), GC(CG)GC (S.*Ngo*VII), and TCACC (S.*Ngo*VIII). Piekarowicz et. al. (19), in attempting to purify DNA methylases from *N. gonorrhoeae*, were only able to isolate three different enzymes. The recognition sequence of one of these was not one of the original eight described above. Also, Piekarowicz et. al. (19) isolated a restriction enzyme that corresponded to one of the nine DNA methylase activities. Sullivan and Saunders (31) showed that pLV155 encodes two genes corresponding to the *Ngo*PII restriction and modification system (S.*Ngo*II). Wilson and Young had been able to purify a restriction enzyme with an S.*Ngo*I specificity (PuGCGCPy) (21a), and Piekarowicz et. al. (18) have purified a restriction enzyme with an S.*Ngo*VIII specificity (TCACC). Duff and Davies (6) have found a *Dpn*I-like restriction enzyme for the methylated adenine at the recognition sequence G^{m}ATC. With the data presented in this paper, six restriction enzymes corresponding to six of the nine reported DNA methylase systems have been isolated. It is possible that all of the methylation activities have corresponding restriction activities that are not always detectable biochemically.

From the DNA sequence of pCBB49.1, two open reading frames were identified (Fig. 3). From the phenotypes of the various deletion mutants, we were able to assign specific functions to the two open reading frames. The molecular mass of the purified restriction enzyme was determined to be 33,000 Da and was in good agreement with the size predicted from the DNA sequence (31,759 Da). The molecular mass of the restriction endonuclease and that predicted for the methylase (35,225 Da) are very similar to those reported for the corresponding genes in other systems (12).

In comparing the amino acid sequences of DNA methyltransferases from several prokaryotes, Lauster (12) identified four highly conserved core sequences, CE I through CE IV. Each of these core sequences is separated by a variable segment of DNA. From the alignments shown in Fig. 4, it can be seen that there are extensive regions of similarity between these proteins. The conserved regions that have been noted among other cytosine-specific DNA methylases are all present (12). This includes the so-called GXPC motif of CE II that is involved in the CH_3 group transfer (33). M.*Ngo*MI contains protein sequences that correspond to the

FIG. 4. Amino acid sequence alignment of cytosine-specific type II DNA methyltransferases from procaryotes and M.*Ngo*MI. Terminal elongation as well as insertions that are unique for individual enzymes have been omitted in order to obtain a comprehensive alignment picture of the four cytosine-specific DNA methylase CE (CE I to CE IV). The consensus sequence is as described by Lauster (12). If an amino acid is always identical at any one position, then the corresponding letter appears in the consensus line. +, position where either only two different residues appear or all residues are related; /, position where part of an individual sequence is omitted. The numbers represent the number of amino acids, as they relate to M.*Ngo*MI.

core sequences, but the size and spacing between these conserved regions are significantly different from those seen in the proposed conserved domains in other cytosine-specific DNA methylases. For example, in M.*Ngo*MI, CE I and II have no intervening protein sequence. In fact, there appears to be some sequence overlap between the two regions (Fig. 4). The proposed target-recognizing domain is located between CE II and CE III and contains certain amino acids, spaced at defined intervals, which provide the backbone for the target recognition. This sequence was found located around a threonine residue at position 214 in all of the DNA methylases analyzed (12). The amino acids immediately surrounding this threonine appear to be similar to the consensus sequence predicted as a backbone, but no homologies are apparent at either end of the target-recognizing domain. For CE III, all the DNA methylases examined to date have a glutamate (E) in the middle of this domain. For M.*Ngo*MI, this residue has been replaced by a methionine (M). Since these residues are not similar, it indicates that the consensus sequence for this domain has been defined too specifically. Comparison of the amino acid sequences of *Ngo*PII and *Ngo*MI indicate that these enzymes are not related. This suggests that these enzymes evolved during separate events.

We were unable to isolate *Ngo*MI from extracts of *N. gonorrhoeae* MS11, but we were able to purify large quantities from clones that expressed this gene. The fact that all gonococcal strains contain multiple DNA methylases, suggests that these enzymes have some important biological function. The ability to regulate the activity of *Ngo*MI and possibly other restriction enzymes (5, 16) also raises the question of whether the restriction of foreign DNA is the only in vivo biological function of these enzymes. We reasoned that, if these enzymes played an important role in regulating cell growth or antigenic variation, derivatives defective in these enzyme functions should have altered growth or antigenic variation processes. Mutants defective in RM.*Ngo*MI gene function were viable and had no easily detectable phenotypic differences with respect to growth rates or frequency of pilus phase variation. This led us to conclude that these enzyme functions are dispensable for the in vitro growth of the organism.

We predict that many gonococcal strains encode restriction activities that are not well expressed and cannot be detected biochemically. Although restriction of parasitic DNA is an important process for a bacterial cell, the redundancy evidenced by the gonococcus suggests that some of these enzymes should have an additional role in controlling gene expression and/or directing DNA recombination. Although we could not detect a phenotype for *Ngo*MI, the creation of an RM.*Ngo*MI mutant strain is a first step in determining what role these enzymes play in gonococcal biology.

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