INFECTION AND IMMUNITY, Mar. 2001, p. 1816–1820 Vol. 69, No. 3
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.3.1816–1820.2001

*Nme*SI Restriction-Modification System Identified by Representational Difference Analysis of a Hypervirulent *Neisseria meningitidis* Strain

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Received 16 August 2000/Returned for modification 21 September 2000/Accepted 28 November 2000

Neisseria meningitidis **is a gram-negative bacterium that may cause meningitis, sepsis, or both. The increase in the incidence of meningococcal disease in various countries in the past 2 decades is mainly due the genotypically related lineage III meningococci. The chromosomal DNA differences between lineage III strains and non-lineage III strains were identified using representational difference analysis. Thus, a 1.8-kb locus that is specific for lineage III meningococci was identified. The locus contains three open reading frames encoding the *Nme*SI restriction-modification system. The methyltransferase gene was cloned and expressed in *Escherichia coli*. Site AGTACT was found to be modified by the enzyme. In conclusion, lineage III strains differ from endemic strains by the presence of a specific restriction-modification system. This restriction-modification system may contribute to the clonal and hypervirulent character of lineage III strains by influencing horizontal gene transfer and transcription.**

Neisseria meningitidis is a gram-negative bacterium that commonly resides in the human nasopharynx. Occasionally, this bacterium causes serious disease, mainly meningitis and sepsis. Since 1980, an increase in the incidence of meningococcal disease has taken place in The Netherlands. Previous studies showed that this increase is due to genotypically related isolates, designated lineage III strains (7). Strains of this cluster have also been isolated in many other western European countries and in Chile and more recently in New Zealand (15). Lineage III strains isolated from patients in various countries over the past 20 years can be recognized as such by both multilocus enzyme electrophoresis (7) and multilocus sequence typing (12). This suggests that the diversifying effect of horizontal gene transfer affecting meningococcal population biology (24) in these lineage III strains is relatively low. The increased incidence of meningococcal disease due to a specific clone may imply that such a clone possesses certain virulence properties that are not present in other isolates (8).

To address the observed differences between lineage III meningococci and other meningococci, we previously used representational difference analysis (RDA) (11) to compare the chromosomal DNA content of lineage III strains with that of two strains that only caused endemic disease. By this method, DNA sequences that are present in one DNA pool (i.e., the lineage III chromosomal DNA) but absent in another DNA pool (i.e., the chromosomal DNA of the endemic strains) are selectively amplified. Parts of the differences and point mutations are expected to go undetected by this method. Recently, we identified three DNA sequences that are present in all lineage III strains tested but absent from a majority of non-lineage III strains (4). Database similarities of the fragments suggested that they formed part of a restriction-modification (RM) locus.

Here we report the identification of the lineage III-specific *Nme*SI RM system and show that this is an isoschizomer of the *Sca*I RM system. Sequence analysis indicates that the *Nme*SI RM system and the *Sca*I RM system may have evolved separately.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and reagents. *N. meningitidis* strains 800615, 882066, 3532, and 830248 were isolated from patients with meningococcal disease and collected by the Netherlands Reference Laboratory for Bacterial Meningitis (Academic Medical Center, Amsterdam, The Netherlands, and the Rijksinstituut voor Volksgezondheid en Milieuhygiëne, Bilthoven, The Netherlands). Strains 800615 and 882066 belong to the hypervirulent lineage III clone. Strains 3532 and 830248 belong to lineage IV, most closely related to lineage III (7), containing isolates from the period 1958 to 1986 causing endemic disease (23). Meningococci were grown on heated blood (chocolate) agar plates at 37°C in a humidified atmosphere of 5% CO_2.

Competent *Escherichia coli* Top10F′ cells and cloning vector pCR2.1 were obtained from Invitrogen (Groningen, The Netherlands). Plasmid-carrying *E. coli* strains were routinely grown in Luria-Bertani medium with 100 μg of ampicillin/ml, supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) if necessary for screening purposes, according to the manufacturer's protocol. Expression vector pSE380 was obtained from Invitrogen. Expression was induced by adding IPTG according to the manufacturer's protocol. Oligonucleotides used in this study were synthesized by Perkin-Elmer Nederland B.V., Gouda, The Netherlands.

DNA techniques. Chromosomal DNA was isolated as described by Akopyanz et al. (1) or using the Puregene kit (Gentra Systems, Minneapolis, Minn.). Plasmid DNA isolations were performed using the QIAGEN kit (Qiagen GmbH, Hilden, Germany) or the Wizard kit (Promega Corp., Madison, Wis.). The concentration of DNA was assessed by measuring the optical density at 260 nm using an Ultraspec 2000 spectrophotometer (Pharmacia, Woerden, The Netherlands). Restriction enzymes and digestion buffers were obtained from Boehringer Mannheim GmbH (Almere, The Netherlands) and used according to the manufacturer's instructions.

Sequence analysis of parts of the *Nme*SI locus. The procedure for inverse PCR (IPCR) was performed as previously described (18, 26). The IPCR

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template was prepared by self-ligation of digested chromosomal DNA from strain 800615. IPCR with primers ABM3 (5′-ATA CAT TCA ATT TAG ATG CTG TAC G-3′) and ABM4 (5′-GGT GGA GAT GTG ATT GTC ATT TGG A-3′) yielded a single 1.2-kb amplicon for *Pst*I-digested and self-ligated chromosomal DNA. Cloning this amplicon in the pCR2.1 vector yielded recombinant plasmid pMP01. Digestion of pMP01 with *Ssp*I and *Eco*RV yielded two fragments. A 600-bp fragment was subcloned in pUC19, yielding pMP10. Self-ligation of the other fragment yielded 4.5-kb plasmid pMP02. Plasmids pMP01, pMP10, and pMP02 were sequenced with the M13 universal primers.

Similarly, IPCR with primers ABM2 (5′-ATT TAG CAG GAT TTT TCA CAT ACC A-3′) and ABM3 yielded a 1.4-kb fragment for *Sph*I and a 2.5-kb product for *Cla*I-digested and self-ligated chromosomal DNA. Cloning these products in pCR2.1 did not yield transformants. We suspected that this was due to the lethality of an expressed gene product of this sequence in *E. coli*; therefore, a second PCR was performed on both ABM2 and -3 amplicons with primers ABM2 and ABM7 (5′-CTC GCC TGC TGG CCT GTC GCT GCA G-3′). By this repeated PCR, we hoped to abolish the lethal effect in *E. coli* by the introduction of a mutation due to the infidelity of *Taq* polymerase. Cloning the ABM2 and -7 IPCR products yielded only a few transformants, two of which were sequenced with the M13 dye primers.

The sequence information obtained from the IPCR product (sub)clones was used to design primers ABM5 to ABM14. Sequences were as follows: ABM5, 5′-TTA AAT GGA TGA TTG AAG AAT TGA G-3′; ABM6, 5′-TCT CCA GAG GCT TAT AGA AGT AAA C-3′; ABM8, 5′-GAG ATT GTC CAA CTT TGT TTA GAT A-3′; ABM9, 5′-CTC ATT CAA AGA AGC ATA CGG CGA T-3′; ABM10, 5′-AAG TCG TTT CGA TAA ATC ATA GGA C-3′; ABM11, 5′-TGT AGC CTG CAT CAA ACC GCG TGC A-3′; ABM12, 5′-GCA TCG ACG CGG TTT GAT GCA GGC T-3′; ABM13, 5′-CGG TAT CTA CCT ACC CCA CCT ATT T-3′; ABM14, 5′-ACC CAA TAG TTT TCC AAA CCG CAT A-3′. PCR products amplified with primer pairs ABM5 and -2, ABM5 and -6, ABM5 and -12, ABM1 and -6, ABM1 and -8, ABM3 and -8, ABM3 and -10, ABM7 and -8, ABM7 and -10, ABM7 and -12, ABM9 and -10, ABM9 and -12, ABM9 and -14, ABM11 and -10, ABM11 and -14, ABM13 and -10, and ABM13 and -14 were sequenced directly using dye terminator chemistry.

DNA sequencing. Automated DNA sequencing was performed with fluorescence dye-labeled universal M13 primers or dye terminators. Analysis was performed on an automatic sequencer (model 373), according to the instructions supplied by Applied Biosystems Incorporated (Foster City, Calif.). Computer analyses of DNA and protein sequences were performed with the programs in the PCGene package and with Genetics Computer Groups programs. Database similarity searches were performed using the BLASTX and BLASTN algorithms (2), and sequence patterns were identified using BEAUTY (BLAST enhanced alignment utility) (29) and using the PROSITE database (3). GC content and codon usage were compared using the CUTG (codon usage tabulated from GenBank) database (17) and the Countcodon program. Pairwise alignments were made using ALIGN (20).

Expression and specificity of the *Nme*SI methyltransferase in *E. coli*. PCR products were generated with primer combination ABM6 and MNCO1 (5′-TAG CAC CAT GGG TTT AGA AAA TTT TCA AT-3′) and with primer combination ABM6 and MNCO2 (5′-AAA TTT CCA TGG ATA CTA TAA GTA GC-3′) and cloned into vector pCR2.1, introducing *Nco*I sites (underlined in the primer sequences). Resulting plasmids pCRT1 and pCRT2 were checked by sequence analysis, after which the *Nco*I-*Spe*I fragments containing the methyltransferase genes of both plasmids were cloned into expression vector pSE380 and transformed to *E. coli* Top10F′, yielding plasmids pT1 and pT2, respectively. To detect expression of M.*Nme*SI, cells containing pSE380, pT1, and pT2 grown with or without IPTG were collected and lysed by boiling at 100°C for 5 min. The lysates were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (11% separating gels), and the gels were stained with Coomassie brilliant blue. To assess the effects of M.*Nme*SI expression, plasmid DNA was isolated from these cells and the sensitivity to digestion with restriction endonucleases with recognition sites of interest was examined. Sequence analysis of the modified site was performed as described above using primers AB1249F (5′-GTG AAA GTA AAA GAT GCT GA-3′) and AB1658R (5′-TGT CAC GCT CGT CGT TTG GT-3′), which target vector sequences present in pSE380.

Nucleotide sequence accession numbers. The nucleotide sequence data are available in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession no. AF123569.

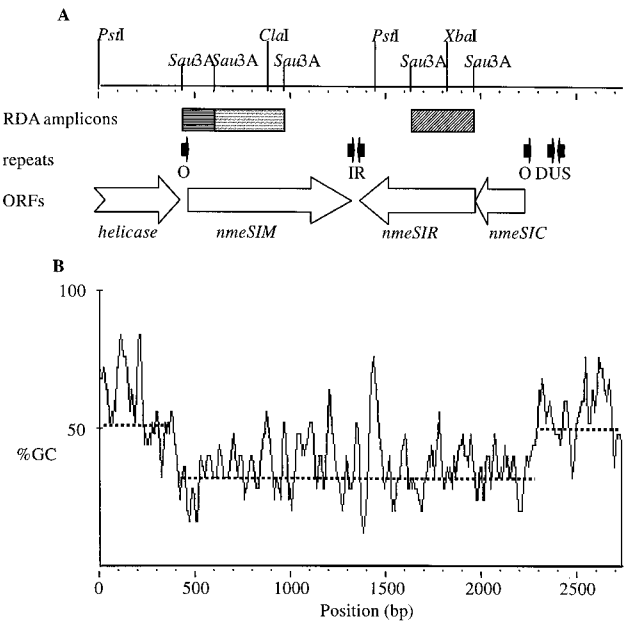

FIG. 1. (A) Schematic representation of the *Nme*SI locus. Relevant restriction sites are indicated. Hatched bars, RDA fragments; open arrows, ORFs; solid arrows, repeats; O, 12-bp direct repeat, putative NmeSIC binding site (5); IR, 10-bp inverted repeat. (B) GC content of the *Nme*SI locus. Sliding windows with a size of 25 bp were used. Dotted lines, mean values.

RESULTS AND DISCUSSION

Sequencing of the flanking sequences of the RDA amplicons. Previously, three DNA fragments that contained sequences specific for the hyperendemic lineage III cluster were identified by RDA using lineage III strain 800615 as the tester and strains 3532 and 830248 as the drivers (4). The sequence of the 3-kb locus containing these sequences was obtained as described in Materials and Methods. The region contains three complete open reading frames (ORFs) as indicated in Fig. 1A.

The predicted protein sequences encoded by two of the ORFs are 53 and 33% identical to the predicted sequences of the *Sca*I methyltransferase and restriction endonuclease (30), respectively. The genes were named *nmeSIM* and *nmeSIR*. The putative target recognition domains (21) of the predicted M.*Nme*SI sequence and the M.*Sca*I methyltransferase are highly conserved between the two proteins. Both sequences contain conserved motif IV sequence TSPP, which is shared by *N*-4-cytosine-specific methyltransferases. Based on the order of

```
NmeSIC MENELVEFGKRVKHYRLLNGWSQEELAEKVGLHRTYIGSVERGERNISLLNISLLAKSFS
       :. . . ... :. :: .:. :::.:::  :::::::::::: :::..: .. .:::...
SmaIC  MDIKEI-LAENVRSYRNINNLSQEQLAETSGLHRTYIGSVERKERNVTLSTLIILAKALN
                                      Helix-turn-helix

NmeSIC INISGTCRRD-------
         ..     :.
SmaIC  TSVPKLLTRQGLKNEQG
```

FIG. 2. Alignment of NmeSIC and SmaIC. The putative helix-turn-helix regions of these short proteins are underlined. The second helices, the recognition helices, are identical and indicated by double underlining (5). Double dots, identical amino acids; single dots, similar amino acids.

the nine conserved motifs of methyltransferases, M.NmeSI belongs to the β group of methyltransferases (13).

The third ORF is 40% identical to the control element of the *Sma*I RM system. An alignment, given in Fig. 2, shows that the recognition helices of the helix-turn-helix regions of the two control element proteins are identical. The *Nme*SI locus is flanked by two 12-bp direct repeats (Fig. 1A), which have been postulated to contain the operator sequences for the control gene product (5). Notably, the *Sca*I RM system does not contain such a regulatory control element.

Upstream (5′ of position 413) and downstream (3′ of position 2281) of the *nmeSI* locus are sequences that are present in both completely sequenced *N. meningitidis* strains (19, 25). Flanking the 5′ end of the region containing the RDA fragments is the 3′ end of an ORF that probably encodes a helicase, which is also present in the sequences from the three *Neisseria* genome projects (19, 25; B. A. Roe, L. Song, S. P. Lin, X. Yuan, S. Clifton, T. Ducey, L. Lewis, and D. W. Dyer, http://www.genome.ou.edu/gono. or http://www.ncbi.nlm.nih.gov/BLAST/ouacgtbl.html). Directly flanking the 3′ end (position 2375) of the *Nme*SI locus are two degenerate *Neisseria* DNA uptake sequences (DUSs), which form a 15-bp inverted repeat (Fig. 1A). DUSs are typical features of *Neisseria* DNA sequences. Sequence analysis suggests that the 3′ flanking region contains repetitive IS*1106* DNA sequences typical for *Neisseria* DNA. This implies that only the *Nme*SI locus is lineage III specific, in contrast to the flanking sequences.

Strikingly, the GC content of the sequence containing the *Nme*SI locus is much lower (33% for both the full sequence and only the coding sequence) than those of the flanking sequences (54% for the partial helicase coding sequence and 50% for the 3′ region) and the expected value for *N. meningitidis* coding sequences (51%), as illustrated in Fig. 1B. The low GC content is not the result of overrepresentation of certain amino acids, as the locus encoding the *Sca*I RM system has a 58% GC content. The lower GC content suggests that the *Nme*SI locus was recently acquired by a lineage III *N. meningitidis* ancestor from an organism with a low GC content. Strikingly, sequences in the region downstream of the helicase in the serogroup A strain Z2491 encode a different RM system (19) and those of serogroup B strain MC58 encode a putative regulatory protein (25). This suggests that the *Nme*SI locus was not inserted, but rather replaced genes. In addition, this demonstrates the plasticity of the neisserial genome at this position.

Specificity of the methyltransferase. Apart from protecting the bacterial chromosome from the action of its own restriction enzymes, DNA methylation also plays an important role in chromosome repair and replication (14). The methylation state of DNA has been shown to influence gene expression, the paradigm of this mechanism being the regulation of uropathogenic *E. coli* adhesin expression by DNA methylation (10, 27). Through the action of the methylase, a lineage III-specific RM system could interfere with expression of *Neisseria* virulence factors and thus be involved in the observed hypervirulent phenotype of these strains. Therefore, we determined the specificity of the methylase gene product as well as its expression in lineage III meningococcal strains.

Since the M.*Nme*SI sequence was highly similar to the M.*Sca*I sequence in the target recognition domain, we expected the specificities of both methylases to be similar as well.

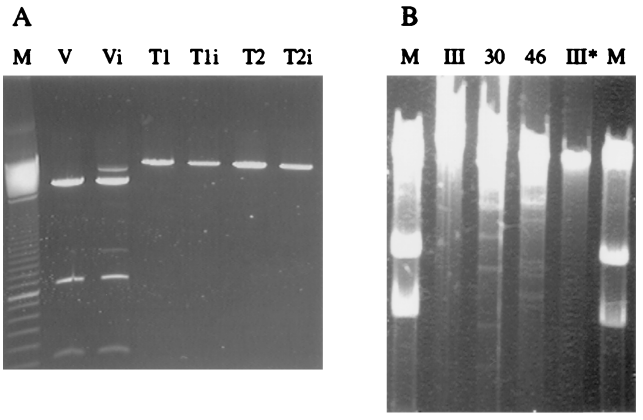

FIG. 3. Digestion of methylated and nonmethylated plasmid DNA. (A) *Sca*I digestion of *Nco*I-linearized plasmid DNA. M, marker (100-bp marker; the lower intense band equals 800 bp); V, vector pSE380; T1, plasmid T1; T2, plasmid T2. The subscript i denotes induction of expression in the *E. coli* culture with IPTG. (B) *Sca*I digestion of *N. meningitidis* chromosomal DNA. M, marker (23.1-, 9.4-, and 6.6-kbp bands); III, lineage III strain 800615; 30, non-lineage III strain 3532; 46, non-lineage III strain 830246, III*, lineage III strain 882066. To visualize the large resulting fragments, a high input of chromosomal DNA was used.

To test this assumption *nmeSIM* was cloned in *E. coli* and plasmid DNA of the transformants was digested with *Sca*I. Since *nmeSIM* contains two possible ATG start codons, two primers (MNCO1 and MNCO2) were developed to clone *nmeSIM* in expression vector pSE380. *E. coli* cells containing recombinant expression systems pT1 and pT2 were grown with IPTG (induced) or without IPTG (uninduced) for 4 h. Expression of the methyltransferase was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE. Both induced Top10F′ (pT1) and induced Top10F′ (pT2) produced a protein of approximately the expected size (approximately 32 kDa), which was not detectable in induced Top10F′(pSE380) (data not shown). From noninduced and induced cultures, plasmids were isolated. The plasmids were digested with *Nco*I, resulting in a linear plasmid for pSE380, pT1, and pT2. This linearization was performed since the *Sca*I restriction endonuclease is known to cut a supercoiled plasmid poorly. As shown in Fig. 3A, pT1 and pT2 were not digested by *Sca*I, in contrast to linearized pSE380. This shows that *Sca*I site 5′-AGTACT-3′ in both pT1 and pT2 was modified by the methyltransferase so that it was no longer a substrate for *Sca*I. Apparently, induction of expression is not required to protect the plasmid DNA from digestion, since plasmids from both noninduced and induced cultures were resistant to digestion with *Sca*I. Also, the six-amino-acid difference at the N termini of the pT1 and pT2 products does not abolish methylation by one of the recombinant proteins. To certify that the observed inhibition of digestion was due to an *N*-4-cytosine modification, the regions containing the *Sca*I sites of pSE380 and pT1 were sequenced using dye terminator chemistry. It was previously shown that template methylation influences the incorporation of fluorescently labeled dideoxynucleoside triphosphates by AmpliTaq FS polymerase (22). As shown in Fig. 4, unmethylated template pSE380 yields lower G signals in the AGTACT sequence than methylated template pT1. This shows that the cytosine of the

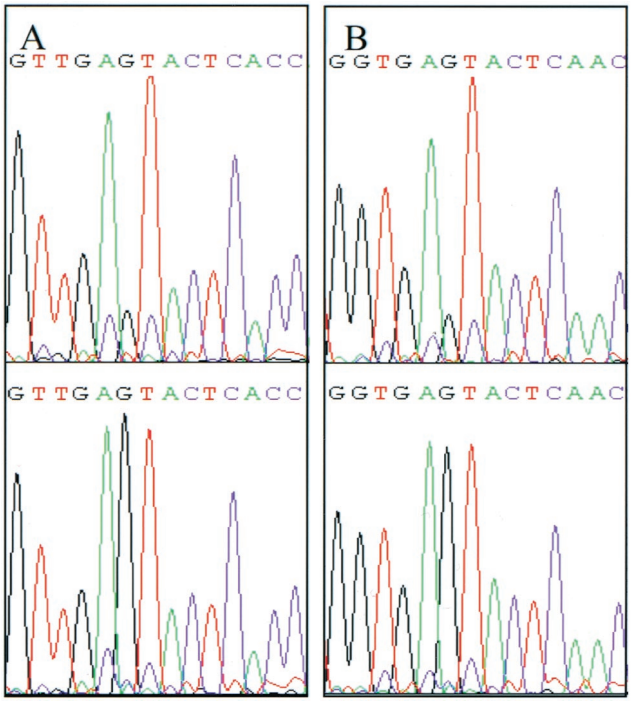

FIG. 4. Dye terminator sequences of methylated and nonmethylated template. (A) Forward sequence obtained using primer AB1249F on nonmethylated template DNA pSE380 (top) and M.*Nme*SI methylated template DNA pT1 (bottom). The G signal in sequence AGT ACT is different. (B) Reverse sequence obtained using primer AB1658R on nonmethylated template DNA pSE380 (top) and M.*Nme*SI methylated template DNA pT1 (bottom). The G signal in the sequence AGTACT is different.

template is modified and rules out N5 methylation, as this results in lower G signals (22). Next, we confirmed that the methyltransferase is expressed and active in lineage III meningococci by incubating chromosomal DNA of two lineage III strains (800615 and 882066) and of two non-lineage III strains (3532 and 830248) with restriction enzymes *Sca*I (recognizing 5′-AGTACT-3′) and *Sph*I (recognizing 5′-GCATGC-3′), respectively. The chromosomal DNA of the four strains was digested by *Sph*I (results not shown), confirming that the DNA was susceptible to restriction endonucleases. Incubation with *Sca*I showed that the DNA from the non-lineage III strains is digested (Fig. 3B). However, the DNA of the lineage III strains is protected from digestion with *Sca*I, indicating that the methylation pattern of lineage III strains differs from that of non-lineage III strains. Possibly, this differential methylation pattern affects transcriptional regulation. Differential methylation of DNA has also been implicated in the frequency of phase variation in meningococci (6). Whether the methylation by M.*Nme*SI contributes to the hypervirulent character of lineage III isolates in either way awaits further investigations.

The population biology of *N. meningitidis* is supposed to be influenced by horizontal gene transfer to a large extent (24). Horizontal gene transfer can be affected by RM systems (16), and this phenomenon has been implicated in maintenance of clonality of hypervirulent *N. meningitidis* clones (9). Therefore, the clonality of lineage III may in part be the consequence of the presence of the *Nme*SI RM system. However, the digestion of chromosomal DNA of the non-lineage III strains with *Sca*I yields large restriction fragments, indicating that sequence AGTACT is underrepresented in these meningococcal genomes (Fig. 3B). This is in accordance with the low number of AGTACT sites (only 65) in the genome sequence of strain MC58 (26). This suggests that *Nme*SI RM may have a limited effect on the clonal character of lineage III via its influence on horizontal gene transfer.

Evolutionary implications. The serine residue in motif IV (SPPY) is conserved in *N*-4-cytosine methyltransferases and is part of the active site of the protein (28). This amino acid is encoded by the codon TCT in *nmeSIM*, whereas it is encoded by AGT in *scaIM*. Mutation of one of these codons to the other requires two point mutations, resulting in a threonine (ACT) or cysteine (TGT) residue after the first mutation. Neither of these residues has been found in any aminomethyltransferase. Possibly either of these residues results in an inactive protein. Therefore, it is unlikely that the two methyltransferases have a recent common ancestor. Moreover, the similarity between the predicted protein sequences of M.*Sca*I and M.*Nme*SI is more concentrated in the putative target recognition domain (TRD) than in conserved domains I to X of methyltransferases. In addition, the genes of the respective systems mentioned above have a large difference in GC content, and the *Nme*SI RM system seems to contain a regulatory protein, in contrast to the *Sca*I system. In conclusion, the similarity of the respective TRDs of the two methyltransferases is likely to be the consequence of convergent evolution.

ACKNOWLEDGMENTS

We acknowledge the Gonococcal Genome Sequencing Project supported by USPHS/NIH grant AI38399.

We thank Wendy Keijzers for expert technical assistance. We acknowledge B. A. Roe, L. Song, S. P. Lin, X. Yuan, S. Clifton, T. Ducey, L. Lewis and D. W. Dyer at the University of Oklahoma for sequence data prior to publication.

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Editor: T. R. Kozel