Temperate *Myxococcus xanthus* Phage Mx8 Encodes a DNA Adenine Methylase, Mox

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Temperate bacteriophage Mx8 of *Myxococcus xanthus* encapsidates terminally repetitious DNA, packaged as circular permutations of its 49-kbp genome. During both lytic and lysogenic development, Mx8 expresses a nonessential DNA methylase, Mox, which modifies adenine residues in occurrences of *Xho*I and *Pst*I recognition sites, CTCGAG and CTGCAG, respectively, on both phage DNA and the host chromosome. The *mox* gene is necessary for methylase activity in vivo, because an amber mutation in the *mox* gene abolishes activity. The *mox* gene is the only phage gene required for methylase activity in vivo, because ectopic expression of *mox* as part of the *M. xanthus* *mglBA* operon results in partial methylation of the host chromosome. The predicted amino acid sequence of Mox is related most closely to that of the methylase involved in the cell cycle control of *Caulobacter crescentus*. We speculate that Mox acts to protect Mx8 phage DNA against restriction upon infection of a subset of natural *M. xanthus* hosts. One natural isolate of *M. xanthus*, the lysogenic source of related phage Mx81, produces a restriction endonuclease with the cleavage specificity of endonuclease *Bst*BI.

Myxococcus xanthus is a representative of the myxobacteria, unicellular prokaryotes that display complex social behaviors. During vegetative growth, *M. xanthus* cells glide in swarms. When starved for nutrients, the swarms undergo a developmental program involving both the morphogenesis of a fruiting structure and the differentiation of a subset of cells into heat-resistant spores. Both gliding motility and development depend on intercellular communication mediated by extracellular signal molecules. The pathways of signal transduction used by *M. xanthus* are simple prokaryotic models for similar pathways that mediate gliding motility and development in higher eukaryotes (5, 9, 21, 32).

The study of *M. xanthus* cell-cell interactions has been facilitated by a variety of prokaryotic genetic methods. Among the most powerful of these methods is transduction, genetic exchange mediated by bacteriophage. *M. xanthus* is the host for both lytic (2) and lysogenic (13) generalized transducing phages. Our work has focused on the study of one of these phages, temperate phage Mx8. Mx8 particles have icosahedral capsids and short tails and encapsulate a terminally repetitious genome derived from 49 kb of unique, linear sequence (reference 13 and unpublished results).

During lysogenic development, Mx8 integrates into the host chromosome at a preferred attachment site, *attB*. The prophage state is stable throughout development (15). Thus, Mx8 has the potential for being engineered as a cloning vector for host genes involved in *M. xanthus* motility and development. In this report, we describe the first step in the construction of specialized transducing derivatives of phage Mx8. We identify a nonessential region of the Mx8 genome that may be deleted to compensate for small insertions without the loss of terminal repetition. This region encodes a DNA adenine methylase, Mox, that is both necessary and sufficient for modifying a small number of target sites on the Mx8 genome.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this work are described in Table 1. *Escherichia coli* strains carrying plasmids were grown in Luria-Bertani medium supplemented with ampicillin (100 µg/ml) and/or kanamycin sulfate (40 µg/ml) (Sigma Chemical Co.). CT liquid medium (4) was used for the routine growth of *M. xanthus*. Derivatives of *M. xanthus* DZ1 with integrated plasmids were grown on CT medium with kanamycin (40 µg/ml). TM buffer is CT medium without Casitone. Natural isolates of *M. xanthus* used in the study are listed in the legend to Fig. 8.

Bacteriophage strains and methods. Phage strains used in this work are described in Table 1. Wild-type Mx8 (Mx8⁺⁺) was reisolated from *M. xanthus* DK883. The supernatant of a culture of strain DK883 grown to exponential density in CT medium at 32°C was plated on a lawn of DZ1 cells by the soft-agar (0.75% agar) overlay method on CT agar plates (1.5% agar), as described by Martin et al. (13). A single turbid plaque-forming phage was designated the wild type.

Low-titer stocks of Mx8 were prepared by inoculating a 5- to 10-ml culture of strain DZ1 at a density of about 2×10^8 cells/ml with a single plaque plugged from a CT agar plate with a Pasteur pipette followed by incubation of the culture at 32°C for 48 to 72 h. Phage were harvested by differential centrifugation; bacterial debris was pelleted by centrifugation at $8,000 \times g$ for 5 min. High-titer phage stocks were grown by inoculating a 200-ml culture of DZ1 at a density of about 2×10^8 cells/ml with a single plaque plugged from a CT agar plate with a Pasteur pipette followed by incubation of the culture at 32°C for 48 to 72 h. Routinely, single plaques of phage were purified three times on host DZ1 prior to stock growth. Phage were harvested by differential centrifugation; bacterial debris was pelleted by centrifugation at $8,000 \times g$ for 5 min, and phage were pelleted by centrifugation at $35,000 \times g$ for 3 h. Pellets were resuspended in 8 ml of TM buffer.

Phage DNA was prepared from 0.4 ml of the low-titer stocks by sequential extraction with equal volumes of phenol and chloroform and then precipitation by additions of potassium acetate to 1 M and a 2.5× volume of ethanol. Phage DNA was made from high-titer stocks by an alternative method. To 400 µl of chloroform-extracted phage, 50 µl of 2.0 M Tris-HCl (pH 8.0), 0.1 M EDTA, 2 µl of diethylpyrocarbonate, and 10 µl of 10% sodium dodecyl sulfate (SDS) were added. The mixture was incubated at 65°C for 5 min to disrupt phage particles, and 50 µl of 5 M potassium acetate was added to precipitate SDS-capsid protein complexes. After incubation at 4°C for 1 h, mixtures were microcentrifuged at $9,000 \times g$ for 15 min. Supernatants were precipitated with a 2.5× volume of ethanol, and pellets were rinsed in 70% ethanol–10 mM Tris-HCl (pH 8.0)–1 mM EDTA–10 mM MgCl₂ (DNA wash solution) for 5 min. (Incubation in DNA wash solution allows an exchange reaction to occur between heavy metal cations that undergo redox reactions in aqueous solution to generate hydroxyl radicals and Mg²⁺ cations and prolongs the stability of phage DNA.) DNA pellets were air dried, resuspended in 200 µl of 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA, and stored routinely at –20°C. DNA extracted from reisolated Mx8 phage gave restriction patterns with endonucleases *Eco*RI, *Sau*3aI, and *Pvu*II identical to those published by Stellwag et al. (24) and those obtained with Mx8 phage from

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TABLE 1. Bacteria, phage, and plasmids

Strain or plasmid	Genotype or description	Source or reference
<i>M. xanthus</i>		
DK879	Natural isolate of <i>M. xanthus</i> ; lysogen of Mx81	13
DK883	Natural isolate of <i>M. xanthus</i> ; lysogen of Mx8	13
DK893	Natural isolate of <i>M. xanthus</i> ; lysogen of Mx82	13
DK6204	$\Delta mglBA-6204$	7
DK6204::pAY703	<i>mgl</i> ⁺	This work
DK6204::pAY720	<i>mgl</i> ⁺ , <i>mox</i> ⁺	This work
DZ1		2
DZ1(pAY50)	<i>attP</i> ⁺ - <i>int</i> ⁺ , <i>imm</i> ⁺ , <i>mox</i> ⁺	This work
DZ1(pAY442)	<i>attP</i> ⁺ - <i>int</i> ⁺ , <i>imm</i> ⁺ , <i>mox</i> -86(Am)	This work
<i>E. coli</i>		
JM107	<i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR7</i> , <i>supE44</i> , <i>relA1</i> , λ^- , $\Delta(lac-proAB)$, [F', <i>traD36</i> , <i>proAB</i> , <i>lacI</i> ^q Δ M15]	34
Phage Mx8		
Mx8 ⁺⁺	<i>mox</i> ⁺	
Mx8 <i>vir1</i>	Virulent derivative of Mx8 ⁺⁺	This work
Mx8 <i>del1</i>	<i>mox</i> deletion derivative of Mx8 ⁺⁺	This work
Plasmids		
pACYC177	Ap ^r Km ^r	3
pBluescriptKSII ⁺	Phagemid vector for DNA cloning and sequencing; ColE1 replicon, <i>lacZ</i> α , Ap ^r	Stratagene, Inc.
pLITMUS28/38	Phagemid vector for DNA cloning and sequencing; ColE1 replicon, <i>lacZ</i> α , Ap ^r	New England Biolabs
pBGS18	Phagemid vector for DNA cloning and sequencing; ColE1 replicon, <i>lacZ</i> α , Km ^r	23
pPLH325	2.2-kb <i>Pst</i> I fragment of pKNS123 ligated to <i>Pst</i> I site of pBGS18	8
pAY50	8.1-kb <i>Sau</i> 3aI- <i>Pvu</i> II fragment of Mx8 ligated to <i>Bam</i> HI- <i>Sca</i> I sites of pACYC177	This work
pAY210	1,761-bp <i>Bss</i> HII fragment of pAY50 ligated to <i>Bss</i> HII site of pLITMUS28	This work
pAY216	542-bp filled-in <i>Ava</i> II fragment of pAY304 ligated to <i>Sma</i> I site of pKSII ⁺	This work
pAY260/261	865-bp <i>Eco</i> RI fragment of pAY50 ligated to pLITMUS28	This work
pAY262/263	Deletion derivative of pAY260/261 made by <i>Aat</i> II cleavage, then ligation	This work
pAY264/265	Deletion derivative of pAY260/261 made by <i>Bss</i> HII cleavage, then ligation	This work
pAY266	232-bp <i>Bss</i> HII fragment of pAY260 ligated to pLITMUS28	This work
pAY277	486-bp <i>Aat</i> II fragment of pAY210 ligated to pLITMUS28	This work
pAY280	431-bp <i>Msc</i> I- <i>Nco</i> I fragment of pAY260 ligated to pLITMUS28	This work
pAY295	103-bp <i>Bsp</i> HI- <i>Nco</i> I fragment of pAY260 ligated to pLITMUS28	This work
pAY296	144-bp <i>Nco</i> I- <i>Eco</i> RI fragment of pAY261 ligated to pLITMUS28	This work
pAY304	2,761-bp <i>Eco</i> RI- <i>Sau</i> 3aI fragment of pAY50 ligated to the <i>Eco</i> RI- <i>Bam</i> HI sites of pBS KSII ⁺	This work
pAY426	2.8-kb deletion derivative of pACYC177 made by <i>Bsp</i> HI cleavage and ligation; the deletion corresponds to coordinates 3645–713 (1,008 bp) of pACYC177	This work
pAY442	Otherwise isogenic derivative of pAY50 with <i>mox</i> -86(Am) mutation	This work
pAY703	Km ^r derivative of pPLH456 with 1,292-bp <i>mgl</i> operon	This work
pAY718	1,136-bp <i>Eco</i> RI- <i>Mfe</i> I subclone of the Mx8 <i>del1</i> deletion derivative ligated to the <i>Eco</i> RI site of pLITMUS28	This work
pAY719	811-bp subclone of the <i>mox</i> gene ligated to the <i>Acc</i> 65I- <i>Hind</i> III sites of pLITMUS28	This work
pAY720	805-bp <i>Acc</i> 65I- <i>Mfe</i> I fragment carrying the <i>mox</i> gene ligated to the <i>Acc</i> 65I- <i>Eco</i> RI sites of pAY703	This work

Larry Shinkets (data not shown). Genomic DNA was prepared from *M. xanthus* strains in a similar way. Cells (0.4 ml at 4×10^8 /ml) were pelleted by low-speed centrifugation and resuspended in TM buffer prior to lysis with SDS.

The virulent mutant phage Mx8 *vir1* was isolated by plating a stock of wild-type Mx8 on a lawn of the immune-defective lysogen DZ1(pAY50). On this host, as on a lysogen of DZ1 carrying Mx8⁺⁺ as prophage, wild-type phage forms plaques with an efficiency of plating (EOP) of 10^{-5} of that on DZ1 alone. DZ1(pAY50) was used instead of DZ1(Mx8⁺⁺) because lawns of the latter host typically include 50 to 5,000 plaques formed by virulent phages released spontaneously from this nondefective lysogen. A plaque recovered from a lawn of the defective lysogen DZ1(pAY50) was purified on host DZ1 and designated Mx8 *vir1*. Mx8 *vir1* plates with equal efficiencies on hosts DZ1, DZ1(pAY50), and DZ1(Mx8⁺⁺).

The spontaneous, clear-plaque-forming mutant Mx8 *c1* was isolated from wild-type Mx8. A single plaque of wild-type phage was resuspended in 1 ml of TM buffer and treated with chloroform. A small aliquot (4 μ l) of the aqueous phase was spotted in the center of a CT plate and then overlaid with a soft-agar lawn of DZ1 to disperse phage. A phage that formed an isolated clear plaque was designated Mx8 *c1*.

The deletion mutant phage Mx8 *del1* was isolated as a large plaque-forming phage released from a lysogen of DZ1 with an oversized prophage. To construct an oversized derivative of Mx8, 1 μ g of Mx8 DNA was digested partially with *Eco*RI and ligated with 1 μ g of kanamycin-resistant (Km^r) plasmid pBGS18 (23) DNA cleaved to completion with *Eco*RI in a total volume of 25 μ l with phage T4

DNA ligase. Ligated DNA was electroporated into host DZ1. After 3 h of outgrowth at 32°C, electroporated cells were plated on lawns of DZ1 and the plates were incubated at 32°C for 48 h. The centers of 500 independent, small plaques were picked into CT plates with kanamycin; 8 of 500 plaques gave rise to Km^r lysogens. Lysogens were purified five times by streaking for single colonies on CT-kanamycin plates and then overlaid with a soft-agar lawn including host DZ1. One of the eight lysogens gave rise to both small and large plaques. One phage from a single large plaque was purified on DZ1 and designated Mx8 *del1*.

Lysogens carrying Mx8 wild-type and Mx8 *del1* prophages were constructed by spotting phage (about 10^8 PFU/ml) on lawns of DZ1 or DK6204. Single colonies were purified by streaking surviving cells from the centers of the spots. Colonies were tested for superinfection immunity by cross-streaking on CT plates against Mx8 *c1* and Mx8 *vir1* phages at 10^8 PFU/ml.

Plasmid constructions and electroporation methods. The insert in plasmid pAY50 (Km^r) was obtained by cleavage of phage Mx8 DNA and is an 8.1-kb *Sau*3aI-*Pvu*II fragment ligated to the *Bam*HI-*Sca*I backbone of plasmid pACYC177. This insert carries the phage-encoded site-specific recombination functions *int* and *attP* as well as a repressor gene, *imm*, necessary for superinfection immunity (unpublished results). All other plasmids, except for pAY703, pAY718, and pAY720, were derived in one or more steps from plasmid pAY50 and are described in Table 1. Methods for manipulating plasmid DNA were similar to those described by Sambrook et al. (17). For electroporation of *E. coli*

and *M. xanthus*, we used the methods of Taketo (26) and Kashefi and Hartzell (10), respectively.

An amber mutation was introduced into the *mox* gene on plasmid pAY50 by using PCR with the mismatch primer 5' GACGAATTCGTGTCTTAGGGCTGGTTGTTGCGGA, the M13 forward primer GTTTCCTCCAGTCACGA, and template plasmid pAY260. The mutant 874-bp *EcoRI* fragment generated by PCR was cloned into the *EcoRI* site of pLITMUS28 to make plasmid pAY441. The mismatch primer replaces the codon for residue glycine-86 of *Mox* with an amber codon within a new, unique *AvrII* site. The smaller *EcoRI* fragment of plasmid pAY441 was substituted for the smaller *EcoRI* fragment of plasmid pAY50 to generate plasmid pAY442. Plasmids pAY50 and pAY442 were electroporated into *M. xanthus* DZ1 to construct the otherwise isogenic *mox*⁺ and *mox* mutant Km^r defective lysogens DZ1(pAY50) and DZ1(pAY442), respectively. The presence of the amber codon in plasmids pAY441 and pAY442 was confirmed by cleavage with *AvrII*.

To determine if *Mox* is sufficient for DNA methylation, we constructed a vector, pAY703, that introduces a pair of unique restriction sites downstream of *mglA* in the two genes of the *mglBA* operon (8), between which the *mox* gene was cloned. To amplify the *mglBA* operon, we used PCR with the M13 forward primer GTTTCCTCCAGTCACGA; a primer, ATCTGGTACCCACCTTCTTGAG, which is complementary to the 3' end of the *mglA* gene and introduces an *Acc65I* restriction site; and template pPLH325 (8). The amplified 1.2-kb *mglBA* operon and plasmid pBGS18 were cleaved with *HindIII* and *Acc65I* and ligated to make plasmid pAY703. The *mox* gene was amplified with primers AAAGGTACCATGAGGAGGCGTAGT and GGGAAGCTTCAATTGTCA GCGCCGAA and plasmid template pAY50. The amplified *mox* gene and plasmid pLITMUS28 were cleaved with *Acc65I* and *HindIII* and ligated to make the intermediate plasmid pAY719. Plasmid pAY719 was cleaved with *Acc65I* and *MfeI*, and the 805-bp DNA fragment containing the *mox* gene was ligated to plasmid pAY703 (cleaved with *Acc65I* and *EcoRI*) to make plasmid pAY720. Plasmids pAY703 and pAY720 were electroporated into *M. xanthus* DK6204, and the Km^r electroporants DK6204::pAY703 and DK6204::pAY720 were selected.

To map the *dell* deletion mutation, phage Mx8 *dell* was cleaved with *EcoRI* and *MfeI*. A 1,136-bp DNA fragment, not seen in Mx8⁺-treated DNA, was gel purified and ligated to the *EcoRI* site of plasmid pLITMUS28 to make plasmid pAY718. The deletion junction defined by *dell* was identified by DNA sequence analysis.

HPLC analysis of the nucleoside composition of phage DNA. Phage DNA (20 µg) was hydrolyzed and dephosphorylated according to the method of Shigenaga et al. (20). The free nucleosides were analyzed by high-performance liquid chromatography (HPLC) with a method similar to those described previously (6, 20). A Perkin-Elmer Series 4 liquid chromatograph was used in conjunction with a Perkin-Elmer LC-95 UV/Vis spectrophotometer and a Supelcosil LC-18-DB 15-cm by 4.6-mm C₁₈ reversed-phase column (Supelco). Initially, 5% (vol/vol) methanol in a 50 mM KH₂PO₄ (pH 5.5) carrier was pumped at 1 ml/min for 20 min followed by 10% (vol/vol) methanol in a 50 mM KH₂PO₄ (pH 5.5) carrier pumped for 40 min. A flush of 65% methanol in water was pumped for 10 min following the carriers to remove any strongly retained materials from the column. The column was reequilibrated with the initial carrier for 20 min following each run. Sample aliquots (50 µl) were injected into a 20-µl sample loop. The spectrophotometer was set at 260 nm, with a response time of 500 msec and sensitivity of 0.01 absorbance units, full scale. Output from the spectrophotometer was acquired and analyzed with a MacLab/2e module and Chart software for the MacIntosh (ADI Instruments). Nucleoside standards including adenosine, N⁶-methyladenosine (N⁶-MeA) and 5-methylcytosine (Sigma) were dissolved in 1 mM deferoxamine mesylate–20 mM sodium acetate (pH 5) and were run at the beginning and end of each set of analysis runs. To quantify the mole fraction of adenosine and N⁶-MeA in each phage DNA, standards were measured at concentrations ranging from 1 to 100 µg/ml and 2 to 200 µg/ml, respectively. Areas from peaks corresponding to these nucleosides in each phage DNA were measured six times, and nucleoside abundance was calculated relative to the peak areas of concentration standards.

DNA sequence analysis. Plasmid templates used for the sequence analysis of the *mox* gene are listed in Table 1. Inserts were sequenced by the method of Sanger et al. (18) with M13 forward and reverse primers, and additional primers when necessary, by Commonwealth Biotechnologies, Inc., Richmond, Va. Sequencing runs were resolved on an ABI Prism model 377 automated sequencing apparatus. The sequence of each strand of each template was determined completely; the identity of each base in the assembled sequence was determined from at least four different primer-template combinations.

Developmental assays. To initiate development, derivatives of DK1622 or DK6204 were grown to a density of 5×10^8 cells/ml in CT medium at 32°C, concentrated by low-speed centrifugation, and resuspended in a 0.1× volume of TM buffer. Multiple spots (20 µl) were made on TM plates (1.5% agar), and the plates were incubated at 32°C for 120 h. To measure viable spores, five 20-µl spots were harvested after incubation at 50°C for 2 h, scraped into 1 ml of TM, and sonicated for 10 s at 20 W on a Fisher cell dismembrator to disperse the spores. Serial dilutions were plated on CT plates and scored for growth after 72 h.

Assay of cell-free extracts of *Myxococcus* strains for restriction endonuclease activities. To assay *Myxococcus* strains for endonuclease activity, cultures were

grown to a density of 5×10^8 cells/ml in CT medium at 32°C, concentrated by low-speed centrifugation, resuspended in a 0.1× volume of restriction buffer (0.2 M potassium acetate, 50 mM Tris-acetate [pH 7.6], 20 mM magnesium acetate, 1 mM 2-mercaptoethanol, 20 µg of bovine serum albumin per ml) supplemented with 1 µM phenylmethylsulfonyl fluoride, and frozen at –20°C. Frozen cell suspensions were thawed on ice and sonicated for 10 s at 20 W. Cell debris was pelleted by microcentrifugation at $15,000 \times g$ for 1 min, and cell supernatants (2 µl) were added to 10-µl reaction mixtures also containing 5 µl of substrate DNA (either phage λ or Mx8 DNA at 50 µg/ml) and 3 µl of H₂O. Reaction mixtures were incubated at 32°C for 20 min, and the products were resolved by electrophoresis through 0.7% agarose gels. Phage λ cI-857 S-7(am) DNA is from New England Biolabs.

Nucleotide sequence accession number. The sequence of the 8.1-kb *Sau3aI*-*PvuII* fragment obtained in this study and the endpoints of the *dell* deletion of this insert have been assigned GenBank no. U64984.

RESULTS

Temperate *M. xanthus* phage Mx8 encodes an enzyme, *Mox*, which modifies DNA. Although plasmid subclones of phage Mx8 DNA are cleaved with the restriction endonuclease *XhoI* (8), DNA isolated from wild-type phage particles is not cleaved with *XhoI* (24). These results suggest that phage Mx8 encodes a function which modifies *XhoI* recognition sites on its genomic DNA, a function which is not expressed from a subset of plasmid subclones of Mx8 DNA. We designated this function *Mox*, for methylation of *XhoI* sites.

A derivative of wild-type Mx8 with a deletion of the nonessential *mox* gene, Mx8 *dell*, was isolated by using a strategy that proved successful for isolating deletion mutants of *Salmonella* phage P22 (31). Because the genome of Mx8 is terminally repetitious (24), like that of P22 (30), phage with oversized genomes form smaller plaques with lower efficiencies because their genomes have reduced terminal repetition. Large plaque-forming derivatives of these small plaque-forming phages carry deletions which compensate for the parental insertion and restore terminal repetition. Therefore, we cleaved Mx8 DNA partially with *EcoRI* and Km^r plasmid pBGS18 DNA completely with *EcoRI*, ligated the DNA, electroporated this DNA into host DZ1, and selected Km^r recombinants. Because pBGS18 has no homology with the *M. xanthus* genome, the only way for a Km^r electroporant to arise is by the integration of a plasmid-phage cointegrate. A simple insertion of the 3.6-kb pBGS18 genome into the Mx8 genome should give rise to a lysogen carrying an oversized prophage that, upon spontaneous induction, gives rise to phage that form small plaques with reduced efficiencies. One such lysogen was recovered; it gives rise at a high frequency to phages that form small plaques and at a low frequency to phages that form larger plaques. One of the larger plaque-forming phages was analyzed and found to carry the deletion mutation *dell*. This mutant is missing 1,533 bp of Mx8 DNA (see Fig. 3). Fig. 1a shows that the Mx8 *dell* genome is cleaved at multiple sites by *XhoI*. Thus, the *dell* deletion mutation inactivates *Mox*, the function required for modification of *XhoI* sites on Mx8⁺ DNA.

***Mox* modifies both *XhoI* and *PstI* sites.** To determine the target specificity of the *Mox* methylase, we cleaved Mx8⁺ and Mx8 *dell* DNA with a variety of restriction endonucleases which recognize and cleave sequences related to the *XhoI* target site, CTCGAG. The results in Fig. 1a also show that although Mx8⁺ DNA also is not cleaved by *PstI*, which recognizes the sequence CTGCAG (24), Mx8 *dell* DNA is cleaved with *PstI*. Neither Mx8⁺ nor Mx8 *dell* DNA is cleaved with *AflIII* (CTTAAG), indicating most likely that Mx8 DNA does not contain occurrences of this A+T-rich recognition site.

The endonucleases *ClaI* (ATCGAT), *SalI* (GTCGAC), and *BstBI* (TTCGAA) recognize sites differing symmetrically from the *XhoI* site (CTCGAG) in the first nucleotide. All three enzymes cleave Mx8⁺ DNA multiple times (data not shown),

FIG. 1. Mox methylates both *XhoI* and *PstI* sites. (a) Treatment of Mx8⁺⁺ and Mx8 *delI* DNA with *XhoI*, *PstI*, and *AflII*. Wild-type DNA is cleaved by none of these enzymes, whereas Mx8 *delI* DNA is sensitive to both *XhoI* and *PstI* cleavage. (b) *SfcI* treatment of Mx8⁺⁺ DNA, Mx8 *delI* DNA, and a mixture of Mx8⁺⁺ and Mx8 *delI* DNAs. DNA isolated from Mx8⁺⁺ phage is susceptible to cleavage by *SfcI*; however, DNA isolated from Mx8 *delI* phage is cleaved at a larger number of sites.

indicating that a GC base pair is required as the first nucleotide of the Mox recognition site. Similarly, the enzymes *NsiI* (ATG CAT) and *ApaI* (GTGCAC), which recognize sites related to the *PstI* site (CTGCAG), cleave Mx8⁺⁺ DNA.

The endonucleases *PmlI* (CACGTG) and *SmaI* (CCCGGG) cleave Mx8⁺⁺ DNA at sites different from the *XhoI* target (CTCGAG) in the second nucleotide. The enzymes *PvuII* (CAGCTG), *SacII* (CCGCGG), and *EagI* (CGGCCG) differ only in the second nucleotide from the *PstI* site (CTGCAG); all three cleave Mx8⁺⁺ DNA (data not shown). Thus, a TA base pair is required as the second nucleotide of the Mox site.

Mox modifies a subset of the target sequences CTNNAG. As shown in Fig. 1b, the enzyme *SfcI*, which recognizes the set of target sequences CTRYAG, cleaves Mx8⁺⁺ DNA at several sites and cleaves Mx8 *delI* DNA at a larger number of sites. These results show that Mox recognizes a proper subset of the target sequences CTNNAG, most likely CTSSAG, where S is G or C. That is, both *XhoI* (CTCGAG) and *PstI* (CTGCAG) sites are modified, whereas only a subset of *SfcI* sites (CTGCAG, CTGTAG, CTACAG, and CTATAG) are modified. Several endonucleases recognize asymmetric sites which differ from the *XhoI* (CTCGAG) and *PstI* (CTGCAG) targets of Mox by only a single nucleotide base pair. These include *BssSI* (CACGAG), *BpmI* (CTCCAG), and *Eco57I* (CTTCAG). All three of these enzymes cleave both Mx8⁺⁺ and Mx8 *delI* DNA to yield similar size distributions of products. The observation that *BpmI* cleaves Mx8⁺⁺ and Mx8 *delI* DNA to similar extents shows that either modification does not prevent cleavage at *BpmI* sites or the sequence CTCCAG is not modified by Mox.

Mox is an adenine methylase. Phage DNA can be modified in two very different ways. Some phages, like *Bacillus subtilis* phages SP10 and SPO1, incorporate modified bases into their genomes, rendering their genomes insensitive to cleavage by a large subset of endonucleases (25, 33). Others, like *B. subtilis*

phages ϕ 3T, SP β , and ρ 11, methylate adenine or cytosine nucleotides within specific target sequences recognized by a small subset of endonucleases (28, 29). Mox falls into the latter category, because it is highly specific in its target recognition.

To determine the precise chemical nature of the Mox-dependent modification, we cleaved DNA isolated from both Mx8⁺⁺ and Mx8 *delI* phage particles with endonuclease P1, treated the mononucleoside phosphates with alkaline phosphatase, and separated the nucleosides by HPLC. As shown in Fig. 2, one of the products of enzymatic hydrolysis of wild-type Mx8 DNA is a nucleoside with the retention time of *N*⁶-MeA. Quantification of the peak corresponding to this nucleoside shows that *N*⁶-MeA represents 0.60% $\pm$ 0.36% of the mole fraction *N*⁶-MeA plus adenosine. This peak is absent from the column profile of Mx8 *delI* DNA, and neither column profile includes a peak corresponding to 5-methylcytosine. These results show that phage Mx8 encodes an adenine methylase, Mox, and that the *delI* mutation inactivates or prevents the expression of Mox.

The predicted product, Mox, is similar in sequence to other adenine methylases. To map the *mox* gene, we determined the position of the *delI* mutation on the physical map of Mx8. The cleavage sites for endonucleases *EcoRI*, *PvuII*, and *Sau3aI* have been ordered on this physical map (Fig. 3 [24]). The *delI* mutation removes a single *EcoRI* site and has endpoints within an 8.1-kb *Sau3aI*-*PvuII* fragment of the Mx8 genome. This 8.1-kb fragment was subcloned from wild-type phage DNA into plasmid vector pACYC177 (3) to construct plasmid pAY50. The position of *mox* was determined by sequencing the 8.1-kb insert in plasmid pAY50 as well as the 1.1-kb insert in an *EcoRI*-*MfeI* subclone of Mx8 *delI* DNA, pAY718, with the *delI* deletion mutation. One of the open reading frames (ORFs) altered by the *delI* mutation is *mox*.

As shown in Fig. 4, *mox* corresponds to an ORF predicted to encode a protein 258 amino acid residues in length. The base composition of this ORF is 63.8% G+C, slightly lower than the G+C composition of the 8.1-kb fragment (69.2%) of the Mx8 genome in which it resides. As is typical for genes from organisms with G+C-rich genomes, *mox* has an unusually high G+C representation in the third codon position (86.7%). The *mox* gene is preceded by the 6-base Shine and Dalgarno sequence, AGGAGG (22), located 7 bp upstream of an ATG start codon, and terminates with a TGA stop codon. Located 32-bp downstream of the stop codon is a potential stem-loop structure that may serve as a transcription terminator (Fig. 4).

The Mox protein is predicted to have a nearly neutral charge (*pI* = 6.77) and a primary sequence most similar to those of the adenine methylases M-*CcrI* (35), M-*MboII* (1), M-*RsrI* (11), and M-*DpnII* (12). The sequences of all adenine methylases share two features seen in Mox (11, 29). The amino terminus has a four-residue motif, Asp-Pro-Pro-Tyr, critical for *S*-adenosylmethionine binding. The carboxyl (C) terminus of Mox includes the second consensus sequence, Phe-X-Gly-X-Gly (Fig. 5).

The *mox* gene is necessary for methylase activity. To show that *mox* is necessary for methylase activity, we constructed an otherwise isogenic derivative of plasmid pAY50, plasmid pAY442, with an amber mutation in *mox*. This plasmid, like pAY50, also carries the Mx8 prophage attachment site (*attP*) and integrase (*int*) genes (27). When pAY50 (or pAY442) is electroporated into *M. xanthus* DZ1, these plasmids integrate into the *attB* site on the *M. xanthus* genome, giving rise to Km^r electroporants with a linear permutation of the plasmid as a defective prophage.

To assay for Mox function in vivo, genomic DNA was prepared from *M. xanthus* DZ1, the defective lysogens DZ1(pAY50)

FIG. 2. HPLC analysis of $Mx8^{++}$ and $Mx8\ del1$ shows that $Mx8\ del1$ DNA has less N^6 -MeA. (a) Elution profile of nucleosides derived from $Mx8^{++}$ DNA (100 μ g/ml). (b) Profiles of nucleosides from $Mx8\ del1$ DNA (300 μ g/ml). The arrows indicate peaks at the retention time of N^6 -MeA (28 min). Note that at 260 nm, the molar extinction coefficient for N^6 -MeA is greater than that for adenosine by a factor of 14.

and DZ1(pAY442), and phage $Mx8^{++}$. As an internal control, supercoiled plasmid pLITMUS28 DNA was added to each genomic DNA preparation. DNA was cleaved with endonuclease *XhoI* or *StuI* or left untreated, and the products were

resolved by agarose gel electrophoresis (Fig. 6). For each restricted DNA sample, pLITMUS28 DNA is completely linearized by *XhoI* or *StuI*, showing that the DNAs in these mixtures have been digested to completion with each endonuclease.

FIG. 3. Position of the *mox* gene on the physical map of $Mx8$. The physical map shows restriction sites for *EcoRI* (E), *PvuII* (V), and *Sau3aI* (S) (24) as well as for *MfeI* (M) and *NotI* (N). The region where headful DNA packaging initiates (*pac*) (24) is also shown. Plasmid pAY50 (shaded) has the 8.1-kbp *PvuII*-*Sau3aI* insert of $Mx8^{++}$ DNA cloned into the *Bam*HI-*ScaI* backbone of plasmid pACYC177. This fragment contains the phage-encoded *imm*, *mox*, and *int* genes. The deletion mutation *del1* removes a 1,533-bp fragment of phage DNA from bp 2279 to 3813 on the sequence and abolishes *mox* function. The position of *mox* was determined by sequencing the 8.1-kbp insert in pAY50; the deletion junction generated by *del1* (black bar) was positioned on this map by sequence analysis of a subclone of $Mx8\ del1$ DNA with the deletion.

1561 GGCCGTGCGCGTCGCTGCAGTTGCGGCGCGCGTACGCGTGCCGTGTGC
 1611 ATGAGGAGACGCACGGCAGCCACACAGAGGAGCGTAGTCATGGCCTGCC
 M A C
 1661 GTCCCTACTACCAACGCGGCGAGCTCACCTCTACCCTGCACGCACGAG
 R P Y Y Q R G D V T L Y H C T H E
 1711 GAGCTTGCCGATGCCCTCCAGGCAGAGTCAGTAGACGCCATCGTCACGGA
 E L G D A L Q A E S V D A I V T D
 1761 CCCGCCCTATGGGAGACGGCGTTGGAGTGGGACAGGTGGCCCGTTGGCT
 P P Y G E T A L E W D R W P V G
 1811 GGCCGGGACTGGTGCGCCCTGTGAAGCGCACCGGCTCCATGTGGTGT
 W P G L V R P L L K R T G S M W C
 1861 TTCGGGTGCTTCAGGATGTGGTGGGACAAGCGAGACGAATTCGTGCCG
 EcoRI
 F G S F R M W W D K R D E F V S G
 1911 CGGCTGGGTGTTGCGGAAGACGTGGTGTGGCGGAAGCAGAACGGCAGCG
 G W V V A E D V V W R K Q N G S
 1961 GCTTCCGCCACCGACAGGTTCGCGCGGTGCATGAGCAGCCGTCCTACTTC
 G F A T D R F R R V H E Q P V H F
 2011 TACCGAGCGGATGCGCGGTGGCGCATGTGTCCACCAAGTGGCCGTCA
 Y R A D A A W R D V F H Q V P V T
 2061 CATGGATGCCAAGGCCCGGACGGTGACGCGACGAGCGACGCCGCCGACC
 NcoI
 M D A K A R T V T R R A Q P P H
 2111 TCGGCGCCATCGCGGCCCAAGTACACGTCCGAGACGGCGGCCCGGC
 L G A I G A H K Y T S E D G G P R
 2161 CTCATGACGTCCGTGCTGGAAGTGCAGAACTGCCAGGGTTCGCCGTCAA
 BspHI
 L M T S V L E V R N C H G F A V N
 2211 CGAGACACAGAAGCCAGTGGCGCTCGTGAACCGCTGGTGCAGCAATGCCT
 E T Q K P V A L V E P L V R N A
 2261 GCCCACCGGGGGCCTCGTCGCGGACTTCTTCGCGGGCTCCGCGACGACG
 C P P G G L V A D F F A G S G S T
 2311 GCGCTGGCTGCCTCGCCACCGCGCCGCTTCATCGGTTGCGACATCCG
 A L A C L A T G R R F I G C D I R
 2361 GGAGGCGCAGTGCAGGCGCGCGCGAGCTCTCGCAGGTACTCCCTT
 BssHII
 E A Q C E A A A R E L S Q V L P
 2411 TGGGGTTCGCGCCCTGACCTCACCTGGGGGTAGATGAGGCGCCTTGA
 L G F G A -
 2461 CTATGCGTCAGCCATGACGCATAGTGTGGCCATAGCGCGCGCTTCAG
 2511 CCGCACTCAAGTGGGGTTGCCATGCAGTTTTTCATTGCCGTGATGGAAG
 2561 ACAACGACTCTGTGCCGACCGCTACGCCATCGACGCGGTACGGAAGAC
 2611 GCTGCGCGCAAGAAGCGGAGCGCTGGCTCCAGCTACCCGCTGGGCGG
 2661 TGGTGGCGTCACGTTGTACCGGGGCACTCCAACGACGAG

FIG. 4. Nucleotide sequence of the *mox* gene. Coordinates are those of the 8.1-kb fragment of Mx8⁺ DNA cloned in pAY50 (GenBank no. U64984). The deduced amino acid sequence of its product is shown below the nucleotide sequence. Selected restriction sites are indicated. The Shine-Dalgarno sequence is double-underlined. A potential stem-loop structure that may be a transcriptional terminator distal to the coding region is single-underlined.

Genomic DNA isolated from the nonlysogenic host, *M. xanthus* DZ1, is cut to a similar extent with *Xho*I and *Stu*I. DNA isolated from the defective lysogen DZ1(pAY50) is protected partially from cleavage with *Xho*I but not from cleavage with *Stu*I. This result shows that plasmid pAY50 expresses the Mox methylase when integrated as a single-copy defective prophage.

DNA isolated from DZ1 and DZ1(pAY442) is cleaved with both *Xho*I and *Stu*I. Thus, an amber mutation in the *mox* gene on plasmid pAY442 abolishes modification in vivo. Similar results were obtained with DNA extracted from a lysogen of DZ1 with Mx8 *del*1 as prophage (data not shown). DNA isolated from Mx8⁺ particles is also resistant to cleavage with *Xho*I but not with *Stu*I. These results show that the *mox* gene is expressed from a promoter that is active during both the lysogenic state and the lytic development of Mx8.

The *mox* gene is the only phage gene required for methylase activity. To show that the *mox* gene is the only phage gene required for methylase activity, we expressed *mox* as part of the constitutive *M. xanthus* *mgl* operon (8). PCR was used to amplify the *mgl* operon, which was cloned into Km^r plasmid vector pBGS18 to introduce a unique *Acc*65I site overlapping the 3' end of the promoter-distal *mglA* gene, near a unique *Eco*RI site in the vector polylinker sequence, and make plasmid pAY703. This plasmid was electroporated into *M. xanthus* DK6204. DK6204 carries a deletion mutation, Δ *mglBA*-6204, that removes sequence between *Bal*I sites within *mglB* and *mglA* and impairs both gliding motility and development (7). Km^r electroporants arise by homologous recombination between the *mglBA* promoter on plasmid pAY703 and the *mglBA* promoter upstream of the deletion in DK6204 and are mero-diploid for this region (Fig. 7a). When integrated into the genome of nonmotile host DK6204 (Δ *mglBA*-6204), plasmid pAY703 should make an MglA protein with the sequence Pro-Ser-Ser-Asn-Ser added to the C terminus. Because Km^r DK6204::pAY703 cointegrates are motile and undergo normal development (data not shown), we conclude that this altered MglA protein is expressed and functional.

We then added the *mox* ORF to pAY703, downstream of *mglA*, to make pAY720. Plasmid pAY720 has a TGA stop codon after the codon for an additional Pro residue at the end of *mglA*. Again, the integration of plasmid pAY720 into the DK6204 genome restores gliding motility. These results show that two different modifications of the C terminus of MglA do not interfere with its function. In plasmid pAY720, the ATG start codon of *mox* is positioned 12 bp downstream of the TGA stop codon of *mglA* and 7 bp downstream of its natural Shine and Dalgarno sequence, AGGAGG, which overlaps the TGA stop codon of *mglA* by 1 bp. As shown in Fig. 7b, the mero-diploid carrying the *mox* gene also expresses Mox methylase, because genomic DNA extracted from DK6204::pAY720 is (at least partially) resistant to cleavage with *Xho*I.

What is the physiological role of Mox methylase? To understand why myxophage Mx8 encodes a site-specific methylase, we explored four potential roles of this methylase in the physiology of the phage. First, because DNA methylases are often genetically linked with their cognate restriction endonucleases, we asked whether Mox might be a part of a phage-encoded restriction-modification system, like the coliphage P1-encoded Mod methylase (16, 19). We were unable to detect endonuclease activity in cell-free extracts prepared from *M. xanthus* DK883, the original source of Mx8, or from extracts prepared from a lysogen of host DZ1 carrying wild-type Mx8 as prophage with DNA prepared from Mx8 *del*1 or coliphage λ particles as substrates. Furthermore, we were unable to detect endonuclease activity in extracts prepared from a second nat-

FIG. 5. The predicted amino acid sequence of Mox has two regions similar to other DNA adenine methylases. Comparison of Mox with adenine methylases from *C. crescentus* (CcrM), *Moraxella bovis* (M.*Mbo*II), and *Streptococcus pneumoniae* (M.*Dpn*II) is shown. (a) The conserved amino-terminal motif (DPPY) is involved in S-adenosylmethionine binding (11, 29). (b) The conserved carboxyl-terminal consensus sequence, FXGXG, is found in both type II and type III adenine methylases (11, 29). Deduced amino acid sequences were aligned by inspection. Identical residues are indicated in bold and by asterisks, and similar residues are indicated by carets below the four sequences.

ural isolate of *M. xanthus*, DK893, which is the source of related phage Mx82. Although DK893 was isolated from a source quite distant geographically from the source for DK883 (13), Mx8 and Mx82 DNAs have identical cleavage patterns with restriction endonucleases *Eco*RI, *Pvu*II, *Sau*3aI, *Sma*I, *Mlu*I, and *Ava*II and are resistant to cleavage with both *Xho*I and *Pst*I, suggesting that they are independent sources of the same prophage which makes Mox. An additional line of evidence supports the hypothesis that Mox is not part of a phage-encoded restriction-modification system. Mutations which inactivate the *mod* gene of coliphage P1, linked with its phage-encoded restriction endonuclease, confer a clear-plaque phenotype (16, 19). In contrast, phage Mx8 *del*1 forms turbid plaques and stable lysogens of both DZ1 and DK6204 (data not shown).

A second hypothesis is that because the *mox* gene is near the *imm* gene, which encodes a primary determinant of superinfection immunity (unpublished results), *mox* might be involved in superinfection immunity. For example, Mox might methyl-

ate either promoter or operator sites involved in the expression of *imm* or in the expression of lytic phage genes controlled by *imm*. To test whether *mox* is involved in superinfection immunity, we measured the EOP for Mx8⁺⁺ and Mx8 *vir*1 phage on *M. xanthus* DZ1 and defective lysogens DZ1(pAY50) and DZ1(pAY442). The EOPs for both phages on otherwise isogenic *mox*⁺ and *mox* mutant strains are identical (1.0 for wild type and 10⁻⁵ for *vir*1), showing that the *mox* gene is not involved in superinfection immunity. Also, the finding that Mx8 *del*1 phage forms turbid plaques and stable lysogens argues against this hypothesis.

The third hypothesis is that because Mox is most closely related to M-CcrI, an adenine methylase involved in the cell cycle control of the G+C-rich gram-negative bacterium *Caulobacter crescentus*, Mox might act in a developmental role. Unlike many other gram-negative hosts for phages, *M. xanthus* can undergo a complex program of multicellular development. Stellwag et al. (24) have shown that Mx8 lysogens are stable

FIG. 6. The *mox* gene is necessary for methylase activity. The engineered *mox*-am68 mutation prevents methylation of *M. xanthus* DZ1 DNA in vivo. Genomic DNA was prepared from *M. xanthus* DZ1, the defective lysogens DZ1(pAY50) and DZ1(pAY442), and phage Mx8⁺⁺. As an internal control, supercoiled plasmid pLITMUS28 was added to each genomic DNA preparation. Sample treatments include untreated DNA (U), *Xho*I-treated DNA (X), and *Sma*I-treated DNA (S). The arrow indicates that a substantial fraction of the genomic DNA extracted from the *mox*⁺ DZ1(pAY50) host and cleaved with *Xho*I migrates at the excluded size limit of the gel; in contrast, no detectable DNA extracted from the otherwise isogenic *mox* mutant DZ1(pAY442) host and cleaved with *Xho*I migrates at this position.

FIG. 7. The *mox* gene is the only phage gene required for methylase activity. (a) Genetic structures of the *mgl* locus in *M. xanthus* strains DK1622, DK6204, DK6204::pAY703, and DK6204::pAY720. *M. xanthus* DK1622 is the wild type, and DK6204 has a deletion (indicated B'/A) extending rightward from the *BalI* site within *mglB* to the *BalI* site within *mglA* (7). Strains DK6204::pAY703 and DK6204::pAY720 are Km^r merodiploids, with plasmids integrated by homologous recombination at the *mgl* locus. Strain DK6204::pAY720 expresses *mox* under the control of the *mgl* promoter. (b) Genomic DNA was prepared from *M. xanthus* strains DK6204, DK6204::pAY703, and DK6204::pAY720 and phages Mx8⁺⁺ and Mx8 *del1*. DNA was either left untreated (U) or cleaved with *XhoI* (X). As an internal control, to ensure complete digestion with *XhoI*, supercoiled plasmid pLITMUS28 DNA was added to each genomic DNA preparation prior to cleavage. The arrow indicates that a substantial fraction of the genomic DNA extracted from the *mox*⁺ DK6204::pAY720 host and cleaved with *XhoI* migrates at the excluded size limit of the gel; in contrast, no detectable DNA extracted from the otherwise isogenic *mox* mutant DK6204::pAY703 host and cleaved with *XhoI* migrates at this position.

throughout the developmental cycle, despite observations that development involves a cascade of gene expression involving the recognition of a series of development-specific promoters by a series of development-specific sigma factors of RNA polymerase, as is the case for the development of *B. subtilis*. Therefore, we investigated whether Mox was required for prophage stability throughout the developmental cycle in two different ways, by testing whether the defective prophages in the otherwise isogenic *mox*⁺ and *mox* mutant strains DK1622(pAY50) and DK1622(pAY442) were stable upon development. Each of these strains was grown to exponential density, starved for nutrients, and assayed for the ability to produce heat-resistant spores in the absence of selection for Km^r. Both strains yield a wild-type complement of spores; these spores yield Km^r vegetative cells upon germination (data not shown). In a similar way, we tested whether the ectopic expression of *mox* could interfere with development. Again, strain DK6204::pAY720, which expresses *mox* from the *mgl* promoter, yields a wild-type complement of heat-resistant spores when subjected to the stress of starvation, as does the control strain DK6204::pAY703.

Finally, we considered the possibility that Mx8 encodes Mox

to protect infecting phage DNA from restriction by a subset of natural *Myxococcus* hosts. This possibility seemed particularly attractive because two species closely related to *M. xanthus*, *Myxococcus stipitatus* and *Myxococcus virescens*, have been shown to produce endonuclease activities (13a, 14). Cell-free extracts were prepared from cultures of *Myxococcus fulvus*, *M. virescens*, and 10 independent natural isolates of *M. xanthus* and screened for endonuclease activity. As shown in Fig. 8, only one of these strains, DK879, makes an endonuclease activity detectable by our assay. This natural isolate is the lysogenic source of phage Mx81, which is related serologically to Mx8 (13). However, this endonuclease activity appears to recognize the target sequence TTCGAA, a specificity which does not overlap that of Mox methylase. As yet, we can assign no necessary physiological role to Mox.

DISCUSSION

We have shown that adenines in the DNA sequences CTC GAG and CTGCAG, recognition sites for endonucleases *XhoI* and *PstI*, respectively, are methylated on the *M. xanthus* phage Mx8 genome. We have identified the gene encoding the DNA

FIG. 8. The natural isolate of *M. xanthus*, DK879, makes an isoschizomer of endonuclease *BstBI*. Ten independent natural isolates of *M. xanthus* were analyzed for endonuclease activity, as described in Materials and Methods. Isolates from Larry Shimkets are MX1012Y, LS-2002, LS-2008, DK853, DK862, and DK870; DK816, DK879, DK883, and DK893 are from Dale Kaiser. Incubation of a cell-free extract from *M. xanthus* DK879 with phage λ DNA yields a pattern of products with sizes identical to those made with endonuclease *BstBI*; we designated the endonuclease activity in this extract *MxaI*. The cleavage products of *Mx8⁺⁺* DNA with *MxaI* and *BstBI* are also similar.

methyltransferase, *mox*, and shown that this phage gene is both necessary and sufficient for DNA modification. The *mox* gene encodes a nonessential function that has no effect on lytic phage growth, superinfection immunity, or the stability of lysogens during the complex multicellular developmental cycle of *M. xanthus*. Although we could assign no necessary physiological role to Mox methylase, we speculate that Mx8 may make Mox to protect infecting phage DNA from restriction by a subset of natural *Myxococcus* hosts. Consistent with this hypothesis, we have shown that at least one natural isolate of *M. xanthus* makes a restriction endonuclease. This strain, DK879, carries the related phage Mx81 as prophage. Perhaps other *M. xanthus* hosts encode endonucleases with targets that are blocked by Mox modification.

The target specificity of Mox methylase appears to be unique among known adenine methylases. Mox methylates $0.60\% \pm 0.36\%$ of the adenines in *Mx8⁺⁺* DNA. If we assume that the G+C content of Mx8 DNA, like that of the 8.1-kb subfragment of its genome that we have sequenced, is 69.2% and the length of the genome is 49 kbp (as we have determined by restriction endonuclease digestion), then we would predict that 91 ± 54 adenines are methylated on the Mx8 genome. Cleavage of Mx8 *delI* DNA with *XhoI* and *PstI* yields 7 and 8 large (>400-bp) restriction fragments, respectively. DNA sequence analysis of the 8.1-kbp region of Mx8 DNA subcloned in plasmid pAY50 and of a subfragment of this region with the *delI* deletion mutation shows that *delI* covers one additional *XhoI* site and that two *XhoI* sites are located 30 bp from one another in the region upstream of *mox*. Thus, there are at least 17 *XhoI* plus *PstI* sites on the Mx8 genome. If we assume that both adenines in each of these sites are methylated, these sites account for only 34 AT bp with methylated adenines. This number of sites,

17, is less than the range of 45 ± 27 sites measured by our HPLC analyses. Cleavage of *Mx8⁺⁺* DNA with endonuclease *BpmI*, which recognizes the asymmetric sequence CTGGAG, yields about 17 large fragments (data not shown). If adenines in these sites also are methylated, then at least an additional 34 adenines should be modified. Thus, the predicted specificity of Mox which best agrees with our data is that Mox recognizes both symmetric and asymmetric sites with the sequence CTSSAG.

To show that the sequenced ORF that we call *mox* encodes this adenine methylase, we introduced an amber mutation within the *mox* gene. The amber mutation results in a loss of function. This result indicates that amber mutations are non-sense mutations in *M. xanthus*, which is indeed the case. The introduction of two different amber mutations into the *mglA* gene abolishes gliding motility (unpublished results), and at least one *M. xanthus* gene, *icl*, which encodes isocitrate lyase, terminates with an amber codon (GenBank no. U81372).

To show that the *mox* ORF is the only phage gene required for methylase activity, we expressed *mox* as part of the *mglBA* operon and found that the low-level constitutive expression of *mox* in single copy from the *mgl* promoter results in partial methylation of the *M. xanthus* chromosome. This result shows that a gene may be expressed ectopically at a low, constitutive level as part of the *mgl* operon.

In conclusion, we have taken the first step in the construction of plaque-forming specialized transducing derivatives of phage Mx8. We have identified a nonessential region of the Mx8 genome, which includes *mox*, that may be deleted to introduce small insertions into this genome without the loss of terminal repetition.

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