Genetics in Methanogens: Transposon Insertion Mutagenesis of a *Methanococcus maripaludis* nifH Gene

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We designed a transposon insertion mutagenesis system for *Methanococcus* species and used it to make mutations in and around a nifH gene in *Methanococcus maripaludis*. The transposon Mudpur was constructed with a gene for puromycin resistance that is expressible in *Methanococcus* species. A 15.6-kb nifH region from *M. maripaludis* cloned in a λ vector was used as a target for mutagenesis. A series of 19 independent Mudpur insertions spanning the cloned region were produced. Four mutagenized clones in and around nifH were introduced by transformation into *M. maripaludis*, where each was found to replace wild-type genomic DNA with the corresponding transposon-mutagenized DNA. Wild-type *M. maripaludis* and a transformant containing a Mudpur insertion upstream of nifH grew on N₂ as a nitrogen source. Two transformants with insertions in nifH and one transformant with an insertion downstream of nifH did not grow on N₂. The transposon insertion-gene replacement technique should be generally applicable in the methanococci for studying the effects of genetic manipulations in vivo.

Genetic approaches to the study of methanogenic Archaea are becoming feasible because of the development of methods for genetic transformation and selection in *Methanococcus* species (3, 6, 10, 14). In both *Methanococcus voltae* (6) and *Methanococcus maripaludis* (12), a puromycin resistance gene can be introduced by transformation. The resistance gene is maintained after integration into the genome by recombination events that are facilitated by the presence of genomic fragments in the introduced DNA. These developments make it possible to produce mutations in cloned genes and to observe the effects in vivo. We have developed a transposon insertion mutagenesis technique that builds upon these advances and have used it to test the effects of mutations in and around a nifH gene of *M. maripaludis*.

MATERIALS AND METHODS

Growth of bacteria. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were maintained at 30°C (MH132 and P2392) or 37°C (DHSaF) in Luria broth (LB) (9) or NZY (0.5% NaCl, 0.2% MgSO₄, 0.5% yeast extract, 1% Casamino Acids [pH 7.5]). Ampicillin and chloramphenicol were used at a concentration of 25 μg/ml unless otherwise stated. The techniques used for growing methanogens were those of Balch et al. (2). *M. maripaludis* was grown at 30°C in medium number 3 (2) with the following modifications. Vitamins, sodium acetate, yeast extract, and Trypticas were omitted. The trace mineral solution was supplemented with NiCl₂·6H₂O (0.025 g/liter), NaSeO₃ (0.2 g/liter), and Na₂WO₄·2H₂O (0.1 g/liter), and the amount of Na₃C₆H₅O₇·2H₂O was increased to 0.1 g/liter as described in reference 15. For maintenance of *M. maripaludis* strains containing Mudpur, puromycin was added to 2.5 μg/ml. Nitrogen-free medium was further modified so that all forms of combined nitrogen were lacking. Fe(NH₄)₂(SO₄)₂ was replaced by FeSO₄·7H₂O (0.01 g/liter), and NH₄Cl and cysteine were omitted. In the trace mineral solution, nitritotriacetic acid was replaced by Na₃citrate·2H₂O (2.1 g/liter). V(III)Cl, (0.01 g/liter) was added, and Na₂WO₄·2H₂O was omitted. Glassware was acid washed in 1 N HCl and rubber stoppers were boiled in 1 N NaOH.

Molecular techniques. Standard protocols (1) were used. Oligonucleotide probes were labeled with [γ-³²P]ATP with T4 polynucleotide kinase and then separated from the unincorporated nucleotides with a Sephadex G-25 spin column. Larger probes were prepared with [α-³²P]dATP with a random label kit (Boehringer Mannheim). A λ library of *M. maripaludis* genomic DNA was constructed by K. Sandbeck in the BamHI site of λDASHI (Stratagene). Hybridization against the λ library was performed with plaques bound to nitrocellulose filters. Southern analysis of DNA digests was accomplished by transfer of the DNA onto a Nitran membrane (Schleicher & Schuell). Filters were prehybridized for at least 4 h at 50°C in 4× SSC (0.15 M NaCl plus 0.015 M sodium citrate−100 mM Tris-HCl (pH 7.4)−0.5% sodium dodecyl sulfate (SDS)−2× Denhardt’s solution). The hybridization was carried out with the same solution with the addition of approximately 2.0 × 10⁶ cpm of labeled probe per ml for more than 16 h at 50°C. The filters were washed at 50°C with 2× SSC−0.1% SDS three times for 20 min each. Autoradiography was done with either X-ray film (Kodak) or with PhosphorImager screens (Molecular Dynamics).

Isolation of the MmpA-1 clone. An oligonucleotide, designated nifHR1 (5′-CCA CCG/A CAT ACA ACG TCC CC 3′), was designed as a nifH-specific probe with the DNA sequences of nifH1 and nifH2 from *Methanococcus* thermolithotrophicus and nifH2 from *M. voltae*. The oligonucleotide was end labeled and used to probe the *M. maripaludis* λ genomic library. Hybridizing λ clones were isolated and then reprobed to confirm the signal. From the 15.6-kb MmpA-1 clone, a 9.7-kb XbaI fragment was subcloned into pBluescript to give pMMP1.

Sequencing and phylogenetic analysis. DNA sequencing was carried out on both strands with either the Sequenase kit (United States Biochemical Corp.) or the SequiTHERM cycle sequencing kit (Epiconcept Technologies) according to the protocols provided. nifH was sequenced on both strands from plasmid pMP1 with the nifHR1 oligonucleotide, vector sequences, or internal sequences as primers. Sequence analysis was carried out with the Sequence Analysis Package of the University of Wisconsin’s Genetics Computer Group. Phylogenetic analysis of nifH sequences was done with programs from the Phylip 3.5c phylogenetic inference suite (5).

Construction of Mudpur. The miniMu derivative on plasmid pPR3 (11) contains the chloramphenicol acetyltransferase and the neomycin phosphotransferase genes between the left and right ends of Mu. The neomycin phosphotransferase gene was removed by digestion of pPR3 with BamHI and religation of the sticky ends with T4 DNA ligase, forming pCB101. The puromycin transacetylase gene cassette was excised from plasmid Mpl1 (6) with EcoRI and then cloned into the EcoRI site of pBluescript KS, creating pBluePUR. The puromycin transacetylase cassette was then removed from pBluePUR by digestion with PvuII and blunt end ligated into the HinII-Sall site of pCB101, forming the transposon Mudpur on the plasmid pMP1 (Fig. 1).

Transposon insertion. *E. coli* MH132 containing pMudpur was grown at 30°C in LB with 10 mM MgSO₄, 0.2% maltose, ampicillin, and chloramphenicol. One to two milliliters of the culture was infected with 10⁶ PFU of MmpA-1 for 20 min without shaking at room temperature. The culture was mixed with 50 ml of prewarmed (42°C) LB with chloramphenicol. The culture was shaken gently at 42°C for 20 min to induce transposition and then was transferred to 37°C and shaken for 3 to 24 h to obtain lysates. Chloroform (1 ml) was added, and cellular debris was pelleted at 6,000 × g for 10 min. The supernatant (transposition lysate) was kept at 4°C in the presence of chloroform.

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Phage with transposon insertions was obtained by a selective plaque assay (8). An overnight culture (0.2 ml) of P2392 cells grown at 30°C in LB with 10 mM MgSO₄ and 0.2% maltose was infected with 10⁹ total phage from the transposon insertion lysate and incubated at room temperature for 20 min without shaking. 

**RESULTS AND DISCUSSION**

Our goal was to develop a system by which any cloned gene of *M. maripaludis* could be mutagenized by transposon insertion and reintroduced into the *M. maripaludis* genome. A useful method should produce insertions that are randomly dis-
sponding to the nifHR1 oligonucleotide probe is shown.

Insertions 1 through 27 were obtained from one transposition lysate, and 29 through 41 were obtained from another. The location of the sequence corresponding to the nifHR1 oligonucleotide probe is shown.

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We designed the system to be used on M. maripaludis genomic fragments cloned into a λ vector and used it to test the functions of a nifH gene and its adjoining sequences.

Cloning, sequencing, and phylogeny of nifH. Using an oligonucleotide probe for nifH, we screened 2,000 plaques and isolated a single positive clone from a λ bank of M. maripaludis DNA. This clone is designated Mmpλ-1 and has an insert with a length of 15.6 kb. A partial restriction map is shown in Fig. 2. The nifH gene was located on this map by a combination of restriction analysis, Southern hybridization with the oligonucleotide probe, and sequencing. The entire nifH gene was sequenced (GenBank accession number U23068). Phylogenetic analysis of the M. maripaludis nifH gene and 36 other genes from Bacteria species and methanogenic Archaea was done by parsimony and distance matrix methods. Both methods gave essentially the same results, which agreed with those from a recent similar analysis (4). The M. maripaludis gene was in the same cluster with several other methanogen genes that have been designated nifH1 and are thought to encode functional nitrogen reductases.

Transposon insertion into Mmpλ-1. The puromycin resistance gene had previously been cloned between the M. voltae methyl reductase promoter and terminator and had been demonstrated to confer puromycin resistance on M. voltae (6) and M. maripaludis (12). By placing the puromycin resistance cassette into a Mud transposon, we obtained a derivative designated Mudpur (Fig. 1) that contains a puromycin resistance marker for selection in Methanococcus species and a chloramphenicol resistance marker for selection in E. coli. Insertions of Mudpur into Mmpλ-1 were obtained as described in Materials and Methods. The procedure produced transposition frequencies of 1.4 × 10^-6 to 2 × 10^-7 recombinant phage per PFU. When the length of transposition induction (42°C treatment) was increased from 20 to 40 or 60 min, a lower phage titer resulted. This may be due to an increased number of insertions in genes essential for lytic functions.

A collection of chloramphenicol-resistant plaques, each representing a putative Mudpur insertion into Mmpλ-1, were picked for further study. The plaques came from either of two original transposition lysates. Plaques were purified, DNA was obtained, and the locations of the transposon insertions were mapped by restriction analysis. Each sample contained a single insertion. Out of 29 mapped insertions, 19 unique sites were identified (Fig. 2). The insertions were distributed throughout the Mmpλ-1 insert, indicating a degree of randomness in insertion targets. No insertions were found in the λ arms, presumably because they would destroy the lytic activity of the phage.

Introduction of nifH::Mudpur insertions into M. maripaludis. Circular DNA containing the appropriate features (a selectable marker and a region of homology with the genome), when introduced into M. voltae or M. maripaludis, usually inserts into the genome by a single homologous recombination event (6, 12). We predicted that in the case of our transposon-mutagenized λ clones, the insert would replace the wild-type locus by a double homologous recombination event, because the DNA is linear and has relatively long stretches of genomic DNA flanking the selectable marker in the transposon. We tested this with clones Mmpλ-1-33, Mmpλ-1-20, Mmpλ-1-18, and Mmpλ-1-29, which contain all of insertions determined by restriction mapping (Fig. 2). Puromycin-resistant transformants, designated Mm33, Mm20, Mm18, and Mm29, respectively, were obtained. Genomic DNA of wild-type M. maripaludis and the four transformants was isolated, each digested separately with PvuII and EcoRI, run on a gel, and probed with the nifHR1 oligonucleotide. In each case, a single hybridizing band was observed (Fig. 3). Mobility shifts corresponding to those one would expect from simple Mudpur insertions in the locations determined by restriction mapping (Fig. 2). (All four insertions had been determined by restriction mapping to lie in the same orientation, i.e., Mu RE to the left.) Similar results were obtained from experiments in which genomic digests were probed with the Pur gene cassette. In some cases, several transformants (different puromycin-resistant colonies) from a given transformation were analyzed, and all gave the same-sized hybridization band. Genomic digests of the transformants were also probed with λ DNA, and no hybridization was seen. These results indicate that in the transformants, the wild-type nifH region had been replaced by DNA containing the
transposon insertions by double homologous recombination events.

Nif phenotypes. The nitrogen-fixing abilities of wild-type M. maripaludis and the four transformants were determined by monitoring growth on N₂. (Acetylene toxicity makes the acetylene reduction assay difficult in methanogens.) N₂-dependent growth was considered to be growth that occurred under a N₂-H₂-CO₂ atmosphere relative to that of an Ar-H₂-CO₂ control. Cultures in which NH₄⁺ had been added were used to confirm that non-N₂-dependent growth was normal. Growth curves are shown for wild-type M. maripaludis (Fig. 4A) and for a representative mutant, Mm18 (Fig. 4B). No growth occurred in either culture in argon controls when neither N₂ nor NH₄⁺ was present. Growth occurred reproducibly on N₂ in the wild-type culture (Nif⁺ phenotype), while Mm18 did not grow on N₂ (Nif⁻ phenotype). Neither the gas phase nor the mutation affected growth when NH₄⁺ was present. Growth end points are shown for wild-type M. maripaludis and all four mutants (Fig. 5A). Wild-type M. maripaludis and Mm33 were Nif⁺, while Mm20, Mm18, and Mm29 were Nif⁻. Total protein determinations confirmed the results of OD measurements (Fig. 5B). Thus, transposon insertions that mapped within or immediately downstream from nifH disrupted nitrogen fixation, while insertion 33, mapping upstream, did not.

In summary, we have constructed and tested a system that allows one to obtain transposon insertion mutants of M. maripaludis from genes contained in a λ bank of M. maripaludis DNA. The system should work as well for M. voltae and may be adaptable to mutagenesis of the entire genome by insertion into a gene library en masse followed by transformation.

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REFERENCES

5. Felsenstein, J. 1993. PHYLIP (Phylogenetic Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle. (Distributed by author.)

FIG. 4. Growth curves showing Nif phenotypes of wild-type M. maripaludis (A) and mutant Mm18 (B). Values are averages of four replicate cultures. Error bars represent 1 standard deviation.

FIG. 5. Final OD₆₆₀ (A) and protein concentrations (B) showing Nif phenotypes of wild-type [wt] M. maripaludis and mutants. Measurements were made after 135 h of incubation. Values are averages of four replicate cultures. Error bars represent 1 standard deviation.


