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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 expressed and selectable 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 N_2 as a nitrogen source. Two transformants with insertions in *nifH* and one transformant with an insertion downstream of *nifH* did not grow on N_2. 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 (6) 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 (DH5αF) in Luria broth (LB [9]) or NZY (0.5% NaCl, 0.2% $MgSO_4$, 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 Trypticase were omitted. The trace mineral solution was supplemented with $NiCl_2 \cdot 6H_2O$ (0.025 g/liter), $NaSeO_3$ (0.2 g/liter), and $Na_2WO_4 \cdot 2H_2O$ (0.1 g/liter), and the amount of $Na_3MoO_4 \cdot 2H_2O$ 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. $Fe(NH_4)_2(SO_4)_2$ was replaced by $FeSO_4 \cdot 7H_2O$ (0.01 g/liter), and NH_4Cl and cysteine were omitted. In the trace mineral solution, nitrilotriacetic acid was replaced by Na_3 citrate $\cdot 2H_2O$ (2.1 g/liter), V(III) Cl_3 (0.01 g/liter) was added, and $Na_2WO_4 \cdot 2H_2O$ 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-^{32}P]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-^{32}P]dATP with a random label kit (Boehringer Mannheim). A λ library of *M. maripaludis* genomic DNA was constructed by K. Sandbeck in the *Bam*HI site of λ-DASHII (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 Nytran membrane (Schleicher & Schuell). Filters were prehybridized for at least 4 h at 50°C in 4× SSC (1× SSC is 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 2.0×10^6 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 Mmpλ-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 Mmpλ-1 clone, a 9.7-kb *Xba*I 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 (Epicentre Technologies) according to the protocols provided. *nifH* was sequenced on both strands from plasmid pMMP1 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 analyses of *nifH* sequences were done with programs from the Phylip3.5c 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 *Bam*HI and religation of the sticky ends with T4 DNA ligase, forming pCB101. The puromycin transacetylase gene cassette was excised from plasmid Mip1 (6) with *Eco*RI and then cloned into the *Eco*RI site of pBluescript KS, creating pBluePur. The puromycin transacetylase cassette was then removed from pBluePur by digestion with *Pvu*II and blunt end ligated into the *Hinc*II-*Sal*I site of pCB101, forming the transposon Mudpur on the plasmid pMudpur (Fig. 1).

Transposon insertion. *E. coli* MH132 containing pMudpur was grown at 30°C in LB with 10 mM $MgSO_4$, 0.2% maltose, ampicillin, and chloramphenicol. One to two milliliters of the culture was infected with 10^9 PFU of Mmpλ-1 for 20 min without shaking at room temperature and then transferred to a flask containing 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 lysis. 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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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Characteristics	Source or reference
E. coli strains		
MH132	F$^+$ *araD* Δ[*ara-leu*::{−Mu *c*61(Ts) Δ(*T-attR*)}]*132*	13
P2392	*hsdR514 hsdM supE44 supF58 lacYI* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* (P2)	Stratagene
DH5αF′	F′ ϕ*80d lacZ ΔM15* Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44*λ$^-$ *thi-1 gyrA relA1*	Gibco BRL
Plasmids		
pPR3	Contains MudIIPR3, Am^r Km^r Cm^r	11
pCB101	1.4-kb *Bam*HI fragment deleted from pPR3; Am^r Cm^r	This study
Mip1	Contains the *pac* (puromycin resistance) gene on an *Eco*RI cassette; Am^r	6
pBluescript KS$^-$ or KS$^+$	Cloning vector; Am^r	Stratagene
pBluePur	*pac* cassette in pBluescript KS; Am^r	This study
pMudpur	Contains transposon Mudpur; Am^r Cm^r	This study
pMMP1	9.6-kb *Xba*I fragment from Mmpλ-1 in pBluescript; Am^r	This study
Phages		
Mmpλ-1	*M. maripaludis* λ genomic library clone containing *nifH*	This study
Mmpλ-1-18	Mmpλ-1 *nifH18*::Mudpur Cm^r	This study
Mmpλ-1-20	Mmpλ-1 *nifH20*::Mudpur Cm^r	This study
Mmpλ-1-29	Mmpλ-1 Ω29::Mudpur Cm^r	This study
Mmpλ-1-33	Mmpλ-1 Ω33::Mudpur Cm^r	This study
M. maripaludis strains		
JJ	Wild type	7
Mm18	JJ *nifH18*::Mudpur (Pur^r)	This study
Mm20	JJ *nifH20*::Mudpur (Pur^r)	This study
Mm29	JJ Ω29::Mudpur (Pur^r)	This study
Mm33	JJ Ω33::Mudpur (Pur^r)	This study

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 $MgSO_4$ and 0.2% maltose was infected with 10^9 total phage from the transposition lysate and incubated at room temperature for 20 min without shaking. NZY-agarose (3 ml; NZY containing 0.7% agarose, melted, and cooled to 48°C) was added and poured onto an LB or NZY plate containing 10 μg of chloramphenicol per ml. Plates were incubated overnight at 37°C and then were incubated for 24 h at room temperature. Medium to large plaques were visible against a very faint lawn of P2392 cells. Phage was isolated by inserting the small end of a Pasteur pipette into the plaque and blowing the agar plug into SM (0.58% NaCl, 0.2% $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin), where phage was allowed to diffuse overnight. The phage suspension was streaked onto an NZY plate with chloramphenicol and an overlay of P2392 cells, and isolated plaques were obtained. Phage DNA was prepared from 10 ml of lysate with polyethylene glycol (1). The locations of the transposon insertions were determined by restriction mapping of the phage DNA with *Xba*I and *Sal*I. Ambiguities were resolved by digestion with *Not*I.

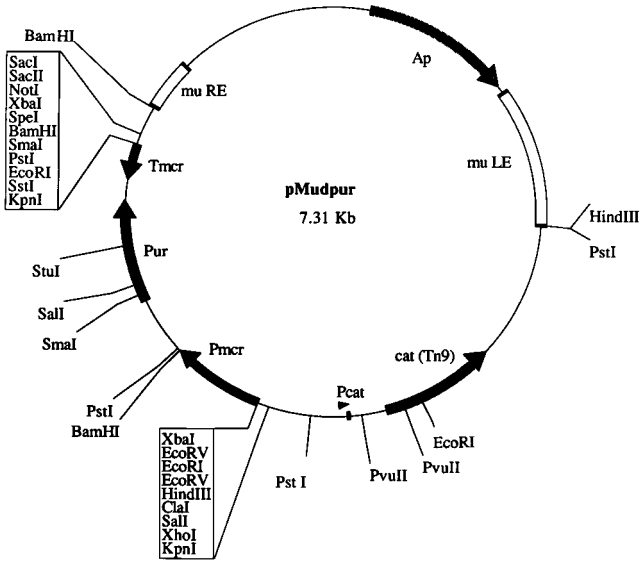

FIG. 1. Plasmid pMudpur containing the transposon Mudpur. Shown are the right (mu RE) and left (mu LE) ends of Mu, the puromycin resistance gene (Pur, puromycin transacetylase, or *pac*) flanked by the promoter (Pmcr) and terminator (Tmcr) from the *M. voltae mcr* (methylreductase) transcription unit, the chloramphenicol resistance gene (cat) with its promoter (Pcat), and the ampicillin resistance gene (Ap).

Transformation of *M. maripaludis* and isolation of genomic DNA. *M. maripaludis* was transformed with 7 μg of phage DNA with a recently developed polyethylene glycol-protoplast procedure (14). Transformants were plated with 2.5 μg of puromycin per ml as described previously (12, 14). Individual colonies were either streaked onto plates or inoculated into liquid medium with puromycin. Genomic DNA of *M. maripaludis* was harvested from 5 ml of liquid culture. Cells were pelleted in a microcentrifuge, lysis was obtained by resuspension of the cell pellet in TE (6), and DNA was purified with phenol-chloroform and precipitated with ethanol.

Determination of the Nif phenotype. Tubes with nitrogen-free medium were gas exchanged three times with 10 lb/in^2 of either N_2-CO_2 (80:20) or Ar-CO_2 (75:25) before autoclaving. One-tenth of a milliliter of each *M. maripaludis* strain (grown to an optical density at 660 nm [OD_{660}] of 0.4 in regular medium) was transferred to 5 ml of nitrogen-free medium. The carryover NH_4^+ from the inoculum was calculated to be 200 μM. As a control, some tubes were supplemented with 10 mM NH_4^+. After inoculation, tubes were gassed to 40 lb/in^2 with H_2-CO_2. Tubes were incubated at 30°C while lying stationary on their sides and were repressurized with H_2-CO_2 to 40 lb/in^2 after 66 and 111 h. The total protein content of cultures was determined by combining 50 μl of Bradford's reagent (Bio-Rad) with 0.2 ml of culture. A_{600} values were compared with those of known standards.

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-

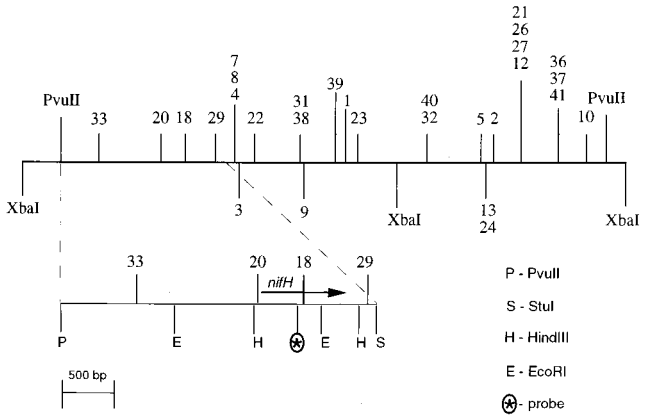

FIG. 2. Map of the Mmpλ-1 insert containing the cloned *M. maripaludis nifH* region. Locations of Mudpur insertions are shown by the numbers at the top. Insertions 1 through 27 were obtained from one transposition lysate, and 29 through 41 were obtained from another. The location of the sequence corresponding to the nifHR1 oligonucleotide probe is shown.

tributed, allow genomic replacement of wild-type DNA with mutagenized DNA, and yield the expected mutant phenotypes. 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 nitrogenase reductases.

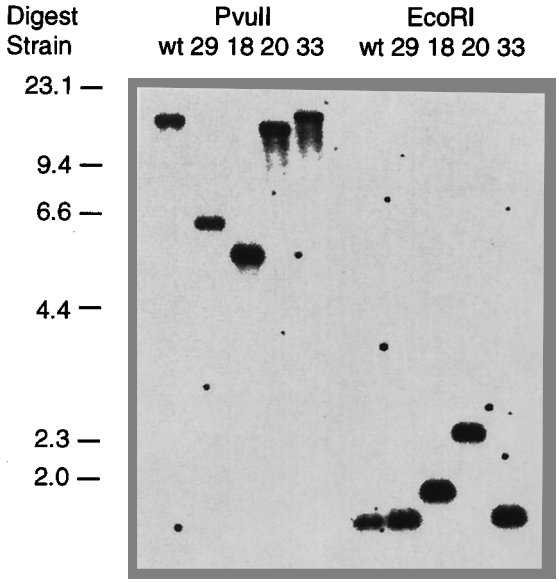

FIG. 3. Southern hybridization of the nifHR1 oligonucleotide to DNA digests from the *M. maripaludis* wild type (wt) and transformants.

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 in or around *nifH* (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 *Pvu*II and *Eco*RI, run on a gel, and probed with the nifHR1 oligonucleotide. In each case, a single hybridizing band was observed (Fig. 3). Mobility shifts corresponded 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

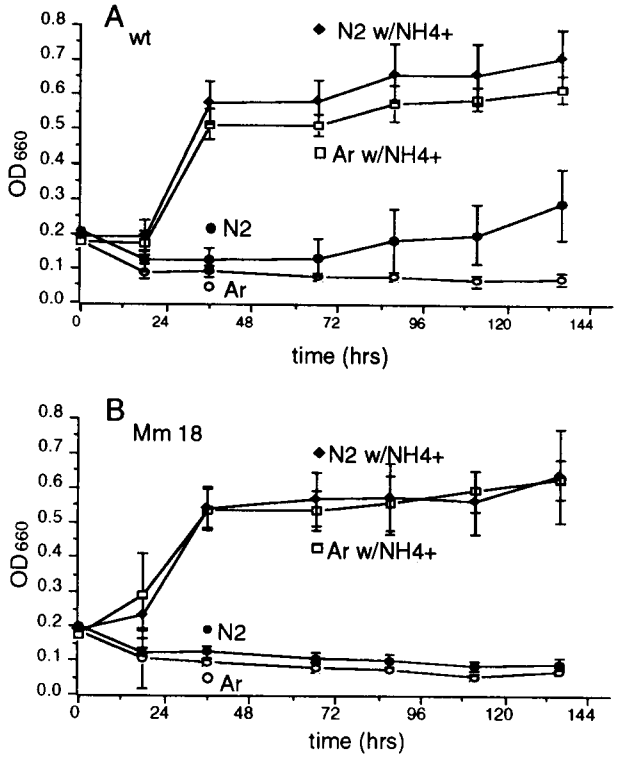

FIG. 4. Growth curves showing Nif phenotypes of wild-type *M. maripaludis* (A) and mutant Mm18 (B). Values are averages of four replicate cultures. Error bars represent 1 standard deviation.

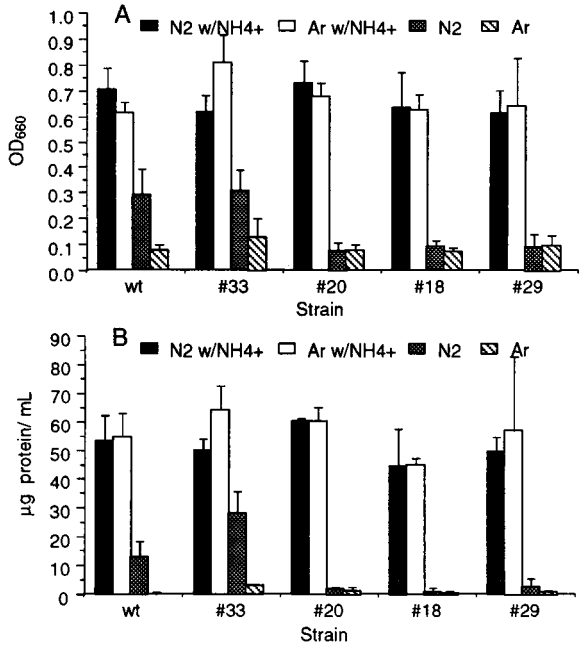

FIG. 5. Final OD_{660} (A) and protein concentrations (B) showing Nif phenotypes of wild-type (wt) *M. maripaludis* and mutants. Measurements were made after 135 h of incubation. Values are averages of four replicate cultures. Error bars represent 1 standard deviation.

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 N_2. (Acetylene toxicity makes the acetylene reduction assay difficult in methanogens.) N_2-dependent growth was considered to be growth that occurred under a N_2-H_2-CO_2 atmosphere relative to that of an Ar-H_2-CO_2 control. Cultures in which NH_4^+ had been added were used to confirm that non-N_2-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 N_2 nor NH_4^+ was present. Growth occurred reproducibly on N_2 in the wild-type culture (Nif^+ phenotype), while Mm18 did not grow on N_2 (Nif^- phenotype). Neither the gas phase nor the mutation affected growth when NH_4^+ 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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