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Molecular Construction and Characterization of *nif* Mutants of the Obligate Methanotroph *Methylosinus* sp. Strain 6

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We describe here a method for constructing mutants in bacteria that are not amenable to mutant isolation by conventional means. A one-step marker exchange procedure was used to construct nitrogen fixation (*nif*) mutants of the obligate methane-utilizing bacterium *Methylosinus* sp. strain 6, using transposon 5 (Tn*5*)-containing *nif* genes cloned into pBR325. The resultant mutants appeared to contain defects in *nif* structural genes, and DNA hybridization analysis showed that although one out of five had apparently been produced as a result of double-crossover homologous recombination, a variety of molecular events had led to the production of the other four mutants.

Obligate methane-oxidizing bacteria (methanotrophs) are bacteria with strong commercial potential in the area of biocatalysis (1). An important step in the realization of this potential is the development and application of genetic techniques. However, obligate methanotrophs are particularly difficult organisms with which to carry out genetic studies. They are characterized by relatively long generation times (6 to 30 h for most strains), they grow poorly on agar plates, and they have restricted metabolic capabilities, growing only on C_1 compounds. In addition, mutant isolation is especially difficult in methanotrophs. Mutation frequencies are low and are not increased by the application of a variety of mutagens, even though the cells are sensitive to these mutagens (8, 23; A. Toukdarian, unpublished data). Transposon mutagenesis is a possible alternative. However, our attempts to isolate mutants with a variety of transposon delivery vehicles have not been successful, apparently due to the low frequency of transposition (D. Nunn and M. Lidstrom, unpublished data). Finally, no generally useful gene transfer systems have been identified in methanotrophs. Broad-host-range (IncP1) conjugative plasmids have been transferred into several methanotrophs (9, 21), but it is not known whether they are capable of transferring genetic material.

The difficulties of carrying out genetic studies in methanotrophs can be largely circumvented by the application of recombinant DNA techniques. In particular, mutants can be constructed by a procedure called marker exchange (15, 16), in which transposon-containing cloned genes are exchanged with the corresponding DNA fragment in the chromosome by homologous recombination. This is normally accomplished in two steps: first, the transposon-bearing genes are transferred into the recipient cell, and then the vector is excluded with a conjugative, incompatible plasmid. Nitrogen fixation (*nif*) provides an ideal system for developing these mutant construction techniques in methanotrophs. The physiological conditions for nitrogen fixation have already been studied in some of these bacteria (4, 11, 19), and genetic probes are available that have allowed the molecular cloning of *nif*-specific DNA from a type II methanotroph, *Methylosinus* sp. strain 6 (19). These tools are prerequisites for the construction of mutants by marker exchange. However, we found that the drug resistance markers present on many conjugative plasmids used for marker exchange were not expressed in *Methylosinus* sp. strain 6; therefore, it was not possible to carry out the conventional two-step procedure. Instead, we have taken advantage of the fact that pBR325 is not maintained in *Methylosinus* sp. strain 6 to develop a one-step marker exchange procedure.

The bacterial strains, plasmids, and phages used in this study are listed in Table 1. Culture conditions, nitrogen fixation assays, sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis of proteins, *Escherichia coli* maxicell analysis, DNA isolation, restriction enzyme analysis, and hybridization conditions have all been described previously (4, 19). Two-dimensional sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis of proteins was performed by the procedure of O'Farrell (12), except that Triton X-100 was used in the first dimension instead of Nonidet P-40, isoelectric focusing gels were prefocused for 20 min each at 200, 300, and 400V, and second-dimension gels contained 12.5% acrylamide. Samples for two-dimensional electrophoresis were prepared from cell pellets obtained from the equivalent of 1 ml of a cell suspension with an optical density at 600 nm of 1.5. Cell pellets were stored at −70°C for a maximum of 1 month before use. Pellets were thawed and then suspended in 25 μl of sodium dodecyl sulfate buffer (0.05 M Tris-hydrochloride [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate, 20 mM $MgCl_2$ in 1.5-ml microcentrifuge tubes, placed in a boiling water bath for 5 min, and then cooled to room temperature. Fifty microliters of cell lysis buffer (9.5 M urea, 1.6% pH 5 to 7 ampholines [LKB Instruments Inc., Rockville, Md.], 0.4% pH 3.5 to 10 ampholines, 5% [vol/vol] β-mercaptoethanol, 8% [vol/vol] Triton X-100) was then added. The insoluble debris was removed by spinning for 10 s in a microcentrifuge, and the soluble material was loaded onto the isoelectric focusing gel.

The recombinant plasmid we used for marker exchange is pAT600, which consists of pBR325 containing a 2.3-kilobase (kb) *Hin*dIII fragment of *Methylosinus* sp. strain 6 DNA. Hybridization analysis has suggested that this fragment contains part or all of the *nifD* gene, and maxicell analysis

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Description	Source or reference
E. coli		
RR1	RecA$^+$ derivative of strain HB101	3
MM294	*pro endA1 hsdR17* (r_k^- m_k^+), *supE44 thi-1*	2
CSR603	*phr-1 recA1 uvrA6 supE44*	17
D6434	*supB*	22
Methylosinus sp.		This study
6	Wild type	
6E	Spontaneous Eth^r mutant	
6E-1A, 6E-1B, 6E-1C, and 6E-1D	Km^r Eth^r Nif$^-$ mutants isolated from marker exchange between strain 6E and pAT600::Tn*5*-1	
6E-10A, 6E-10B, 6E-10C, and 6E-10D	Km^r Eth^r Nif$^-$ mutants isolated from marker exchange between strain 6E and pAT600::Tn*5*-10	
6E-14A, 6E-14B, 6E-14C, 6E-14D, and 6E-14E	Km^r Eth^r Nif$^-$ mutants isolated from marker exchange between strain 6E and pAT600::Tn*5*-14	
6E-37A, 6E-37B, 6E-37C, and 6E-37D	Km^r Eth^r Nif$^-$ mutants isolated from marker exchange between strain 6E and pAT600::Tn*5*-37	
6-13C	Km^r Nif$^+$ mutant isolated from marker exchange between strain 6 and pAT600::Tn*5*-13	
Plasmids and phages		
pAT600	Ap^r Cm^r 2.3-kb *Hin*dIII fragment of strain 6 DNA in pBR325	19
pAT600::Tn*5*-1, -10, -14, -37, and -13	Ap^r Cm^r Km^r Tn*5* insertion derivatives of pAT600	This study
pBR325::Tn*5*	Tc^r Ap^r Cm^r Km^r	This study
RP4	Ap^r Tc^r Km^r IncP1 *tra*	5
pVK100	Tc^r Km^r IncP1 *rlx*	10
pRK2013	Km^r ColE1 with RK2 *tra*	6
λ::Tn*5*	*b*221 *rex*::Tn*5* *c*I857 *O*am29 *P*am80	22

has shown that it encodes two polypeptides of 57,000 and 34,000 molecular weight (19). pAT600 was mutagenized with transposon *5* (Tn*5*) as described by Weaver et al. (22), and ca. 2,000 to 5,000 Km^r colonies were obtained per plate. Plasmid DNA was isolated from colonies on four plates of each infection and used to transform competent *E. coli* RRI cells. Ap^r Km^r transformants were selected on LB agar containing 40 μg of ampicillin per ml and 20 μg of kanamycin per ml. Plasmid DNA was isolated from individual colonies, and the site of Tn*5* insertion was precisely mapped by restriction with *Bam*HI, *Bam*HI plus *Eco*RI, *Xho*I, and *Hin*dIII plus *Sst*I (Fig. 1).

A three-way filter mating procedure was used to mobilize the pAT600::Tn*5* derivatives (Fig. 1) into *Methylosinus* sp. strain 6 recipients. Mid-log-phase cultures of donor and recipient strains were mixed in a ratio of 1:1:5 and then filtered onto a prewetted 0.45-μm Millipore filter. The filter was washed with 10 ml of NMMS, moved to an NMMS agar plate supplemented with 0.1% NH_4Cl and 0.05% Casamino Acids, and incubated at 30°C under an atmosphere of 80% methane and 20% air for 48 h. Filters were then incubated under methane and air on an NMMS agar plate supplemented with 5 μg of kanamycin per ml. After 3 weeks, Km^r colonies of *Methylosinus* sp. strain 6 or 6E appeared on top of a background lawn at the frequencies shown in Table 2.

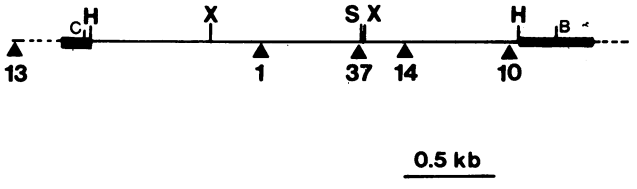

FIG. 1. Tn*5* insertions in pAT600. Map of the 2.3-kb *nif* insert of pAT600 showing the location of Tn*5* insertions 1, 37, 14, and 10. Tn*5*-13 is located in the pBR325 region of pAT600, approximately 1.6 kb from the *Cla*I site. Restriction sites shown are *Hin*dIII (H), *Xho*I (X), *Sst*I (S), *Bam*HI (B), and *Cla*I (C).

TABLE 2. Transfer frequency of various plasmids into *Methylosinus* sp. strains 6 and 6E

Donor	Frequency (no. of Km^r colonies per 10^9 recipient cells)[a]
None	0 (2)
E. coli MM294(pRK2013)	0 (1)
E. coli MM294(pRK2013) × *E. coli* RR1(pBR325::Tn*5*)	0 (4)
E. coli MM294(pRK2013) × *E. coli* RR1(pAT600::Tn*5*-1)	1–12 (5)
E. coli MM294(pRK2013) × *E. coli* RR1(pAT600::Tn*5*-10)	1.3–3.7 (6)
E. coli MM294(pRK2013) × *E. coli* RR1(pAT600::Tn*5*-14)	1.4–6 (3)
E. coli MM294(pRK2013) × *E. coli* RR1(pAT600::Tn*5*-37)	2.9–3.6 (2)
E. coli MM294(pRK2013) × *E. coli* RR1(pAT600::Tn*5*-13)	0.2 (3)[b]
E. coli J53(RP4)	31–286 (6)
E. coli MM294(pRK2013) × *E. coli* MM294(pVK100)	6–21 (4)

[a] The number of matings performed is shown in parentheses.
[b] Only one Km^r *Methylosinus* sp. strain 6 colony was obtained from all three matings.

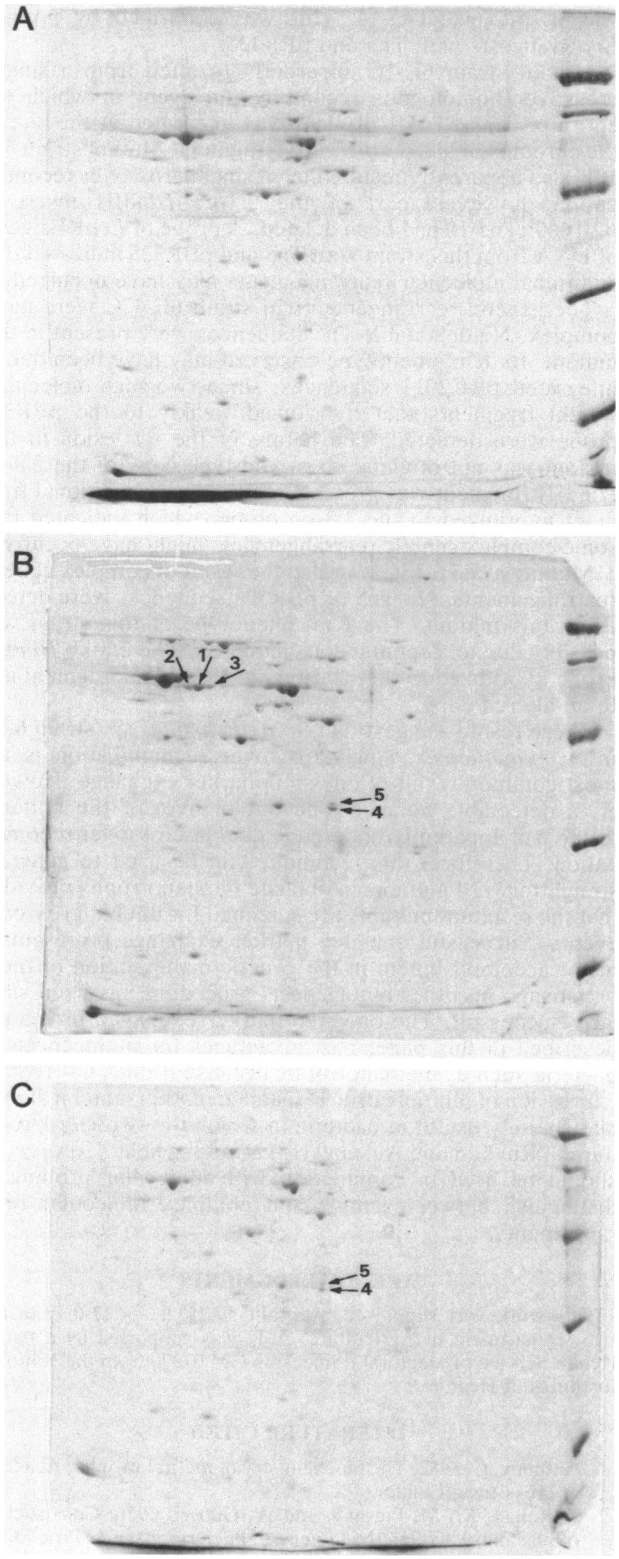

FIG. 2. Two-dimensional polyacrylamide protein gel electrophoresis of *Methylosinus* sp. strain 6 and Nif$^-$ mutants derepressed for *nif*. Cells were grown with (A) nitrate or (B) dinitrogen as the sole nitrogen source. (B) Arrows mark five NH_4^+-repressible polypeptides which were only present in cultures with acetylene reduction activity. When the eight Km^r Nif$^-$ mutants tested were grown under conditions which derepressed acetylene reduction activity in the parental strain, only two of the five polypeptides could be detected on gels. (C) Pattern for one of the mutants, strain 6E-14E.

The frequency of transfer of other plasmids which encode kanamycin resistance from *E. coli* to *Methylosinus* sp. strain 6 or 6E are also shown for comparison. The frequency of pBR325 mobilization by pRK2013 between *E. coli* strains was approximately 10^{-2} to 10^{-3}.

The Km^r colonies could have resulted from a number of events, including (i) double-crossover homologous recombination between the *nif* region contained in pAT600:Tn*5* and the chromosomal *nif* region, resulting in exchange of the Km^r marker; (ii) integration of the entire pAT600:Tn*5* plasmid into the chromosomal *nif* region via single-crossover homologous recombination; (iii) a nonhomologous recombination event between pAT600::Tn*5* and the *Methylosinus* sp. genome; (iv) transposition of Tn*5* from pAT600::Tn*5* into the *Methylosinus* sp. genome; (v) maintenance by integration or as independent replicons of either pRK2013 (which also encodes resistance to kanamycin) or pAT600::Tn*5* in *Methylosinus* sp. or (vi) from spontaneous resistance of *Methylosinus* sp. to kanamycin. The last three possibilities were not likely to account for the majority of the Km^r colonies, since the frequency of spontaneous kanamycin resistance and of Km^r colonies resulting from matings with pRK2013 or pRK2013 and the pBR325::Tn*5* derivative were at least an order of magnitude lower than those of Km^r colonies resulting from matings with the various pAT600::Tn*5* derivatives (Table 2). Of the remaining possibilities, some of the recombinational events should have resulted in Nif$^-$ mutants.

As expected, the single Km^r colony obtained when the pAT600 clone containing Tn*5* inserted in the vector (pAT600::Tn*5*-13) was used in the matings was phenotypically Nif$^+$. However, all of the Km^r *Methylosinus* sp. strain 6 colonies tested from the matings involving pAT600::Tn*5*-1, pAT600::Tn*5*-10, pAT600::Tn*5*-14, and pAT600::Tn*5*-37 showed a Nif$^-$ phenotype. They were unable to grow on either liquid or solid media without a source of combined nitrogen, they could not be derepressed for acetylene reduction activity under conditions in which derepression was achieved in the wild type, and they lacked three of five major polypeptides which were identified on two-dimensional protein gels as repressible by ammonia or nitrate (Fig. 2).

These Tn*5*-derived *nif* mutants apparently represent *Methylosinus* sp. strain 6 nitrogenase structural gene mutants. Since the *nifD* gene from *Klebsiella pneumoniae* shows the strongest homology to the smaller of the *Hin*dIII-*Sst*I fragments in the insert of pAT600 (19) (right-hand end in Fig. 1), the four Tn*5* insertions shown in Fig. 1 appear to be located in or near *nifD*. In addition, the three proteins missing in the mutants have a mobility on two-dimensional gels corresponding to a molecular weight of 57,000 and a pI range of 6.5 to 7, which is similar to the mobility of the *nifK* and *nifD* gene products of a number of diazotrophs (7, 14, 18).

An attempt was made to correlate the missing polypeptides with the two polypeptides encoded by the 2.3-kb *Hin*dIII fragment found in pAT600. A sample of [^{35}S]methionine-labeled *E. coli* CSR603(pAT600) was run on two-dimensional gels either alone or mixed with samples of *Methylosinus* sp. cells which had been derepressed for *nif*. After electrophoresis in the second dimension, the gels were stained with Coomassie blue and then treated for autoradiography.

The 34,000-molecular-weight pAT600-encoded polypeptide focused to a single spot at a pI of ca. 7.0, which did not

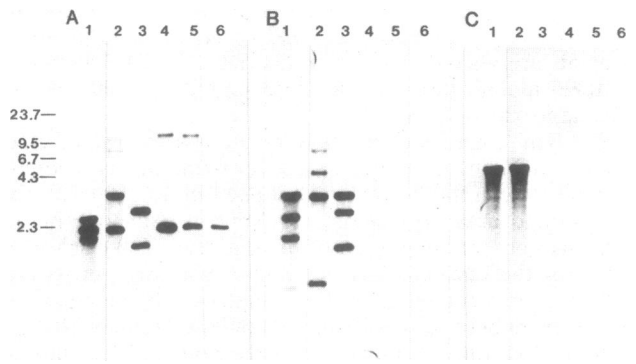

FIG. 3. Hybridization analysis of DNA isolated from five KM^r *Methylosinus* sp. mutants. *Hin*dIII digests of DNA isolated from *Methylosinus* sp. strain 6E-1C (lane 1), 6E-10B (lane 2), 6E-14E (lane 3), 6E-37C (lane 4), 6-13C (lane 5), and the wild type (lane 6) were probed with ^{32}P-labeled (A) 2.3-kb *Hin*dIII *nif* insert of pAT600; (B) Tn*5* (the two internal *Xho*1 fragments); and (C) pBR325. Size markers were lambda DNA digested with *Hin*dIII. The same blot was used for hybridization with all three probes. The faint bands seen in lane 2 (A) and panels A and B, lanes 2, (B), represent partial digests.

correspond to any protein found in *Methylosinus* sp. strain 6 (Coomassie stained), suggesting that it represented a truncated protein produced by a partial gene present in the insert. The 57,000-molecular-weight protein did not focus into a single spot, but ran as a streak in the pI range encompassing all three of the ammonia-repressible polypeptides of molecular weight 57,000. This streaking was not corrected by pretreatment with DNase and RNase or by the addition of solid urea before sample loading.

The lack of focusing with known proteins made it impossible to identify either of the polypeptides conclusively. However, the evidence presented here and elsewhere (19) suggests that the 57,000-molecular-weight protein corresponds to the *nifD* gene product. If the organization of the *nif* structural genes is the same in *Methylosinus* sp. strain 6 as in *K. pneumoniae*, then the truncated polypeptide would correspond to the amino terminus of the *nifK* gene product and the *nifH* gene would lie upstream from *nifD* (13). This model is supported by evidence that the direction of transcription of the insert in pAT600 is from right to left (19), but further experimentation will be necessary to test this hypothesis.

Whenever a marker exchange procedure is used, it is important to determine the molecular nature of the recombinational events which led to the production of the mutants. A study in *Agrobacterium tumefaciens* that used a one-step marker exchange procedure similar to the one described here indicated that single crossovers were the initial recombinational event (20). However, Ruvkun and Ausubel (15) have shown in *Rhizobium meliloti* that with the two-step marker exchange procedure, double-crossover homologous recombination was the most likely event. To test this in the *Methylosinus* sp. strain 6 mutants, DNA was isolated from strains 6E-1C, 6E-10B, 6E-14E, 6E-37C, and the Nif$^+$ strain 6-13C, restricted with *Hin*dIII, and hybridized to ^{32}P-labeled nick-translated pBR325, Tn*5* (the two internal *Xho*I fragments), or the 2.3-kb *nif* insert of pAT600.

The results indicated that a variety of molecular events had led to the *nif* mutations (Fig. 3). Of the five mutants, only one, strain 6E-14E, appeared to result from a double-crossover homologous recombination event, with the transposon inserted in the chromosome in the same location as it was in pAT600::Tn*5*-14. This was confirmed by probing *Eco*RI digests with Tn*5* and pBR325.

Mutant strain 6E-1C apparently resulted from a single-crossover homologous recombination event in which the entire pAT600::Tn*5*-1 plasmid was integrated at the site of the chromosomal 2.3-kb *Hin*dIII fragment. Mutant strain 6E-10B also apparently resulted from single-crossover recombination; however, part of the 2.3-kb *Hin*dIII insert of pAT600::Tn*5*-10 had been deleted. A probe of *Eco*RI digests of DNA from this strain with Tn*5* and pBR325 indicated that additional molecular rearrangements may have occurred.

The genetic rearrangements in strain 6E-37C were more complex. No detectable Tn*5* sequences were present in this mutant. Its Km^r phenotype observed may have been due to integrated pRK2013 sequences, since two high-molecular-weight fragments that hybridized weakly to the pBR325 probe were detected. The nature of the *nif* lesion in this mutant was not obvious, as a wild-type copy of the 2.3-kb *Hin*dIII fragment was present. However, an additional fragment hybridized to the 2.3-kb probe, which indicated that some complex genetic rearrangements might have occurred.

Mutant strain 6-13C was also the result of complex genetic rearrangements. No Tn*5* or pBR325 sequences were detected in this mutant. The Km^r phenotype of this strain was possibly due to a spontaneous mutation. The 2.3-kb *Hin*dIII probe hybridized to the wild-type copy of this fragment and to an altered copy.

These results suggest that in *Methylosinus* sp. strain 6, as in *A. tumefaciens*, single-crossover recombination is the most common result of one-step marker exchange. However, a detectable number (one out of five) of the mutants tested had apparently undergone double-crossover recombination. Therefore, this technique can be used to generate straightforward mutants in obligate methanotrophs provided that the resultant mutants are screened for double-crossover events. Successful one-step marker exchange represents a major accomplishment in the genetic manipulation of these organisms; no other mutagenesis procedure has been similarly successful. The one-step marker exchange procedure described in this paper has advantages for mutagenesis in bacteria such as methanotrophs because it does not require expression of multiple drug resistance markers, and it should be generally useful in bacteria in which the two-step procedure of Ruvkun and Ausubel (15) is not feasible. However, it should be used in conjunction with molecular probing to distinguish between simple and complex molecular rearrangements.

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