Genetic Transformation System in the Archaeobacterium *Methanobacterium thermoautotrophicum* Marburg

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A wild-type strain of *Methanobacterium thermoautotrophicum* Marburg was transformed by DNA from strains resistant to 5-fluorouracil. Recipient cells were grown without selection on gellan gum (GELRITE) plates with DNA. Drug-resistant cells were recovered by replica plating the resulting colonies onto drug plates. Transformation required high-molecular-weight DNA with appropriate markers and was not observed on agar or in liquid media under a variety of conditions.

A significant amount of molecular information has been uncovered about methanogenic archaeobacteria with the use of methanogen DNAs that have been cloned into eubacterial hosts (7, 9). A method for performing genetic experiments in methanogens is necessary so that the cloned genes can be used as starting points for meaningful physiological studies. Techniques for mutant selection and methods for introducing methanogen genes into their original backgrounds are the minimal requirements for a genetic system. Some progress has been made in this direction for several species of archaeobacteria. Several drug-resistant and auxotrophic mutants have been reported in various methanogen species (3, 8, 10, 11, 18, 20, 22). Among the halophilic archaeobacteria, transfer mechanisms based on conjugation and transfection with DNA from a lytic phage have been demonstrated (6, 17). Genetic transformation of the mesophile *Methanococcus voltae* PS in liquid culture was reported recently (3).

We present evidence for a natural transformation system in the thermophilic methanogen *Methanobacterium thermoautotrophicum* Marburg. A marker for 5-fluorouracil (FU) resistance was transformed into the wild-type strain via DNAs from drug-resistant mutants.

MATERIALS AND METHODS

Strains and media. Table 1 lists the strains used in this study. *Halobacterium volcanii* was grown in medium 97 at 37°C (5). *Escherichia coli* was grown in LB broth at 37°C. *M. thermoautotrophicum* strains were grown on mineral medium 2 as described previously (1, 24). Mineral medium plates contained 0.8% GELRITE gellan gum (Kelco Division of Merck & Co., Inc., San Diego, Calif.) plus a cation mixture composed of 1 g each of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per liter. The amount of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was reduced 10-fold to a final concentration of 0.006%. Mineral medium agar plates contained 2% Noble agar (Difco Laboratories, Detroit, Mich.) with or without the cation mixture. All plating operations, including preparation of plates after sterilization of anoxic medium, were done in an anaerobic chamber under an atmosphere of 95% N_2 and 5% H_2 (2). Additions were made by spreading liquid supplements on the solidified medium with a bent glass rod or a sterile plastic loop. Plates were incubated in pressure vessels (modified from references 1 and 2) under an atmosphere of H_2 - CO_2 - H_2S (80:19:1 [vol/vol]) at 200 to 300 kPa for 2 to 4 days at 60°C. To score colonies, the gas phase was exchanged with

nitrogen, and the vessels were brought into the anaerobic chamber.

DNA purification. *M. thermoautotrophicum* cells were suspended in SE solution (0.15 M NaCl, 0.01 M sodium EDTA, pH 8.0; 1 g [wet wt] per 7 ml of buffer) and passed once through a French pressure cell at 40 or 130 MPa. This lysis technique produced a broad size distribution of DNA fragments (0.5 to 20 kilobase pairs). Lysates that were prepared as described by Meile et al. (16) yielded uniformly high-molecular-weight DNA fragments. *H. volcanii* cells were lysed osmotically by suspension in SE solution. *E. coli* (in SE solution) was frozen at -20°C, thawed, and treated with lysozyme (1 mg/ml, 5 min, 4°C). The DNA was purified by the Marmur technique after lysis (12). Purified DNAs were stored at -20°C in 0.1× SSC buffer (0.015 M NaCl, 1.5 mM trisodium citrate, pH 7.0). The size of *M. thermoautotrophicum* DNA was determined by agarose gel electrophoresis with HindIII-cut phage lambda DNA as a molecular weight standard. Low-molecular-weight DNAs (size, <0.5 kilobase pairs) were produced by shearing high-molecular-weight samples three times with a French pressure cell.

Genetic transformation. DNA samples (2 ng to 100 µg) were placed at the centers of plates, and inocula (0.05 to 0.2 ml) containing 100 to 200 CFU were spread over the DNA-containing regions with sterile, disposable plastic loops. In some experiments, enough cells were added to the DNA plates to cause confluent growth; however, this modification had no effect on the results. Controls (plates lacking cells or DNA) were run with every experiment. After 2 to 4 days of incubation at 60°C, the resulting colonies were replica plated onto selective media. Replica plating was done with wooden applicator sticks (2-mm diameter) held perpendicular to the plates. Each inoculum was replica plated by impressing the end of an applicator stick onto the gellan gum surface so as to maintain the organization of the original colony. Any sectorized distribution of drug-resistant cells in a colony was preserved on the selective plates. In several of the early experiments, a random sample of transformants was tested for the ability to produce methane at 60°C to ensure that they did not represent contaminating organisms.

Transformation in liquid medium was attempted as follows. Cultures containing 1.1×10^7 cells per ml were incubated with DNA (0.9 µg/ml from strain RTAE-1 or 51 µg/ml from the wild type) for 6 h with gentle shaking or for 48 to 72 h with or without shaking. A sample of 1×10^7 to 2×10^7 cells from each of these conditions was plated on FU. In some experiments, DNA was added with polyethylene

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TABLE 1. Strains

Strain	Proposed genotype ^a	Phenotype	Source or reference
<i>M. thermoautotrophicum</i> Marburg			
DSM 2133	<i>fus</i> ⁺	FU ^s	H. Hippe
RTAE-1	<i>fus</i> -1	FU ^r	DSM 2133; 18
RTVW-1	<i>fus</i> -1 <i>mps</i> -1 ⁺	FU ^r MP ^r	RTAE-1; this work
<i>E. coli</i> ATCC 8739 ^b			K. Kealy
<i>H. volcanii</i> ATCC 29605			ATCC

^a The *fus* locus encodes sensitivity or resistance to FU. The *mps*-1 allele encodes resistance to 6-mercaptopurine.

^b ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

glycol (PEG) 6000 (final concentration, 20% [wt/vol]) or PEG 6000 and the cation mixture that was used in the gellan gum plates (1 g each of MgSO₄ · 7H₂O and CaCl₂ · 2H₂O per liter). No attempt was made to convert the culture into protoplasts when PEG was included in the medium (14). The presence of transformants was measured by plating the cells on selective plates.

RESULTS AND DISCUSSION

The drug resistance phenotype FU^r was used to assay for transformation of *M. thermoautotrophicum*. This phenotype was attractive, because it appeared at low frequencies by spontaneous mutation ($<1.6 \times 10^{-8}$). The FU^r strain RTAE-1 arose as a spontaneous mutant with a defect in uracil phosphoribosyltransferase (18). Strain RTVW-1 was selected from a culture of strain RTAE-1 which spontaneously grew in the presence of 6-mercaptopurine (500 µg/ml; Table 1).

Genetic transformation of *M. thermoautotrophicum*. We tried several protocols for transformation of *M. thermoautotrophicum* cells with DNA from FU^r strains RTAE-1 and RTVW-1. Liquid cultures were exposed to DNA in the presence of 20% PEG and cations or in the absence of these supplements. PEG in the liquid inoculum protected the plated cells from inhibition by FU, but both methods failed to yield drug-resistant cells at frequencies greater than those of spontaneous mutation. We then reasoned that competence could be associated with a particular physiological state that might not be accessible to most cells in liquid cultures (19, 23). Therefore, we tried transformation experiments on plates on the assumption that cells might pass through a spectrum of physiological states which might include competence (13, 19). In our first experiments, strain RTAE-1 DNA was placed on the surfaces of gellan gum plates, and wild-type cells were allowed to grow into confluent patches over the DNA region. We tested several hundred regions of these confluent lawns and found FU^r cells in 3% of the samples when the cells were exposed to FU^r DNA but not wild-type DNA. Drug-resistant cells were not recovered from plates that lacked DNA (data not shown). FU^r cells were recovered from the confluent patches from the DNA plates only if transformation was allowed to proceed in the absence of selection. As expected, it was important to allow nonselective growth in the presence of FU^r DNA before challenging the cells with the drug to allow time for the development of competence, transformation, and expression of the drug resistance allele.

The transformation experiments were repeated with smaller inocula so that single colonies formed on the DNA plates. The number of colonies which contained a subset of FU^r cells was dependent on the amount of FU^r DNA added (Fig. 1). Transformation was expressed as the percentage of colonies that contained drug-resistant cells. No evidence of transformation was seen in the absence of DNA (825 colonies tested). With as little as 2 ng of DNA from strain RTAE-1, FU^r cells were found in 1.1% of colonies; the frequency of resistant cells reached a plateau value at 10 ng of DNA per plate. When colonies were replica plated so as to preserve the original distribution of sibling cells, we found that transformants were almost always at the edges of the colonies. We never recovered colonies that contained a majority of drug-resistant cells. This result implied that transformation rarely if ever occurred early in the development of a colony.

The appearance of FU^r cells was specific for FU^r DNA from *M. thermoautotrophicum* (Table 2). DNAs from the archaeobacterium *H. volcanii* or the eubacterium *E. coli* did not produce drug-resistant *M. thermoautotrophicum* cells. Only one colony containing FU^r cells appeared in 760 that were tested when wild-type (FU^s) strain Marburg was the

FIG. 1. Transformation of wild-type *M. thermoautotrophicum* with strain RTAE-1 DNA. DNA from FU^r strain RTAE-1 was placed at the centers of mineral medium plates containing GEL-RITE gellan gum. The region with DNA was spread with cells of *M. thermoautotrophicum* Marburg. At least 125 colonies were tested at each concentration of DNA. (A) Transformation data for low concentrations of DNA on an expanded scale. (B) Data for the complete range of DNA concentrations tested.

TABLE 2. Effects of media and DNA source on transformation

Medium and source of DNA	No. of colonies tested ^a	No. of colonies with FU ^r cells
GELRITE		
RTAE-1, RTVW-1 (FU ^r)	1,062	14
RTAE-1 (sheared)	154	0
Marburg (wild type)	760	1
<i>H. volcanii</i>	99	0
<i>E. coli</i>	216	0
None	825	0
Agar		
RTAE-1	395	0
Marburg	378	0
None	389	0

^a Colonies were grown on minimal plates under nonselective conditions for 2 to 4 days and then replica plated onto minimal and FU (100 µg/ml)-containing plates. FU^r was confirmed by replating.

source of transforming DNA. This isolate probably arose by spontaneous mutation. The dependence of transformation on an appropriately marked *M. thermoautotrophicum* DNA implies that drug-resistant cells arose as a result of recombination and not from the induction of SOS or error-prone repair systems. If exposure of the cells to DNA induced mutation, we would expect DNA from any source to induce drug-resistant pseudotransformants.

Efficient transformation was dependent on high-molecular-weight DNA. The DNA listed in line 1 of Table 2 had an average size of about 20 kilobase pairs; fragments of about 0.5 kilobase pairs did not support transformation (Table 2, line 2).

Gellan gum-dependent transformation. GELRITE gellan gum (15) was used in our initial experiments because of its ability to absorb relatively large volumes of water from supplements and because it remained dry after incubation at elevated temperatures. We attempted transformation experiments on the surface of Noble agar but obtained no transformants even at high amounts of DNA per plate (Table 2). The magnesium-calcium cation mixture was added to the agar in some plates to see whether high concentrations of cations could enhance transformation, but this modification inhibited growth of the cells. However, transformation was obtained in experiments with different lots of gellan gum and several preparations of marked DNA made by different methods and performed by different investigators. The reasons for our success with GELRITE gellan gum and not with Noble agar are unclear. It was not likely that the Mg-Ca cation mixture in gellan gum medium was solely responsible for the transformation, since it could not induce transformation in liquid or on agar plates. The only other variable was the use of gellan gum versus agar to solidify the DNA plates. Therefore, it is possible that the structure of the gellan gum matrix is involved in transformation. Gellan gum is a linear polymer of tetrasaccharide that contains glucuronic acid (21). The stiff helical structure of the polymer in solution (4) and the regular alignment of negative charges in the solidified gel might influence the conformation of the DNA or the cell wall to enhance competence.

The transformation system for the pseudomurein-containing *M. thermoautotrophicum* (1) on gellan gum plates is simple, reproducible, and specific for high-molecular-weight DNA with the appropriate marker. No special techniques, such as protoplast formation, heat shock, or starvation, are required. The results suggest that, with auxotrophic mutants and vectors already available, progress on the genetic un-

derstanding of pseudomurein-containing methanogens will accelerate.

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