APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2001, p. 2538–2544 Vol. 67, No. 6
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.6.2538–2544.2001

Chromosomal Gene Inactivation in the Green Sulfur Bacterium *Chlorobium tepidum* by Natural Transformation

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Received 12 December 2000/Accepted 18 March 2001

Conditions for inactivating chromosomal genes of *Chlorobium tepidum* by natural transformation and homologous recombination were established. As a model, mutants unable to perform nitrogen fixation were constructed by interrupting *nifD* with various antibiotic resistance markers. Growth of wild-type *C. tepidum* at 40°C on agar plates could be completely inhibited by 100 μg of gentamicin ml^{-1}, 2 μg of erythromycin ml^{-1}, 30 μg of chloramphenicol ml^{-1}, or 1 μg of tetracycline ml^{-1} or a combination of 300 μg of streptomycin ml^{-1} and 150 μg of spectinomycin ml^{-1}. Transformation was performed by spotting cells and DNA on an agar plate for 10 to 20 h. Transformation frequencies on the order of 10^{-7} were observed with gentamicin and erythromycin markers, and transformation frequencies on the order of 10^{-3} were observed with a streptomycin-spectinomycin marker. The frequency of spontaneous mutants resistant to gentamicin, erythromycin, or spectinomycin-streptomycin was undetectable or significantly lower than the transformation frequency. Transformation with the gentamicin marker was observed when the transforming DNA contained 1 or 3 kb of total homologous flanking sequence but not when the transforming DNA contained only 0.3 kb of homologous sequence. Linearized plasmids transformed at least an order of magnitude better than circular plasmids. This work forms a foundation for the systematic targeted inactivation of genes in *C. tepidum*, whose 2.15-Mb genome has recently been completely sequenced.

Green sulfur bacteria are strictly anaerobic phototrophs which occur in sulfide-rich aquatic environments (21). They form a coherent phylogenetic group and are not closely related to other bacteria (13). Current areas of interest in this group of bacteria include photosynthetic electron transport (involving the reaction center, cytochromes, quinones, etc.); organization of and energy transfer in the light-harvesting antennae (the FMO protein and chlorosomes); biosynthesis and function of chlorophylls (bacteriochlorophylls *a*, *c*, *d*, and *e* and chlorophyll *a*), carotenoids, and isoprenoid quinones; lithotrophic oxidation of sulfur compounds; CO_2 fixation (which occurs via the reverse tricarboxylic acid cycle); and other attributes contributing to their ecological and evolutionary significance (2).

Chlorobium tepidum is a moderately thermophilic green sulfur bacterium. It grows rapidly on a defined medium and makes a suitable model for genetic, biochemical, and physiological studies of the green sulfur bacteria (3, 22). The 2.15-Mb genome of *C. tepidum* has recently been sequenced and reveals about 2,284 open reading frames of which about 50% have been assigned a known function (J. A. Eisen et al., unpublished data).

A powerful way to investigate the function of the genes of an organism is by targeted gene inactivation by homologous recombination (20). The success of such an approach typically depends on the availability of an antibiotic which effectively inhibits the growth of wild-type cells and to which spontaneously resistant mutants are not easily formed, on a selection marker which confers resistance to this antibiotic, and on a method to introduce DNA into the cells. Transformation of *Chlorobium* species using natural transformation (5, 12), chemical transformation (10), and electroporation (9) has previously been reported. Spectinomycin and streptomycin were the only antibiotics used for selection in these transformations (5, 9, 12). Ampicillin and chloramphenicol resistance markers have also been reported to be useful for selection in conjugation studies of *C. tepidum* (24). At present, the only genes in any *Chlorobium* species that have been reported as targets for inactivation encode chlorosomal proteins CsmC and CsmA (5), reaction center cytochrome c_{551} PscC (9), and Rubisco subunit RbcL (T. E. Hanson and F. R. Tabita, personal communication). Of these, only the *csmC* (5) and *rbcL* mutants (T. E. Hanson and F. R. Tabita, personal communication) fully segregated.

C. tepidum is a nitrogen-fixing organism (23), and in the present study the *nifD* gene, which encodes a subunit of nitrogenase (15), was studied as a general model for gene inactivation. This allowed facile detection of true transformants as mutants that had a known phenotype, namely, the inability to grow diazotrophically. Several antibiotics and resistance markers were tested, and three markers were found to be suitable for use in *C. tepidum*: a spectinomycin-streptomycin resistance marker, a gentamicin resistance marker, and an erythromycin resistance marker. Natural transformation of *C. tepidum* was also characterized as a general method for gene inactivation by homologous recombination.

MATERIALS AND METHODS

Organisms and growth conditions. The strain of *C. tepidum* used for transformation was WT2321 (24), which is a plating strain derived from *C. tepidum* strain ATCC 49652 (22). Growth conditions were essentially as previously described (24). All growth and manipulations of *C. tepidum* were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) which had an atmosphere of 10% CO_2 and 5% H_2 balanced with N_2. *C. tepidum* was grown at 40°C throughout the study.

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All cloning was carried out with *Escherichia coli* DH5α grown in Luria-Bertani medium. Antibiotic selection conditions for *E. coli* were obtained with 100 μg of ampicillin ml^{-1} combined with 10 μg of gentamicin, 100 μg of spectinomycin, or 10 μg of chloramphenicol ml^{-1}. Ampicillin, gentamicin, and kanamycin were obtained from U.S. Biochemicals (Cleveland, Ohio), and other antibiotics were obtained from Sigma (St. Louis, Mo).

One liter of liquid medium for *C. tepidum* (CL) was made of 20 ml of salts A (0.64 g of $Na_2 \cdot EDTA \cdot 2H_2O$, 10 g of $MgSO_4 \cdot 7H_2O$, 2.5 g of $CaCl_2 \cdot 2H_2O$, and 20 g of NaCl per liter), 20 ml of salts B (25 g of NH_4CH_3COO, 20 g of NH_4Cl, and 115 g of $Na_2S_2O_3 \cdot 5H_2O$ per liter), 20 ml of buffers (25 g of KH_2PO_4 and 105 g of MOPS [3-{*N*-morpholino}propanesulfonic acid] per liter), 1 ml of trace elements (22), 50 μl of 10-mg ml^{-1} resazurin, and 20 μl of 1-mg ml^{-1} vitamin B_{12}. After the medium was autoclaved at 121°C for 20 min, a freshly made, filter-sterilized solution of 0.6 g of $Na_2S \cdot 9H_2O$ and 2.0 g $NaHCO_3$ in 50 ml of water was added. The pH of the medium was usually within the desired range of 6.9 to 7.0 without further treatment but otherwise was aseptically adjusted with 1 M NaOH or 1 M HCl.

One liter of plating medium for *C. tepidum* (CP) made about 25 plates and contained 20 ml of salts A, 20 ml of salts B, 20 ml of buffers, 1 ml of trace elements, 50 μl of 10-mg ml^{-1} resazurin, 20 μl of 1-mg ml^{-1} vitamin B_{12}, and 0.36 g of L-cysteine. The pH was adjusted to 7.6 with 10 M NaOH, and 15 g of agar (Bacto Agar; Becton Dickinson, Sparks, Md.; used as supplied) per liter was added before autoclaving. The medium was cooled to 50°C in a water bath before addition of antibiotics and the pouring of the plates. The plates were poured in a cold room to allow rapid solidification and moved to the anaerobic chamber in less than 20 min to prevent excessive oxidation of the cysteine. The final pH of the plates was approximately 6.8 to 7.0. Ammonium-free plating medium (CPNF) for diazotrophic growth was made by substituting salts BNF (26.5 g of $NaCH_3COO$ and 115 g of $Na_2S_2O_3 \cdot 5H_2O$ per liter) for salts B and leaving out cysteine. Once inoculated, the plates were placed in an anaerobic jar (BBL GasPak 100 system; Becton Dickinson) without palladium catalyst but containing one disposable H_2-CO_2-generating envelope (BBL GasPak; Becton Dickinson) and a small tube with approximately 0.1 g of thioacetamide; H_2S generation from the thioacetamide was activated by adding approximately 1 ml of 1 M HCl. The jars were kept in dim light for 1 to 2 h before transfer to the appropriate temperature and illumination conditions. In addition to ambient illumination, a single 100-W incandescent light bulb was placed 0.4 to 0.8 m from the jars. The jars were always kept inside the anaerobic chamber.

Transformation of *C. tepidum*. Unless otherwise stated, the standard protocol for agar plate transformation was as follows. Cells from 100 μl of an overnight culture of *C. tepidum* in the late exponential growth phase (approximately 3×10^9 to 6×10^9 cells ml^{-1}) were harvested in a microcentrifuge tube, resuspended in 20 μl of CL medium containing 1 μg of DNA, and spotted on a nonselective CP plate in an area with a diameter of 6 to 8 mm. The plate was placed in a jar and kept in the dark for 1 to 2 h prior to incubation in the light at 40°C for 18 to 20 h. The cell patch was then scraped off and suspended in 300 μl of CL. This suspension and dilutions thereof were spread on selective and nonselective CP plates and incubated for 5 to 6 days to allow single colonies to appear. The mutation frequency was calculated as the number of antibiotic-resistant mutants counted on the selective plates divided by the total number of viable cells counted on the nonselective plates. All transformation frequencies represented in the figures and tables represent the means of up to four separate experiments in which the standard deviations were less than 50% of the means.

Preparation of plasmid and genomic DNA. Plasmids were prepared by alkaline lysis (19). Digested plasmids to be used for transformation were extracted with an equal volume of chloroform-isoamyl alcohol (24:1 by volume), precipitated with 0.1 volume of 3 M ammonium acetate and 1 volume of isopropanol at −20°C, washed with 70% (vol/vol) ethanol, and redissolved in sterile water.

Genomic DNA from *C. tepidum* and *Synechococcus* sp. strain PCC 7002 was prepared using an unpublished method developed by Dexter Chisholm (DuPont, Wilmington, Del.). Cells from 5 to 20 ml of culture were harvested and incubated in 500 μl of TES buffer (5 mM Tris, 5 mM EDTA, 50 mM NaCl, pH 8.5) containing 10 mg of lysozyme ml^{-1} for 0.5 to 1 h at 37°C. Sodium Sarkosyl (50 μl of a 10% [wt/vol] stock solution) was added, and the suspension was extracted twice with 600 μl of buffered phenol. RNase was added to a final concentration of 100 μg ml^{-1}, and the mixture was incubated for 30 min at 37°C. To this solution, NaCl (100 μl of a 5 M stock solution) and hexadecyltrimethylammonium bromide (CTAB; 100 μl of a stock solution of 10% [wt/vol] CTAB–0.7 M NaCl) were added; the mixture was then extracted twice with 600 μl of chloroform-isoamyl alcohol (24:1 by volume). DNA in the aqueous phase was precipitated with 600 μl of isopropanol, washed with 70% (vol/vol) ethanol, and redissolved in sterile water. Genomic DNA for PCR analysis was isolated from cells grown on plates. Genomic DNA for Southern hybridization analysis was isolated from cells grown to the late exponential phase in liquid medium because it appeared to be difficult to completely digest DNA isolated from cells grown on plates.

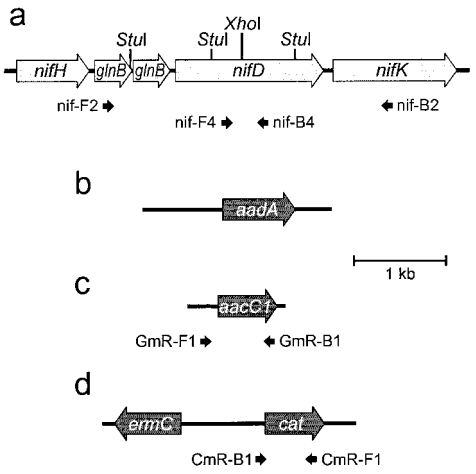

FIG. 1. (a) Map of part of the *nif* region in *C. tepidum*; (b) map of the *aadA* streptomycin-spectinomycin resistance cassette from pHP45Ω; (c) map of the *aacC1* gentamicin resistance cassette from pMS266; (d) map of the *ermC-cat* erythromycin and chloramphenicol resistance cassette from pRL409. The positions of some of the primers discussed in the text are also shown.

PCR conditions. Oligonucleotide primers were designed based on the genomic sequence (J. A. Eisen et al., unpublished data) using MacVector software, version 6.5 (Genetics Computer Group, Madison, Wis.) and synthesized at the Nucleic Acid Facility, The Pennsylvania State University. The PCR conditions were as follows: initially 5 min at 95°C, and then 35 cycles of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C for PCR products <2 kb, and ultimately 10 min at 72°C. For PCR products of 2 to 3 kb, the elongation time at 72°C was increased to 3 min.

Construction of plasmids. Figure 1a shows a map of a portion of the *nif* region of *C. tepidum* based on the complete genomic sequence (J. A. Eisen et al., unpublished data). A 2.93-kb fragment was amplified from genomic DNA using primers nif-F2, 5′-*GGAATTC*GCGTCGGCGATGTGGTCTAT, and nif-B2, 5′-*GGAATTC*GTCGGAGGTGTCTGGGAA. (In the primer sequences, heterologous bases are italicized and *Eco*RI recognition sites are underlined.) Plasmid pTN1 was produced by digesting this PCR product with *Eco*RI and cloning the product into the *Eco*RI site of pUC19 (Table 1). Plasmid pTN1G4 was made by inserting the *aacC1* cassette from pMS266 (Table 1 and Fig. 1c) into the *Xho*I site of *nifD* in pTN1 (Fig. 2a). Similar plasmid constructs with *nifD*::*aacC1* but with shorter flanking regions of *C. tepidum* DNA were also made: pTN2G1 (primers nif-F3, 5′-*GGAATTC*AGGGCGTGGTTCTTGGTCC, and nif-B3, 5′-*GGAATTC*GAGTTCGGCTTTGCTCTTT) and pTN3G11 (primers nif-F5L, 5′-*GGGAATTC*GCTGGTCACCACATCGCAA, and nif-B5L, 5′-CCATGGAATTCCGTACTTGGTCTC) were made by PCR with pTN1G4 and by cloning the PCR products into the *Eco*RI site of pUC19 (Fig. 2a and Table 1). pTN1G4, pTN2G1, and pTN3G11 linearized with *Ahd*I were used for transformation and are depicted in Fig. 2b to d. pTN1S3 was made by inserting the *aadA* cassette from pHP45Ω (Fig. 1b) into the *Xho*I site of pTN1, and pTN1CE1 was made by inserting the *ermC-cat* cassette from pRL409 (Fig. 1d) into the *Xho*I site of pTN1 (Table 1).

Genomic DNA analysis of *C. tepidum* transformants. Detection of wild-type *nifD* and cassette-interrupted *nifD* was performed by PCR with primers nif-F4, 5′-CACCACATCGCAAACAAC, and nif-B4 5′-GCAGGAACCTCTTCGGCAATC (Fig. 1a). PCR detection of the *aacC1* cassette was performed with primers GmR-F1, 5′-GTGACGCACACCGTGGAAAC, and GmR-B1, 5′-TCCCGTATGCCCAACTTTGTA (Fig. 1c). Southern hybridization was carried out as described previously (19) with [α-^{32}P]dATP-labeled probes (Random Primed DNA labeling kit; Boehringer Mannheim, Indianapolis, Ind.). Hybridizing DNA fragments were detected with a PhosphorImager 445-SI (Molecular Dynamics, Sunnyvale, Calif.). A 413-bp probe for *nifD* was made by PCR with primers nif-F4 and nif-B4 (Fig. 1a). A 559-bp probe for the *ermC-cat* cassette was made by PCR with primers CmR-F1, 5′-ACGGGGGCGAAGAAGTTGTC and

TABLE 1. Plasmids used in this study

Name	Size (kb)	Relevant characteristics	Phenotype[a]	Source or reference
pMS266	3.7	Contains a 1.1-kb *aacC1* gentamicin resistance cassette	Ap^r Gm^r	1
pHP45Ω	4.4	Contains a 2.1-kb *aadA* streptomycin and spectinomycin resistance cassette	Ap^r Sm^r Sp^r	16
pRL409	5.4	Contains a 2.8-kb *ermC-cat* erythromycin and chloramphenicol resistance cassette	Ap^r Em^r Cm^r	7
pTN1	5.6	Derived from pUC19; contains a 2.93-kb fraction of the *C. tepidum nif* region	Ap^r	This study
pTN1G4	6.7	Similar to pTN1, but *nifD* is interrupted by the *aacC1* cassette from pMS266	Ap^r Gm^r	This study
pTN2G1	4.8	Similar to pTN1G4 but contains only a 1.08-kb fraction of the *C. tepidum nif* region	Ap^r Gm^r	This study
pTN3G11	4.0	Similar to pTN1G4 but contains only a 0.29-kb fraction of the *C. tepidum nif* region	Ap^r Gm^r	This study
pTN1S3	7.7	Similar to pTN1, but *nifD* is interrupted by the *aadA* cassette from pHP45Ω	Ap^r Sm^r Sp^r	This study
pTN1CE1	8.4	Similar to pTN1, but *nifD* is interrupted by the *ermC-cat* cassette from pRL409	Ap^r Em^r Cm^r	This study

[a] Ap^r, ampicillin resistant; Gm^r, gentamicin resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant.

CmR-B1, 5′-CGGGCGTATTTTTTGAGTTATCG (Fig. 1d). A 1.1-kb probe for *aadA* was excised from plasmid pSRA2, which contains an *aadA* cassette derived from pHP45Ω (N.-U. Frigaard and D. A. Bryant, unpublished data).

RESULTS AND DISCUSSION

Antibiotic sensitivity. Because the antibiotic resistance markers used in this study originate from mesophiles, the temperature used in the transformation experiments was lower than the optimum 47 to 48°C growth temperature of *C. tepidum*. To test the antibiotic sensitivity of wild-type *C. tepidum*, cells from 100 μl of a late-exponential culture were plated on CP plates with increasing concentrations of antibiotics and incubated at 40°C. The following concentrations were found to inhibit growth completely, and these concentrations were used throughout the present study: gentamicin, 100 μg ml^{-1}; erythromycin, 2 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}; tetracycline, 1 μg ml^{-1}. Streptomycin and spectinomycin only efficiently inhibited growth when combined. Therefore, streptomycin (300 μg ml^{-1}) and spectinomycin (150 μg ml^{-1}) were used in combination, since the *aadA* cassette from pHP45Ω confers resistance to both antibiotics. Spontaneous mutants resistant to gentamicin, erythromycin, chloramphenicol, tetracycline, or streptomycin-spectinomycin were not observed when the wild-type culture was plated on plates with the stated concentration of antibiotic. Kanamycin and ampicillin did not completely inhibit growth on plates at concentrations below 100 μg ml^{-1}.

Previous work (11) with other *Chlorobium* species suggests that amoxicillin, nalidixic acid, vancomycin, mitomycin C, and colistin might also efficiently inhibit *C. tepidum*. However, these antibiotics were not tested in this study.

Optimization of transformation. Figure 3 shows the number of transformants and the transformation frequency when *C. tepidum* cells from a 100-μl late-exponential-phase culture were incubated with 1 μg of *Ahd*I-digested pTN1G4 on a CP plate for various times. The transformation frequency reached about 2×10^{-7} to 3×10^{-7} at 10 h and increased only slightly thereafter. (This corresponds to a yield of approximately 10^2 transformants per μg of DNA at 10 h.) This suggests that most of the transformation events occurred at the beginning of the experiment (before 10 h) and that the transformation events were stable.

Transformation was also attempted in a liquid suspension with the same amount of cells and DNA as described above for transformation on an agar plate. The cells were washed and incubated in 100 μl of fresh CL medium containing 1 μg of DNA for various periods between 1 and 25 h and then plated on selective CP plates. The highest transformation frequency obtained was about an order of magnitude lower than the transformation frequencies obtained on agar plates (data not shown). When the cells and DNA are spotted on a solid agar surface and allowed to dry, the cells and DNA may interact

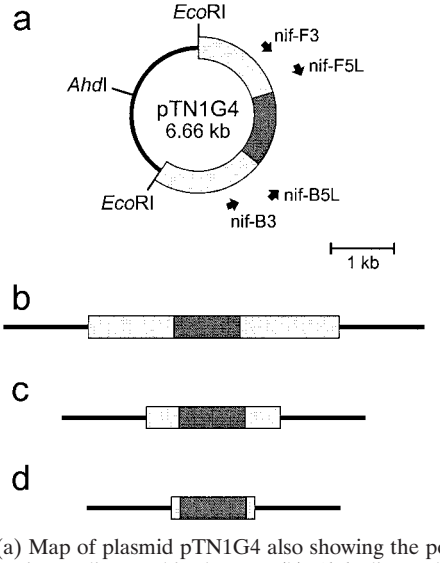

FIG. 2. (a) Map of plasmid pTN1G4 also showing the positions of some of the primers discussed in the text; (b) *Ahd*I-digested pTN1G4; (c) *Ahd*I-digested pTN2G1; (d) *Ahd*I-digested pTN3G11. Line, pUC19 DNA; light grey, *C. tepidum* DNA; dark grey, gentamicin resistance marker.

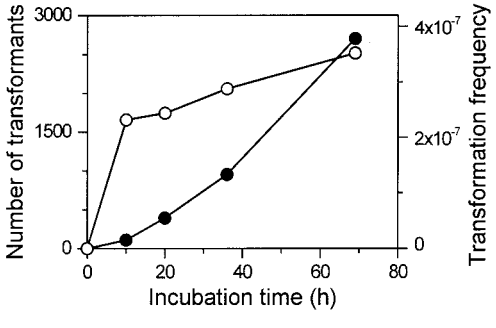

FIG. 3. Transformation of *C. tepidum* incubated for various periods. Solid circles, numbers of gentamicin-resistant transformants; open circles, transformation frequencies. See text for details.

TABLE 2. Transformation with *Ahd*I-digested pTN1G4 in various amounts

DNA amt (μg)	Transformation frequency[a]
0	0
0.1	$(4 \pm 1) \times 10^{-8}$
1	$(1.0 \pm 0.4) \times 10^{-7}$
10	$(1.4 \pm 0.1) \times 10^{-7}$

[a] Values are means ± standard deviations of two independent experiments.

differently than in a liquid suspension. This may allow an increased uptake of DNA by the cells and thus increase the transformation frequency.

Previous work with *Chlorobium limicola* strain 8327 showed that these cells are competent in both the exponential and stationary growth phases (12). The exact growth state of the cells used for transformation was also not critical in our transformation protocol for *C. tepidum*. When equal volumes of an overnight culture in the late exponential growth phase and a 4-day-old culture in the stationary growth phase were used in a 20-h transformation, the stationary cells gave about one-half as many transformants as the late-exponential-phase cells (data not shown). In both cases, the spotted cells exhibited visible growth and sulfur formation during the 20-h incubation on nonselective plates.

Transformation with increasing amounts of linearized DNA resulted in an increased transformation frequency (Table 2). Increasing the DNA amount 100-fold from 0.1 to 10 μg only increased the transformation frequency about 3-fold; this suggests that 10 μg of DNA is close to a saturating amount of DNA for the number of cells used. The transformation frequency with 1 μg of linearized plasmid (*Eco*RI- or *Ahd*I-digested pTN1G4) was about an order of magnitude higher than that for the same amount of circular plasmid (undigested pTN1G4) (data not shown). The reason for this difference is not clear but probably results from the DNA binding and uptake mechanisms of the cells. These observations suggest that at least 1 μg of linearized plasmid is suitable for routine transformation of *C. tepidum*.

Effect of variation in length of homologous flanking DNA. When a plasmid construct is made for gene inactivation by homologous recombination, it is usually advantageous to include a large region of homologous DNA to increase the probability of homologous recombination. However, restriction endonuclease sites and toxic gene products may impose practical restrictions on the length of homologous DNA that can easily be cloned. To determine the effect of the length of homologous flanking DNA on transformation of *C. tepidum*, three constructs for *nifD* inactivation were made; these contained a total of 2.93, 1.08, and 0.29 kb of flanking homologous DNA in which the *aacC1* gentamicin resistance marker was inserted approximately in the middle (Table 1 and Fig. 2). These constructs were digested with either *Ahd*I, which cuts the plasmids only once and which leaves flanks of pUC19 DNA (Fig. 2), or with *Eco*RI, which cuts twice and which excises all of the pUC19 DNA (Fig. 2a). The transformation frequencies with 2.93 kb of homologous DNA were similar regardless of whether the plasmid was digested with *Eco*RI or *Ahd*I (Table 3). But with 1.08 kb of homologous DNA, the transformation frequency was an order of magnitude lower when the plasmid was digested with *Eco*RI than when the plasmid was digested with *Ahd*I. No transformation was observed with only 0.29 kb of homologous DNA regardless of the enzyme used for linearization. Some bacteria partially degrade absorbed DNA via exonuclease activity (14), and this may be the case for *C. tepidum* as well. Therefore, it may be advantageous to include dispensable DNA at the ends of the linearized DNA used for transformation. Such exonuclease activity might explain the difference in transformation frequency with pTN2G11 depending on the enzyme used for linearization (Table 3).

TABLE 3. Transformation with 10 μg of DNA with various lengths of homologous regions

DNA	Total length of homologous DNA (kb)	Transformation frequency[a]
*Ahd*I-digested pTN1G4	2.93	$(1.3 \pm 0.4) \times 10^{-7}$
*Eco*RI-digested pTN1G4	2.93	$(4 \pm 1) \times 10^{-7}$
*Ahd*I-digested pTN2G1	1.08	$(1.1 \pm 0.3) \times 10^{-7}$
*Eco*RI-digested pTN2G1	1.08	$(7 \pm 3) \times 10^{-9}$
*Ahd*I-digested pTN3G11	0.29	0
*Eco*RI-digested pTN3G11	0.29	0

[a] Values are means ± standard deviations of four independent experiments.

These observations suggest that a region of homologous flanking DNA of about 1 kb should be suitable for routine transformation experiments and that it may be advantageous to linearize plasmids with a restriction enzyme that leaves dispensable flanking DNA at the ends of the fragment. Separate fragments of nontransforming DNA produced by an enzyme digest (e.g., the pUC19 vector residue excised by *Eco*RI from pTN1G4) may be disadvantageous because this nontransforming DNA may compete with the transforming DNA for uptake into the cells. Inhibition of transformation by competing DNA was demonstrated by another observation. Addition of 20 μg of sonicated chromosomal DNA from *Synechococcus* to a transformation mixture of *C. tepidum* containing 1 μg of linearized DNA (*Ahd*I-digested pTN1G4) decreased the transformation frequency an order of magnitude (data not shown).

Various selection markers. Three constructs for *nifD* inactivation were made with different antibiotic resistance markers, pTN1G4, pTN1S3, and pTN1CE1 (Table 1). The transformation frequencies were about the same when the *aacC1* gentamicin resistance marker and the *ermC-cat* erythromycin-chloramphenicol resistance marker were used (Table 4.) Antibiotic-resistant mutants were only obtained with the *ermC-cat* marker when erythromycin was used as the selective agent and not when chloramphenicol was used. The obtained Em^r mutants were not Cm^r even though the marker contains both the *cat* and *ermC* genes (Fig. 1d). Southern hybridization analysis con-

TABLE 4. Transformation with 10 μg of DNA with different antibiotic resistance markers

DNA	Drug(s) used for selection[b]	Transformation frequency[a]
*Ahd*I-digested pTN1G4	GM	$(3 \pm 1) \times 10^{-7}$
*Ahd*I-digested pTN1CE1	EM	$(6 \pm 3) \times 10^{-7}$
*Ahd*I-digested pTN1S3	SP and SM	$(3 \pm 1) \times 10^{-3}$

[a] Values are means ± standard deviations of three independent experiments.
[b] GM, gentamicin; EM, erythromycin; SP, spectinomycin; SM, streptomycin.

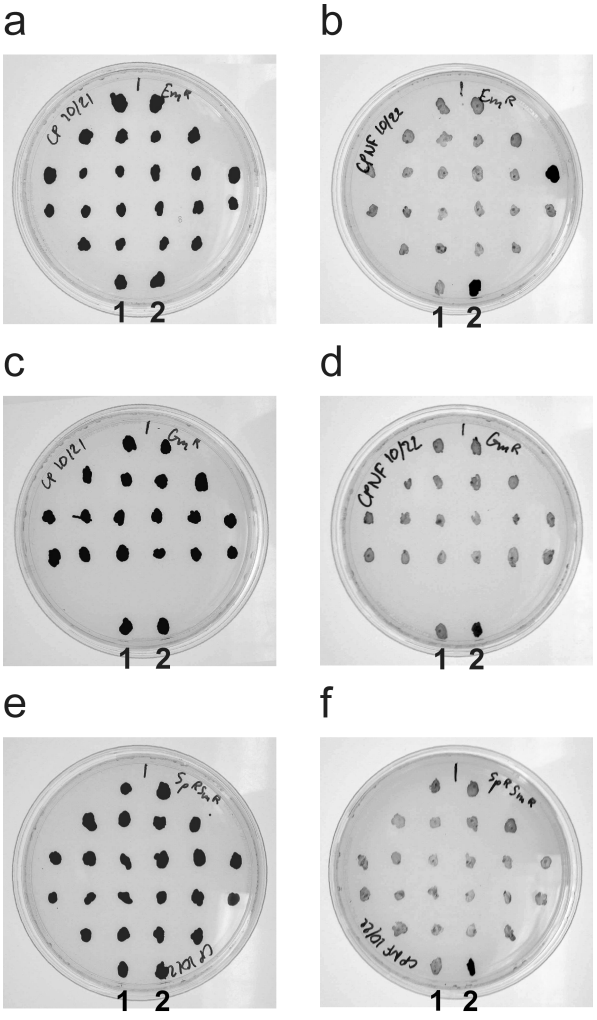

FIG. 4. Growth of wild-type *C. tepidum* and antibiotic-resistant transformants spotted on nonselective CP plates (a, c, and e) and on nonselective CPNF plates (b, d, and f). (a and b) 22 Em^r transformants; (c and d) 18 Gm^r transformants; (e and f) 22 Sm^r/Sp^r transformants. As controls, wild-type *C. tepidum* (labeled 2) and a confirmed Nif^- transformant (labeled 1) were also spotted on all plates.

firmed that the *cat* marker was present in the Em^r mutants (see below; Fig. 4). Thus, this *cat* marker did not function in *C. tepidum*, probably either because the expressed Cat protein is not functional in *C. tepidum* or because the *cat* promoter is too weak in *C. tepidum*. Our failure with the *cat* marker is in contrast to the conjugation studies by Wahlund and Madigan (24), who successfully used a *cat* marker similar to ours for selection in *C. tepidum*. Previous work with the tetracycline resistance marker (*tet*) from pBR325 suggested that this marker does not work in *C. tepidum* (24), and this marker was not investigated further in this study. It is possible that both the *tet* and *cat* markers may work in *C. tepidum* if their promoters are replaced, e.g., with the promoter from *ermC* in pRL409 or from *aacC1* in pMS266 or with a strong indigenous promoter such as that for *csmCA* (4, 5, 8).

A transformation frequency 4 orders of magnitude higher than that obtained with the *aacC1* and *ermC* markers was observed with the *aadA* marker (Table 4). Analysis of the obtained mutants resistant to the combination of streptomycin and spectinomycin (Sm^r/Sp^r mutants) showed that all of 22 mutants analyzed were incapable of diazotrophic growth and that *nifD* therefore was inactivated (see below). The reason for this increased transformation frequency is not clear, but the genomic sequence of *C. tepidum* may hold some clues. In contrast to the *aacC1* and *ermC-cat* markers, the *aadA* marker from pHP45Ω contains a 59-bp recombinational hot spot immediately downstream of *aadA* (17). This hot spot is recognized by IntI-like integrases (6, 18), and the *C. tepidum* genome contains an integrase (CT0176) with high homology to this class of enzymes (J. A. Eisen et al., unpublished data). The hot spot in the *aadA* marker and the indigenous integrase CT0176 in *C. tepidum* may be related to the high transformation frequency of *C. tepidum* with *aadA*-containing plasmid pTN1S3. However, regions of *C. tepidum* DNA were necessary to obtain high transformation frequencies with the *aadA* marker. Transformation with a linear DNA fragment encoding only the *aadA* marker (*Pst*I-digested pHP45Ω) resulted in a transformation frequency of only approximately 3×10^{-8}, which is 5 orders of magnitude lower than the transformation frequency obtained with linearized pTN1S3 (Table 4). The *C. tepidum* genome also contains a gene (encoding CT1017) with a downstream 59-bp recombinational hot spot (J. A. Eisen et al., unpublished data). CT1017 has no significant sequence similarity with any protein in GenBank, but the gene seems to encode a cytoplasmic protein and could be a novel antibiotic resistance-encoding gene (18).

Test of transformants. The expected phenotype of the *C. tepidum nifD* transformants is the inability to reduce dinitrogen. Several mutants obtained by transformation with the three different markers (Table 4) were transferred to selective CP plates three times and then transferred to nonselective CP and nonselective CPNF plates to check for diazotrophic growth (Fig. 4). Wild-type *C. tepidum* grew on CPNF plates although slightly slower than on CP plates. All Gm^r mutants (23 tested), all Sm^r/Sp^r mutants (22 tested), and nearly all Em^r mutants (23 out of 24 tested) failed to grow on the CPNF plates. These results confirm that the mutants had lost the ability to perform nitrogen fixation and that the mutations had segregated completely.

Genomic DNA was isolated from five Gm^r mutants and analyzed by PCR. The results from one of the mutants are shown in Fig. 5a. As expected, PCR with primers specific for *nifD* (nif-F4 and nif-B4) amplified a 0.41-kb fragment in the wild type and a 1.46-kb fragment in the mutants. PCR with primers specific for *aacC1* (GmR-F1 and GmR-B1) did not produce a PCR product in the wild type but amplified a 0.75-kb fragment in the mutants.

Genomic DNA was also isolated from three Em^r Nif^- mutants and analyzed by PCR and Southern hybridization. As expected, PCR with primers nif-F4 and nif-B4 did not produce a 0.41-kb fragment in the mutants. PCR with primers specific for *cat* (CmR-F1 and CmR-B1) did not produce a PCR product in the wild type but amplified a 0.56-kb fragment in the mutants. PCR with primers CmR-B1 and nif-B4 did not produce a PCR product in the wild type but amplified a 1.3-kb fragment in the mutants, which confirms the insertion of the *ermC-cat* marker in *nifD* in the expected orientation. Figure 5b shows a Southern hybridization analysis of *Stu*I-digested

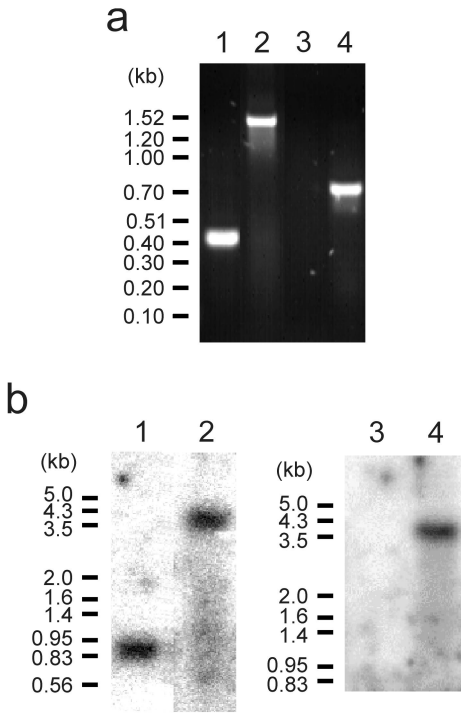

FIG. 5. (a) PCR analysis of wild-type *C. tepidum* (lanes 1 and 3) and a Gm^r mutant (lanes 2 and 4). Primers specific for *nifD*, nif-F4 and nif-B4, were used in lanes 1 and 2, and primers specific for the gentamicin resistance cassette (*accC1*), GmR-F1 and GmR-B1, were used in lanes 3 and 4. (b) Southern hybridization analysis of wild-type *C. tepidum* (lanes 1 and 3) and an Em^r mutant (lanes 2 and 4). A probe specific for *nifD* was used in lanes 1 and 2, and a probe specific for the erythromycin-chloramphenicol resistance cassette (*ermC-cat*) was used in lanes 3 and 4.

genomic DNA from an Em^r Nif^- mutant. As expected, a *nifD* probe hybridized with a 0.9-kb fragment in the wild type and with a 3.6-kb fragment in the mutant. A *cat* probe did not hybridize with wild-type genomic DNA but hybridized with a 3.6-kb fragment in the mutant. PCR analysis of genomic DNA isolated from the only Em^r mutant that exhibited diazotrophic growth showed the presence of wild-type *nifD* using primers nif-F4 and nif-B4 and the absence of *cat* using primers CmR-F1 and CmR-B1. This Em^r Nif^+ mutant could therefore represent a spontaneously resistant mutant or an Em^r transformant produced by an illegitimate recombination event.

Six Sm^r/Sp^r transformants were likewise analyzed by digesting genomic DNA with *Stu*I and performing a Southern hybridization analysis (data not shown). As expected, the *nifD* probe hybridized with a 0.9-kb fragment in the wild type and with a 2.9-kb fragment in the mutants. The *aadA* probe did not hybridize with wild-type genomic DNA but hybridized with a 2.9-kb fragment in all six transformants.

Conclusion. Genes in *C. tepidum* can be insertionally inactivated by natural transformation and homologous recombination. Markers for resistance to gentamicin (*aacC1* from pMS266), erythromycin (*ermC* from pRL409), and streptomycin-spectinomycin (*aadA* from pHP45Ω) were successfully used in the present study to inactivate *nifD*. The *aadA* marker gave a significantly higher transformation yield than the two other markers. We suggest the following general guidelines for routine gene inactivation by natural transformation: (i) cells from at least 100 μl of a late-exponential liquid culture should be used; (ii) linearized DNA (1 to 10 μg) with sequences of at least 0.5 kb of homologous DNA flanking each side of the selection marker should be used; (iii) transforming cells should be spotted on an agar surface and incubated for 10 to 20 h at 40°C; shorter incubation times can probably be used, especially if the incubation temperature is higher. Finally, in its simplest form, transformation may be performed by scraping cells off a plate and incubating a mixture of these cells and transforming DNA on a nonselective plate overnight. The cells should then be restreaked on selective plates the next day.

ACKNOWLEDGMENTS

N.-U.F. was supported by The Danish Natural Science Research Council. This work was supported by U.S. Department of Energy grant DE-FG02–97ER20137 to D.A.B.

We thank the Institute of Genomic Research for prepublication access to the genome sequence of *C. tepidum*.

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