Extremophiles (2001) 5:53–60
Digital Object Identifier (DOI) 10.1007/s007920000173

ORIGINAL PAPER

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Liposome-mediated DNA uptake and transient expression in *Thermotoga*

Received: October 12, 1999 / Accepted: December 1, 2000 / Published online: February 1, 2001

Abstract We report here the successful application of a PCR-based method to detect genetic transformation of *Thermotoga neapolitana* and *Thermotoga maritima*. Plasmid vectors were constructed using pRQ7, an 846-bp plasmid found in *Thermotoga* species strain RQ7, which replicates by a rolling circle mechanism. The vector pJY1 was constructed by placing a gene encoding a thermostable chloramphenicol acetyltransferase from *Staphylococcus aureus* under the control of the *tac* promoter and joining this with pRQ7 in a pBluescript vector. A second vector, pJY2, was similarly constructed using a gene encoding a kanamycin nucleotidyltransferase previously engineered for thermostability. Genetic transformation of *T. neapolitana* and *T. maritima* spheroplasts was achieved using cationic liposomes. The transforming DNA was detected in cells grown in liquid cultures using polymerase chain reaction amplification of the *cat* or *kan* genes. *T. neapolitana* could maintain pJY1 for at least 25 generations in liquid medium containing chloramphenicol. The pJY2 vector conferred kanamycin resistance to *T. maritima* cells grown in liquid culture. Isolation of stable transformants on solid media after 2–3 days of incubation at 77°C was not possible with either vector, probably because of the instability of both vectors and antibiotics under these conditions. However, this transformation procedure provides, for the first time, a method to introduce DNA into this hyperthermophilic bacterium for potential applications such as targeted gene disruption analyses.

Key words Transformation · Vector · Plasmid · Hyperthermophile · *Thermotoga*

Communicated by J. Wiegel

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Introduction

Genetic methods for the study of hyperthermophilic Archaea and Bacteria are at relatively early stages of development because unique technological barriers are posed by the thermophilic and, in some cases, strictly anaerobic nature of these organisms (see Aagaard et al. 1996; Mai and Wiegel 1999; Noll and Vargas 1997 for reviews). Despite these challenges, it is important to develop the ability to genetically manipulate these organisms to provide powerful tools for their further study and technological application. Members of the genus *Thermotoga* are of particular interest for basic research because they represent the deepest lineage of heterotrophs in the domain Bacteria and can provide information about ancestral traits of bacteria (Fitz-Gibbon and House 1999; Woese et al. 1990).

Thermotoga species are fermentative strict anaerobes that grow optimally at 75°–90°C and are found in geothermally heated sediments worldwide (Huber and Stetter 1992). These strains are of biotechnological interest in part because of the large variety of hydrolytic enzymes they possess.

Searching for a means to introduce DNA into any new organism confronts the investigator with a number of experimental variables. Slight variations in experimental parameters can drastically affect the success of a transformation procedure. This restriction is especially true in the case of extreme thermophiles in which a paucity of thermostable selectable markers and the chemical instability of selective agents make detection of a successful transformation difficult. In this study, we attempted to approach the problem by

Table 1. Plasmids used in these studies

Plasmids	Size (kb)	Description	References
pRQ7	0.85	Cryptic miniplasmid from *Thermotoga* strain RQ7	(Harriott et al. 1994)
pHV1248	8.4	Source of the *cat* gene from *S. aureus* plasmid pC194	(Petit et al. 1990)
pMV82	4.59	*cat* gene and RQ7 DNA in pBluescript; Ap^r	This study
pBT	3.25	*tac* promoter DNA cloned between *Bam*HI and *Eco*RI sites in pBluescript; Ap^r	This study
pJY	3.97	*cat* gene cloned between *Eco*RI and *Cla*I sites in pBT; Ap^r, Cm^r	This study
pJY1	4.81	RQ7 DNA cloned between *Bam*HI and *Xba*I sites in pJY; Ap^r, Cm^r	This study
pJY2	4.93	Km^r gene cloned between *Eco*RI and *Cla*I sites in pBT and then RQ7 DNA cloned between *Bam*HI and *Xba*I sites; Ap^r, Km^r	This study

at first measuring transformation without relying on successful expression of the genetic marker. This approach provides the means to optimize transformation methods independent of the selection methodology.

We developed a vector system using the cryptic *Thermotoga* miniplasmid pRQ7, which we discovered (Harriott et al. 1994). This 846-bp plasmid, found in *Thermotoga* species strain RQ7, encodes a single protein that plays a role in its rolling circle mode of replication (Yu and Noll 1997). We demonstrate here successful genetic transformation of *Thermotoga neapolitana* and *Thermotoga maritima* using vectors derived from this plasmid.

Materials and methods

Materials and strains

Chemicals of reagent grade were purchased from Sigma (St. Louis, MO, USA) or Fisher Scientific (Springfield, NJ, USA). Plasmids pBluescript KS(–) and pBluescript II KS(+) came from Stratagene (La Jolla, CA, USA) and pKK223-3 from Pharmacia-LKB (Piscataway, NJ, USA). Polymerase chain reaction (PCR) reagents were obtained from Perkin Elmer (Norwalk, CT, USA). Genius systems (Boehringer Mannheim, Indianapolis, IN, USA) were used for chemiluminescent detection of Southern hybridizations. *Escherichia coli* strain DH10B was used for constructing and maintaining vectors. *Thermotoga* strains were grown in TB-HEPES medium, which is TB medium (Childers et al. 1992) containing (w/v) 0.05% yeast extract, 0.1% glucose, 0.4% starch, and 20 mM HEPES (hydroxyethylpiperazine ethanosulfonic) instead of peperazine-*N*,*N*'-bis[2-ethanesulfonic acid] (PIPES) buffer.

Antibiotic stability

The stability of antibiotics (carbenicillin, penicillin, chloramphenicol, hygromycin, thiostrepton, and kanamycin) was assessed by dissolving each drug in TB-HEPES medium at a concentration of 0.5 mg/ml and incubating these media under nitrogen atmospheres at 77°C and 20°C for 48 h. Aliquots were removed from each sample at 0, 24, and 48 h, and fourfold serial dilutions were made into the growth medium for the test organism (0.15–250 μg/ml). *E. coli* strain LE392 was used as the test organism, except with thiostrepton, in which case *Staphylococcus aureus* was used. An aliquot of an exponentially growing culture of the test organism was inoculated into the growth media and incubated at 37°C for 24 h. The optical density of each culture (including a control without antibiotics) was read at 620 nm, and the results of duplicate measurements were averaged. The potency of each antibiotic was assessed as the fold-increase in the MIC (minimal inhibitory concentration, the lowest concentration that gave an $OD_{620} < 0.05$ after 24 h) when the 0-, 24-, and 48-h incubation intervals were compared.

Construction of vectors

Thermotoga sp. strain RQ7 was grown under strictly anaerobic conditions in a complex medium as previously described (Harriott et al. 1994), and plasmid pRQ7 was isolated from this strain by a modified alkaline lysis method (Lee and Rasheed 1990). In vitro recombinant DNA manipulations were performed using standard protocols (Sambrook et al. 1989). Plasmid pHV1248 containing the chloramphenicol acetyltransferase (*cat*) gene from *Staphylococcus aureus* plasmid pC194 was from the Bacillus Genetic Stock Center at the Ohio State University (Petit et al. 1990) (Table 1). *Thermus thermophilus* plasmid pPP442, containing a kanamycin nucleotidyltransferase gene (*kan*) engineered for increased thermostability, was a generous gift from Dr. T. Hoshino (University of Tsukuba, Japan) (Maseda and Hoshino 1996).

Plasmid pRQ7 has a 22-bp region downstream of its open reading frame (ORF) that, except for three stop codons, is unlikely to contain information essential to plasmid functions (Harriott et al. 1994). Synthetic oligo-

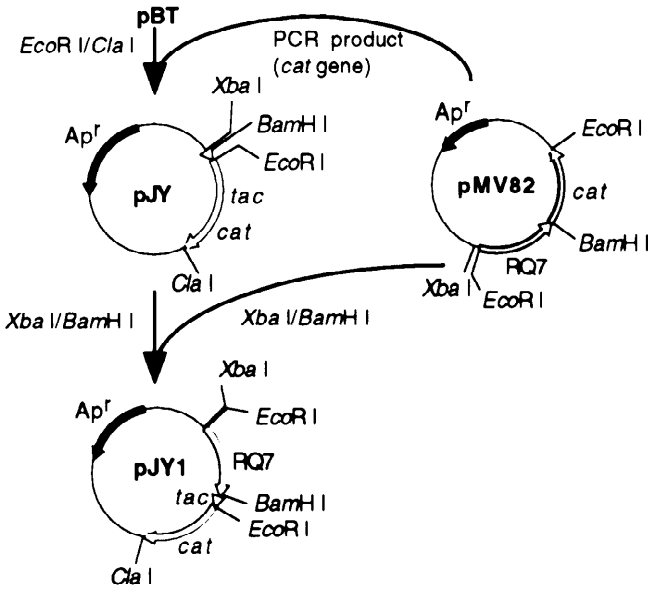

Fig. 1. Construction of plasmid pJY1

nucleotides complementary to this region were designed to maintain two stop codons and introduce adjacent *Bam*HI and *Eco*RI sites, and these were used as PCR primers to generate a modified form of pRQ7 containing these cloning sites. These primers were 5′-GGGAATTCGAATGTGGTTAGTGTGATTAG-3′ and 5′-GGGGATCCGTTAACCATATCCCACTAGTTC-3′. PCR primers were also designed to introduce *Bam*HI and *Eco*RI sites flanking the *cat* gene from pHV1248. These primers were 5′-GGGGATCCAGTAATATTGACTTTTAAAAAAGG-3′ and 5′-GGGAATTCATATTATAAAAGCCAGTCATTAGG-3′. Both PCR products were digested with *Bam*HI, ligated, and digested with *Eco*RI. This product was ligated to *Eco*RI-digested pBluescript KS(–) to give plasmid pMV82. Plasmid pMV82 has the orientation *lacI*-RQ7-*cat*-*lacZ* (Fig. 1).

The native promoter of the *cat* gene was replaced with the *tac* promoter from pKK223-3. To do so, plasmid pBT was constructed with a 285-bp, *Bam*HI/*Eco*RI fragment of pKK223-3 (containing the *tac* promoter), which was cloned into the *Eco*RI and *Bam*HI sites of pBluescript II KS(+) (Fig. 1). From this, plasmid pJY was constructed by cloning a 620-bp fragment containing the *cat* gene derived by PCR amplification from pMV82 using the primers 5′-GGGAATTCAGGCATATCAAATGAACTT-3′ and 5′-CCATCGATATATTATAAAAGCCAGTCATTA-3′ into the *Eco*RI and *Cla*I sites of pBT. Plasmid pJY1 was constructed by inserting an 860-bp fragment of pMV82 that contained pRQ7 into the *Bam*HI and *Xba*I sites in pJY. To construct pJY2, the *cat* gene was replaced in pJY1 with the *kan* gene from pPP442. An 818-bp fragment of pPP442 containing *kan* was amplified using the primers 5′-CCACACCCGAATTCGAGCTA-3′ and 5′-CCATCGATTCATCGTTCAAAATGGTATGCG-3′, which introduced flanking *Cla*I and *Eco*RI sites. The *cat* gene was removed from pJY1 using *Cla*I and *Eco*RI and was replaced with the amplified *kan* gene.

Transformation

For liposome-mediated transformation, all procedures were carried out under strictly anaerobic conditions inside an anoxic chamber. Once opened, the DOTAP liposome reagent (Boehringer Mannheim, Indianapolis, IN, USA) was stored under a nitrogen atmosphere. To form spheroplasts, a 100-ml culture of *Thermotoga* cells grown to early stationary phase was harvested and resuspended to a final volume of 1 ml in a solution of 4.5 mM NH_4Cl, 0.3 mM $CaCl_2$, 0.34 mM K_2HPO_4, 22 mM KCl, 2 mM $MgSO_4$, 340 mM NaCl, 20 mM HEPES, pH 7.4, and 300 µg/ml lysozyme. The cell suspension was incubated for 30 min at 37°C and then for 5 min at 77°C, and the formation of spheroplasts was monitored microscopically. A DNA:liposome mixture was prepared by adding 1 µg DNA to 50 µl 20 mM HEPES buffer (pH 7.4), then adding 20 µl DOTAP with thorough mixing, diluting the mixture with 30 µl 20 mM HEPES buffer (pH 7.4), and then incubating the mixture for 15 min at room temperature. The DNA:liposome mixture was then added to the spheroplast suspension and incubated for 1 h at 37°C. A 0.5-ml portion of the spheroplast suspension was transferred to 10 ml complex medium and incubated at 77°C for cell recovery (3–6 h).

To monitor the stability of the plasmid during growth, an aliquot of recovered cells was inoculated into growth medium (3%–5%, v/v), with or without selection, and following incubation for the specified periods, 1-ml samples were removed for PCR analyses. For plating, transformed spheroplasts were allowed to recover for 6 h in 10 ml complex growth medium at 77°C, and then aliquots were plated inside an anoxic chamber onto medium with antibiotics using an overlay method. In this overlay technique, a cell suspension (10–200 µl) was added to 1 ml of an anoxic 0.3% Gelrite, 0.4% glucose solution, which was then poured onto a bottom layer of 5 ml 0.7% Gelrite prepared with complex medium containing 200 mM HEPES (pH 7.5) in a 50 × 9 mm petri dish. Following solidification of the overlay, the plates were sealed in wide-mouthed canning jars and incubated at 77°C for 2–3 days. It was necessary to incubate the plates in an upright position for 24 h to allow the overlays to firmly attach to the bottom layers: the jars were then rotated to put the plates in an upside-down position for the remaining 1–2 days.

PCR was used to perform qualitative analyses for the presence of transforming DNA. In the growth experiments, 1-ml aliquots of cells were removed as previously described, and cells were collected by centrifugation in a microcentrifuge for 2 min. The cell pellets were resuspended in 200 µl lysis buffer (0.1% Triton X-100 in 10 mM Tris-HCl, pH 7.5). When the suspensions cleared, the cell lysates were extracted with the same volume of phenol/chloroform, and 5 µl of the cleared lysates were used as templates for PCR analyses. The 100-µl PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM dNTPs

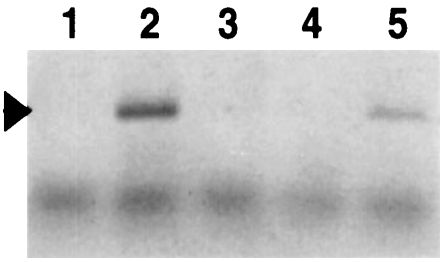

Fig. 2. DNase treatment of *T. neapolitana* cells transformed with pJY1. Cells were collected 20 h after incubation following transformation with pJY1 as described in Materials and methods. Lanes *1* and *3* are amplifications of DNA extracted from *T. neapolitana* cells treated with liposomes without DNA. Lanes *2*, *4*, and *5* are amplifications of DNA extracted from *T. neapolitana* cells transformed with pJY1. Cells in lanes *3* and *5* were treated with DNase before cell lysis. Cells in lane *4* were DNase treated after cell lysis. The image is an inverted image of an ethidium bromide-stained agarose gel. *Arrowhead* indicates the location of pJY1

(denucleoside triphosphates), 40 pmole each 5′ and 3′ primers, 2.5 U *Taq* polymerase (Perkin Elmer), and 3 mM $MgCl_2$.

To further identify the plasmid in transformed *T. neapolitana* cells, we isolated total genomic DNA from 100-ml cultures of *T. neapolitana* cells grown either after transformation with pJY1 or without transformation. Each of these cultures had been transferred through three successive transfers in media with or without 150 μg/ml chloramphenicol. DNA isolated from these cultures was separately digested with *Cla*I and *Eco*RI, resolved by agarose gel electrophoresis, and blotted by Southern transfer for hybridization with a digoxygenin- (DIG-) labeled *cat* gene probe.

DNase treatment of cells

To determine whether DNA was taken up by cells treated with pJY1 DNA, these cells were subjected to treatment with DNase I and then used as a source of DNA template for PCR amplifications. Cells treated with the pJY1/liposome mixture were used as an inoculum and grown for 20 h with selection as described previously. Aliquots of 1 ml were removed, and cells were collected by centrifugation in a microcentrifuge for 2 min. Those cells left untreated (Fig. 2, lanes 1 and 2) were suspended in 0.2 ml lysis buffer and the cleared lysate extracted with phenol/chloroform. Those to be treated with DNase before cell lysis (Fig. 2, lanes 3 and 5) were suspended in 0.4 ml Tris-HCl (pH 8.0) containing 1 mM $MgCl_2$ and DNase I (50 U/ml; Sigma) and incubated at 37°C for 1 h. These cells were then collected by centrifugation, washed twice in 10 mM Tris buffer (pH 8.0) containing 2 mM EDTA (ethylenediaminetetraacetic acid), resuspended in 0.2 ml lysis buffer, and the DNA extracted as already described. One aliquot of cells (lane 4, Fig. 2) was suspended in 0.2 ml lysis buffer; the DNA extracted with phenol/chloroform; resuspended in 10 mM Tris, 1 mM $MgCl_2$ buffer (pH 8.0); treated with DNase I as previously; and the DNA extracted with phenol/chloroform. In each case, a 5-μl aliquot of the phenol/chloroform extract was used for the PCR template.

Results

Chloramphenicol and kanamycin as selective agents

The choice of selectable markers for use with hyperthermophiles is limited. Vectors containing markers derived from chromosomal genes present problems of recombination of the vector into the genome; dominance; and the necessity, in some cases, to derive mutant host strains. Antibiotic-resistant markers are much more versatile, but thermal stability of both the antibiotic and the enzyme conferring the resistance is necessary (Mai and Wiegel 1999). Genes conferring thermally stable resistance to chloramphenicol, kanamycin, and, more recently, hygromycin are available (Cannio et al. 1998; Liao et al. 1986; Mai et al. 1997; Soutschek-Bauer et al. 1987).

We tested the stability of various antibiotics in *Thermotoga* liquid medium at 77°C and also their effectiveness at inhibiting the growth of *T. neapolitana*. We found that penicillin and thiostrepton lost detectable activity against the tester organisms after 24 h (Table 2). Carbenicillin, hygromycin, chloramphenicol, and kanamycin were fourfold less potent after 48 h. Unlike the other antibiotics, chloramphenicol and thiostrepton were unaffected by incubation under reducing conditions at 20°C for 48 h. These thermostability results are consistent with those reported by others (Peteranderl et al. 1990). Our results suggest that carbenicillin, chloramphenicol, and kanamycin would be sufficiently stable during short-term liquid medium cultivation (e.g., overnight growth) but may be less effective for longer incubations (e.g., growth on solid medium). The growth of *T. neapolitana* is sensitive to chloramphenicol, but not kanamycin. *T. maritima* is sensitive to kanamycin, but not carbenicillin. In liquid culture, growth of *T. neapolitana* from a 3% (v/v) inoculum measured after 24 h is completely inhibited by 100 μg/ml chloramphenicol. On solid medium, some spontaneously resistant mutants begin to form colonies after 3 days of incubation at this concentration. Higher concentrations (200–300 μg/ml) slow or prevent the growth of such colonies. *T. maritima* will not grow overnight in liquid medium containing 100 μg/ml kanamycin. These results suggest the use of chloramphenicol and kanamycin as selective agents for use with *T. neapolitana* and *T. maritima*, respectively.

Vector construction

We constructed a vector, pMV82, by introducing *Bam*HI and *Eco*RI sites into the *Thermotoga* species strain RQ7 plasmid pRQ7 and introducing this into the *E. coli* plasmid pBluescript (KS–) (see Fig. 1). As a selectable marker, the

Table 2. Antibiotic stability in TB-HEPES medium at different temperatures

Antibiotic	MIC prior to incubation[a]	MIC after incubation for 24 h at 77°C	MIC after incubation for 48 h at 77°C	MIC after incubation for 48 h at 20°C
Carbenicillin	3.9	3.9	15.6	15.6
Penicillin	62.5	>250	>250	>250
Chloramphenicol	15.6	62.5	62.5	15.6
Hygromycin	62.5	62.5	250	250
Thiostrepton	0.24	>250	>250	0.24
Kanamycin	3.9	15.6	62.5	62.5

[a]MICs are the concentrations of antibiotic (μg/ml) that gave an $OD_{620} < 0.05$ of the tester organism after 24-h incubation. "Incubation" refers to incubation of the antibiotic in TB-HEPES medium under anaerobic conditions

cat gene from pHV1248 was placed adjacent to the pRQ7 derivative. However, chloramphenicol resistance was relatively poorly expressed in *E. coli* (resistance only to 15–20 μg/ml). To improve expression of the *cat* gene in both *T. neapolitana* and *E. coli*, both these components were introduced into pBluescript II (KS+) and the native *cat* promoter from pHV1248 was replaced with the *tac* promoter (Fig. 1). The *tac* promoter is coincidentally identical to the consensus promoter for *T. maritima* ribosomal protein genes L1, L10, L11, and L12; some tRNA genes; and the RNA polymerase β-subunit (Liao and Dennis 1992). This construct, pJY1, improved expression of *cat* in *E. coli* so that *E. coli* strain DH10B harboring pJY1 was resistant to 50 μg/ml chloramphenicol in the absence of isopropyl thiogalactoside (IPTG) and 300 μg/ml chloramphenicol in the presence of IPTG. Vector pJY2 was constructed for kanamycin selection in *T. maritima*. The *kan* gene encoding a thermostable kanamycin nucleotidyltransferase was put in the place of the *cat* gene of pJY1, and its expression was controlled by the *tac* promoter. *E. coli* strain DH10B harboring pJY2 was resistant to as much as 250 μg/ml kanamycin in the presence of IPTG.

Liposome-mediated transformation

A liposome-mediated transformation method was modified from the protocol used for the archaeon *Methanosarcina* (Metcalf et al. 1997). It was necessary to convert *Thermotoga* cells into spheroplasts before applying the liposome:DNA mixture because transformation was not successful when the mixture was applied directly to resuspended cells. *T. neapolitana* spheroplasts were prepared, mixed with a pJY1 DNA:liposome mixture, and a small portion (typically 3%, v/v) of this mixture was inoculated into growth medium following a period of recovery. Because the stability of chloramphenicol for the several days needed for colony growth could be problematic, we chose to first test transformation methods in liquid cultures. PCR was used to monitor the fate of the plasmid (specifically the *cat* gene) in treated cells. PCR has been used as a method to qualitatively detect the presence of transforming DNA in liquid cultures of hyperthermophiles and mesophiles in which genetic selection is problematic (Aagaard et al. 1996; Rachek et al. 1998). By this method, DNA uptake by *T. neapolitana* could be detected. We detected the *cat* gene following treatment for up to 20 h of incubation in media containing 150 μg/ml chloramphenicol (Fig. 3, lanes 6–9). Transformed cells could be transferred in two successive transfers and the plasmid was still detectable (Fig. 3, lanes 10–13). Transformed cells could not grow in media containing 200 μg/ml or 300 μg/ml chloramphenicol. Controls showed that no DNA could be amplified from untreated cells using these primers and PCR conditions (Fig. 3, lanes 2–5).

Selective marker expression and plasmid stability

When we transformed *T. neapolitana* with pJY, a plasmid lacking the pRQ7 portion of pJY1, we detected the *cat* gene in cells 3 h after inoculation into growth medium but not after 20 h (data not shown). Because the plasmid lacks pRQ7 DNA, it apparently does not replicate in *T. neapolitana* cells, thus demonstrating the necessity of pRQ7 DNA for plasmid maintenance.

If cells treated with the pJY1/liposome mixture transported the DNA, the added DNA should be protected from extracellular DNase added to the cell suspension, a test commonly used to assess transformation. We collected, after 20 h of growth, 1 ml of *T. neapolitana* cells treated with pJY1 and treated them with DNase before or after deliberate lysis of the cells (Fig. 2). When cells were lysed before DNase treatment, pJY1 DNA was not detectable (Fig. 2, lane 4). However, when intact cells were treated with DNase, pJY1 DNA was detectable (Fig. 2, lane 5). The band in lane 5 may be fainter than that in lane 2 because some cell lysis may have occurred during DNase treatment, some EDTA may have carried over to the PCR reaction, or a fraction of the DNA might have remained accessible to

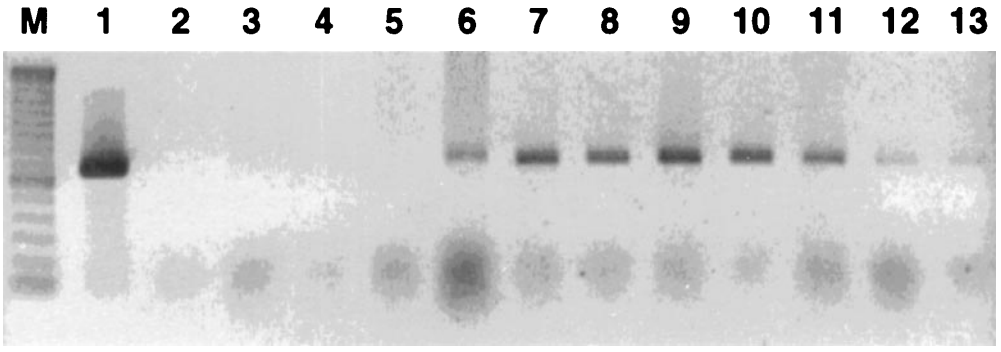

Fig. 3. Polymerase chain reaction (PCR) assay using primers to detect the *cat* gene from transformed *Thermotoga neapolitana* cells. *M*, 100-bp DNA ladder; *lane 1*, PCR product of the *cat* gene. After incubating the cell suspension for 6 h to allow recovery following treatment with the liposome mixture, an aliquot of cells was removed for PCR amplification (0-h lanes, lanes *2* and *6*), and another aliquot was inoculated into fresh medium (5% v/v) either without chloramphenicol (lanes *2–5*) or with 150 μg/ml chloramphenicol added after 3-h incubation (lanes *6–13*). Aliquots of 1 ml were removed from these cultures at the indicated intervals, and DNA was extracted for PCR amplification. DNA was extracted and amplified from cells treated with liposomes but without DNA and grown for 3, 6, and 20 h (lanes *3*, *4*, and *5*, respectively) without chloramphenicol. DNA was also extracted and amplified from cells treated with a pJY1/liposome mixture and grown for 3, 6, and 20 h (lanes *7*, *8*, and *9*, respectively) with chloramphenicol. Cells from the culture shown in lane *8* were inoculated (3%) into fresh medium (with chloramphenicol), and samples were removed after 6 and 20 h incubation (lanes *10* and *11*, respectively). Cells from the culture shown in lane *10* were inoculated (3%) into fresh medium (with chloramphenicol) and samples were removed after 6 and 20 h incubation (lanes *12* and *13*, respectively). The image is an inverted image of an ethidium bromide-stained agarose gel

DNase digestion. Clearly, however, a significant portion of plasmid pJY1 was taken up by *T. neapolitana* cells or bound in a DNase-inaccessible state.

To demonstrate that this DNA genetically transformed the cells, we attempted to measure maintenance of the plasmid in a culture of cells after several generations. *T. neapolitana* cells were transformed with pJY1 and grown in liquid media with or without 150 μg/ml chloramphenicol. From the recovered cell suspension (see Materials and methods), a 3% (v/v) inoculation was made into medium. After growth overnight, cells were reinoculated each day (3% inoculum) into fresh medium. A 1-ml aliquot of cells was removed each day for PCR analysis. The cells maintained pJY1 up to 3 days in medium without chloramphenicol and up to 5 days in medium with chloramphenicol (Fig. 4). The latter represents approximately 25 generations of growth. By contrast, untransformed cells did not grow overnight in medium containing 150 μg/ml chloramphenicol with a 3% inoculum. Thus, the presence of the plasmid conferred the expected phenotype, and so the cells were genetically transformed.

This experiment also demonstrated that the PCR detection method detected replicating DNA and was not simply detecting the DNA originally mixed with the cells. Because we used 3% inocula, the amount of the added DNA in the 10-ml culture after six transfers could be at most 8.1×10^{-7} ng. As only 1/400 of this culture was used for the PCR reaction, at most only 2.0×10^{-9} ng of added pJY1 would be available for the PCR reaction. We determined that no less than 2×10^{-6} ng of pure target DNA can be detected under these PCR conditions. Therefore, the detected amount of crude DNA extracted from cells was at least 1,000 fold greater than that which could have remained from the original transformation mixture. These data eliminate the possibility that the plasmid DNA detected in cells may have been sequestered (perhaps intraperiplasmically) in a DNase-resistant, nonreplicating form.

We verified that the plasmid DNA isolated from transformed cells was pJY1 by isolating total DNA from cultures of *T. neapolitana* cells grown either after transformation with pJY1 or without transformation and subjecting this DNA to restriction digestion and hybridization with labeled *cat* gene probe. These experiments showed that the DNA isolated from transformed cells contained a plasmid that had the *cat* gene located on restriction fragments of the predicted size for pJY1. No hybridization was observed to DNA extracted from cells that were subjected to the transformation procedure without pJY1 DNA (data not shown). These results demonstrate that transformed cells, but not untransformed cells, possess pJY1 containing the *cat* gene.

We also attempted to detect transformation by the traditional method of plating the transformation mixture and selection for transformants. However, plating transformed cells on solid medium gave inconsistent results. Untreated cells gave no colonies after 3 days of incubation when 150 μg/ml chloramphenicol was added to the media, but at lower concentrations and 1 week of incubation, spontaneously resistant mutants formed colonies. After 3 days on medium containing 150 μg/ml chloramphenicol, cells treated with DNA sometimes gave rise to colonies, but we could not detect the plasmid in these cells by PCR, nor could they grow after inoculation into liquid medium containing chloramphenicol. This result may be explained if transformed cells frequently give rise to progeny that have lost the plasmid. These plasmidless progeny may be able to overgrow the transformed cells if the concentration of

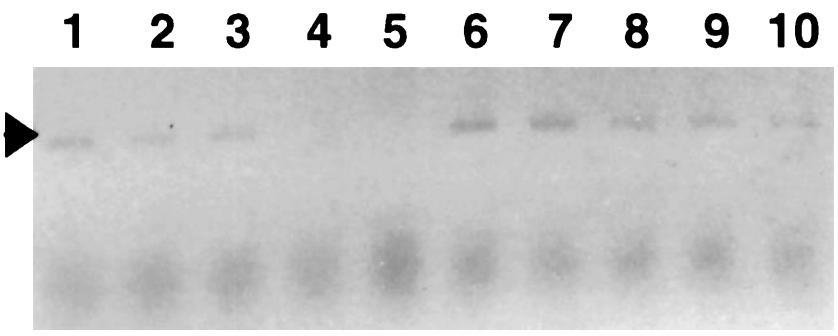

Fig. 4. PCR detection of the *cat* gene to monitor maintenance of pJY1 in transformed *T. neapolitana* grown with and without selection. Cells were collected each day and reinoculated into fresh medium as described in Materials and methods. Lanes *1–10* are amplifications of DNA extracted from *T. neapolitana* cells from cultures grown 1–5 days following transformation with pJY1. Transformed *T. neapolitana* cells were grown in media without (lanes *1–5*) or with (lanes *6–10*) 150 μg/ml chloramphenicol. The image is an inverted image of an ethidium bromide-stained agarose gel. *Arrowhead* indicates the location of pJY1

Table 3. Growth of *T. maritima* cells transformed with pJY2

Time of incubation (h)	Cells, no kanamycin	Cells, with kanamycin	Cells, with pJY2 and kanamycin
0	0.032	0.037	0.087
3	0.152	0.048	0.129
20	0.975	0.074	0.221

Values shown are optical densities at 600 nm. *T. maritima* cells were treated with the liposome mixture with or without pJY2 and these cells were allowed to recover for 3 h in the absence of kanamycin. A 5% (v/v) inoculum of these cells was added to medium with (for cells treated with pJY2) or without 120 μg/ml kanamycin

effective chloramphenicol decreases as a result of its thermal instability during the prolonged incubation necessary for colony development.

We constructed pJY2 to allow use of kanamycin for genetic selections with *T. maritima*. Similar to our results with pJY1 and *T. neapolitana, T. maritima* transformed with pJY2 grew in 20-h liquid cultures containing 120 μg/ml of kanamycin as compared with cultures of cells subjected to the transformation procedure without pJY2 DNA (Table 3). This finding suggests that the thermostable kanamycin nucleotidyltransferase was expressed in transformed *T. maritima* cells. To measure the transformation frequency, we plated transformed *T. maritima* cells on solid medium containing 120 μg/ml kanamycin using the overlay plating method. When we plated nontransformed *T. maritima* cells (approximately 10^8 cells), 10–40 colonies grew, whereas approximately 1,000 colonies grew when the same number of transformed cells was plated. As was the case with *T. neapolitana* transformed with pJY1, we could not detect the transforming plasmid in the cells of these colonies nor could these cells grow in liquid medium containing kanamycin, again suggesting a combined effect of plasmid and antibiotic instabilities.

Discussion

We have embarked on the systematic development of the necessary tools to make *Thermotoga* species amenable to genetic analysis. The demonstration of transformation of these *Thermotoga* species is a crucial first step to provide the genetic tools to learn how these organisms live under their extreme conditions of growth. We have shown here that not only can DNA be introduced into these *Thermotoga* species, but that chloramphenicol resistance and kanamycin resistance markers can be expressed in cells in liquid cultures. However, their utility is limited by the fact that stable transformants cannot be isolated on solid media. The instability of the plasmid is unlikely to be caused by restriction activity because it takes several generations to remove the markers from the population. As the plasmids replicate by a rolling circle mechanism, their stability may be impaired by the presence of additional DNA introduced into the parent plasmid, as has been observed for engineered rolling circle replicating plasmids from gram-positive bacteria (Bron et al. 1988; Gruss and Ehrlich 1988). The inability to obtain colonies of transformants is also likely to be caused by the instability of these antibiotics under the required growth conditions for the 3 days necessary for colony development.

In the present state of development, the transformation method may allow targeted replacement of chromosomal genes by homologous recombination. Although we were unable to obtain transformant colonies, transformed cells can grow in liquid culture under selective conditions in which wild-type cells do not grow. These markers could allow an initial enrichment of transformants to provide more efficient screening for mutants if these markers are used to disrupt chromosomal genes by insertional mutagenesis, for example. Because we have shown that a nonreplicating plasmid (pJY) can be introduced into cells, a standard *E. coli* cloning vector containing one of these selectable markers and a deletion derivative of a chromosomal gene could be introduced into cells to create a

deletion mutation. Alternatively, the use of a replicating plasmid (pJY1 or pJY2) may be advantageous because the single-stranded intermediates they produce may be more recombinogenic than double-stranded plasmid DNA. Homologous recombination of such single-stranded DNA in *T. maritima* is likely as it has RecA (Wetmur et al. 1994).

The availability of the full genome sequence for *T. maritima* provides directed targeting of genes (Nelson et al. 1999). Methods to target specific genes for deletion mutations are currently under development. The construction of genetic tools for the study of *Thermotoga* species will open numerous possibilities to take advantage of the accumulating physiological, biotechnological, and genomic information regarding these organisms.

Acknowledgments This work was supported by grants from the National Science Foundation (MCB-9418197), the U.S. Department of Energy (DE-FG02-93ER20122), and the University of Connecticut Research Foundation. The authors thank Mark Clauss, Paul Betts, and Susan Childers for assistance with this work. The UConn Biotechnology Center provided facilities for and assistance with oligonucleotide synthesis, DNA sequencing, and PCR.

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