

Construction of a Shuttle Vector for, and Spheroplast Transformation of, the Hyperthermophilic Archaeon *Pyrococcus abyssi*

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Our understanding of the genetics of species of the best-studied hyperthermophilic archaea, *Pyrococcus* spp., is presently limited by the lack of suitable genetic tools, such as a stable cloning vector and the ability to select individual transformants on plates. Here we describe the development of a reliable host-vector system for the hyperthermophilic archaeon *Pyrococcus abyssi*. Shuttle vectors were constructed based on the endogenous plasmid pGT5 from *P. abyssi* strain GE5 and the bacterial vector pLitmus38. As no antibiotic resistance marker is currently available for *Pyrococcus* spp., we generated a selectable auxotrophic marker. Uracil auxotrophs resistant to 5-fluorotic acid were isolated from *P. abyssi* strain GE9 (devoid of pGT5). Genetic analysis of these mutants revealed mutations in the *pyrE* and/or *pyrF* genes, encoding key enzymes of the pyrimidine biosynthetic pathway. Two *pyrE* mutants exhibiting low reversion rates were retained for complementation experiments. For that purpose, the *pyrE* gene, encoding orotate phosphoribosyltransferase (OPRTase) of the thermoacidophilic crenarchaeote *Sulfolobus acidocaldarius*, was introduced into the pGT5-based vector, giving rise to pYS2. With a polyethylene glycol-spheroplast method, we could reproducibly transform *P. abyssi* GE9 *pyrE* mutants to prototrophy, though with low frequency (10^2 to 10^3 transformants per μg of pYS2 plasmid DNA). Transformants did grow as well as the wild type on minimal medium without uracil and showed comparable OPRTase activity. Vector pYS2 proved to be very stable and was maintained at high copy number under selective conditions in both *Escherichia coli* and *P. abyssi*.

Pyrococcus species are anaerobic hyperthermophilic archaea that grow optimally at 100 C, with a maximum at around 105 C. They belong to the *Thermococcales* order (genera *Pyrococcus* and *Thermococcus*), the most important order (with 25 species currently described) of the second archaeal phylum, the *Euryarchaeota* (52). All of them are heterotrophic sulfur reducers, fermenting mostly proteinaceous substrates (some species are able to use oligo- and polysaccharides) in the presence or absence of elemental sulfur (S^0) as the terminal electron acceptor (leading to H_2S production). Several laboratories have chosen *Pyrococcus* spp. among other anaerobic hyperthermophiles as model organisms because these versatile organisms are relatively easy to handle and can be grown to high cell density for biochemical analyses. These microorganisms are of major interest in basic research as a model for investigating diverse aspects of life under extreme conditions and because of their evolutionary significance; they also have a great biotechnological potential because of the large variety of thermostable enzymes that they possess.

The genomes of three of them, *Pyrococcus horikoshii* (22) (<http://www.bio.nite.go.jp/>), *Pyrococcus abyssi* (<http://www.genoscope.cns.fr/Pab/>), and *Pyrococcus furiosus* (<http://www.genome.utah.edu/>) have been completely sequenced, and the data are publicly available. Although this unique situation for archaeal representatives allowed meaningful analyses of the speciation

mechanisms at the genome level (27) and led to the identification of the first archaeal replication origin region, *oriC* (33), bioinformatic studies are limited, as typically about half of the open reading frames in these genomes remain without known or predicted function. Genetic techniques, representing some of the most powerful investigative tools in biology, will certainly play an important role in elucidating the function of these genes and in unraveling their mechanisms of modulated expression. Thus, it is now a priority to develop suitable gene transfer systems for this particular class of microorganisms.

For *Archaea*, such genetic tools were first developed in the extreme halophiles (essentially, *Halobacterium* and *Haloferax* spp.) (9, 10, 12) and later for mesophilic methanogens of the genera *Methanococcus* and *Methanosarcina* (43, 49). These genetic systems benefit now from efficient transformation methods and diverse genetic tools (including shuttle vectors and integrative expression vectors, composite transposons, and reporter genes) and have led to investigations on the expression of genes involved in the synthesis of bacteriorhodopsin (29) and gas vesicles (19, 39) in halophiles and in hydrogen metabolism, nitrogen fixation, and flagellin assembly in methanogens (for a review, see references 26 and 45).

In contrast, genetic methods for the hyperthermophilic archaea are still at early stages of development, mainly because of the unique technological barriers imposed by the thermophilic and, in some case, the strictly anaerobic nature of these organisms (for a review, see reference 38). Indeed, most progress has been obtained so far within the genus *Sulfolobus*, whose members are sulfur-metabolizing aerobes belonging to the Crenarchaeota phylum. Many of them were shown to pos-

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sess extrachromosomal elements, conjugative plasmids, and viruses (51), such as the virus SSV1, which served as the basis for the construction of the first generation of shuttle vectors (6, 40). In addition, a variety of analogue-resistant and auxotrophic mutants (18, 21, 25) and a system of exchange and recombination of chromosomal markers by conjugation have been described (17).

Our model organism, *P. abyssi*, was isolated from a deep-sea hydrothermal vent at 2,000 m depth in the North Fiji Basin (southwest Pacific) (16). It is typical of other pyrococci: it grows fast (doubling time of 35 min) under optimal conditions (96 to 100°C, pH 6.8, 3% NaCl), and growth is stimulated by the presence of elemental sulfur. An important characteristic of *P. abyssi* is its ability to grow well on a completely defined medium consisting of a mixture of amino acids and vitamins (47). This property allowed us to isolate the first auxotrophic mutants by plating on medium solidified with the thermostable gellum gum called Gelrite (15, 46, 48).

Another important feature is the presence of a small cryptic plasmid, pGT5 (3.5 kb), in the type strain *P. abyssi* GE5 (13). It is the first described and so far the only plasmid of *Thermococcales* to be studied at the molecular level (14). In particular, it was shown that this plasmid replicates via a rolling-circle mechanism and that this process is specifically initiated by a Rep protein encoded by the largest open reading frame (ORF) of pGT5 (29, 30). This plasmid was fused with pUC19 to create a potential shuttle vector between *Escherichia coli* and both *P. furiosus* and *Sulfolobus acidocaldarius*. The chimerical plasmid could be introduced in both organisms by CaCl₂ treatment followed by heat shock (1). Later, this construct was completed by introduction of a potential selectable marker (as discussed below). The resulting shuttle vector was shown to be maintained, though in low copy number, in liquid cultures of these archaea for several generations after transformation (3).

A genetic marker to score for successful transformation events is mandatory for the set-up of an efficient cloning vector. Genes conferring resistance to growth-inhibitory compounds (generally an antibiotic) are the most convenient selectable markers and have been widely used in mesophilic bacteria but are scarce in mesophilic archaea. The choice is even more restricted for hyperthermophilic archaea, mainly because both the candidate antibiotic and the corresponding resistance-conferring gene product have to be thermostable. At the time we started this work, only two thermostable genetic markers were available: a thermostable derivative of an *E. coli* hygromycin phosphotransferase that had allowed selection of *Sulfolobus solfataricus* transformants on plates (6, 7), and an alcohol dehydrogenase (*adh* gene) from *S. solfataricus* that conferred resistance to butanol in liquid cultures of transformants in *S. acidocaldarius* and *Pyrococcus furiosus* (1, 3).

Early tests that we performed with *Pyrococcus* strains showed that *P. abyssi* strains are naturally resistant to high level of hygromycin (MIC > 300 µg/ml), which precluded the use of this antibiotic for selection of transformants. Moreover, transformation of *P. abyssi* with the vector pAG21 (and similar constructs) carrying the *S. solfataricus adh* marker (3) did not permit the selection of transformants on plates (S. Lucas and G. Erauso, unpublished data).

As an alternative, we searched other genetic markers such as those resulting in auxotrophy. The orotate phosphoribosyl-

transferase (OPRTase) gene (*pyrE*) and the orotidine 5'-monophosphate decarboxylase gene (*pyrF*), involved in pyrimidine biosynthesis, have unique characteristics. Strains deficient in one of these genes become resistant to 5-fluorouracil (5-FOA), which inhibits growth of the wild-type strains. Thus, 5-FOA has been used successfully for positive isolation of uracil auxotrophs in many microorganisms, and their *pyrE* and *pyrF* genes have been used as genetic markers (19, 35, 50). We have chosen this approach to develop a selection system for our model organism, *P. abyssi*.

In this article, we report the isolation and characterization of uracil auxotrophic mutants of *P. abyssi* strain GE9. We describe the construction of a shuttle vector, pYS2, based on the pyrococcal plasmid pGT5 and harboring the *pyrE* gene of *Sulfolobus acidocaldarius* as a selective marker. We show that we could reproducibly transform *P. abyssi* cells with a polyethylene glycol (PEG)-mediated spheroplast method and that the vector pYS2 complemented *pyrE* auxotrophs, leading to transformants that could be selected on plates. Furthermore, we show that pYS2 replicates efficiently and is very stable in both *E. coli* and *P. abyssi*, demonstrating its utility as a shuttle vector.

MATERIALS AND METHODS

Bacterial and archaeal strains, media, and growth conditions. *E. coli* strains TOP10 (Invitrogen) and SURE Km^r (Stratagene) were used for vector construction and propagation and for cloning of the *pyrE* gene from *P. abyssi*, respectively. *E. coli* strains were grown at 37°C in a medium containing 5 g of yeast extract, 5 g of tryptone, and 10 g of NaCl per liter (37). Ampicillin was used at 100 µg/ml and kanamycin at 50 µg/ml in both liquid and solid media.

P. abyssi strains were from our laboratory culture collection. *P. abyssi* type strain GE5 (16) was used as the source of plasmid pGT5, and *P. abyssi* strain GE9 (31) is a prototrophic strain used as the wild type, from which all the auxotrophs described in this study were derived. They were cultivated at 90°C (unless otherwise specified) with strict anaerobic procedures as previously described (16). Two types of media were used for the propagation of *P. abyssi* strains. Both had the same mineral base, which had the following composition (per liter of deionized water): 30 g of sea salts (Sigma), 3.3 g of PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 2 ml of a 5% (wt/vol) K₂HPO₄ solution, 1 ml of a 2% (wt/vol) CaCl₂ · 2H₂O solution, 1 ml of a 25 mM FeCl₃ solution, and 1 ml of a 10 mM Na₂WO₄ solution. Resazurin (0.1 mg/liter) was added as redox indicator, and the pH was set to 6.8.

A defined medium, TAA, contained in addition 0.1 g each of the 20 natural amino acids as the sole carbon and energy source and 10 ml of a vitamin solution (4). It was sterilized by filtration to prevent degradation of thermolabile amino acids. This medium was used for general propagation, plating, and preparation of competent cells. A rich medium, YP, consisted of the mineral base supplemented with 0.1% (wt/vol) yeast extract and 0.4% (wt/vol) tryptone. The latter medium was sterilized by autoclaving; it was used for the regeneration of spheroplasts (see Transformation procedures, below). The media were dispensed into sterile Hungate tubes or glass bottles containing elemental sulfur at 1% (wt/vol) and closed with butyl rubber stoppers. After the gas phase had been replaced by N₂ (100 kPa), anaerobiosis was achieved by injection of 1/100th volume of a 3% (wt/vol) Na₂S · 9H₂O solution.

Plating of *P. abyssi* and isolation of uracil auxotrophs. Plating of *P. abyssi* was done essentially as described (15) with some modifications (W. Zillig, personal communication). Briefly, solid media were prepared by including 0.8% (wt/vol) Phytigel (Gellan gum; Sigma) and poured, 30 ml per 9-cm-diameter plastic petri dish. The polysulfide solution which previously served as the sulfur source was replaced with a saturating suspension (10 g per 100 ml of deionized water) of colloidal sulfur (Riedel-de Haën; distributed by Sigma-Aldrich); 100 µl of this suspension and 100 µl of inoculum were added to 800 µl of overlay medium containing 0.25% (wt/vol) Phytigel in a glass tube kept at 90°C in a heating block. The mixture was quickly homogenized with a Vortex mixer and immediately poured and evenly spread on a plate containing the supporting gel. Culture dilutions were prepared in anaerobic TAA medium. When needed, uracil was added both to the supporting gel and in the soft overlay at a concentration of 50 µg/ml from a filter-sterilized anaerobic stock solution.

TABLE 1. Transformation methods tested on *P. abyssi* mutants PA9-13 and PA9-20 with pYS2

Transformation method	Survival ^a (%)	Transformation frequency ^b	Transformation efficiency ^c
CaCl ₂	ND ^d	<1.0 × 10 ^{-8e}	<1.0 ^e
Electroporation ^f	9 ± 2	<1.0 × 10 ^{-8e}	<1.0 ^e
PEG/whole cells ^g	32 ± 6	2.3 × 10 ⁻⁸ ± 1.4 × 10 ⁻⁸	4 ± 3
PEG/spheroplasts ^g	10 ± 4	1.5 × 10 ⁻⁶ ± 0.8 × 10 ⁻⁶	280 ± 168

^a Ratio of CFU obtained for cells subjected to the transformation treatment over the total CFU determined by plating on TAA medium supplemented with uracil; standard deviations are given.

^b Calculated as the ratio of the number of transformants over the CFU of cells that had gone through a mock spheroplasting/transformation procedure counted on TAA medium supplemented with uracil.

^c Number of transformants extrapolated for 1 µg of DNA.

^d ND, not determined.

^e No transformants were recovered; the detection limit is given.

^f Average of two independent experiments under the best conditions: in anaerobiosis, 200Ω, 25 µF, 1.5 kV.

^g Numbers given are the averages of at least three independent transformation experiments.

For the isolation of uracil auxotrophs, 6-cm-diameter petri dishes were employed, and plates consisted of two layers of supporting gel: a first layer consisting of 10 ml of TAA medium at 0.8% (wt/vol) Phytigel and a second layer of 4 ml of TAA medium at 0.8% (wt/vol) Phytigel containing in addition uracil (50 µg/ml) and 5-FOA (800 to 1,200 µg/ml) (Sigma). A preweighed amount of 5-FOA was dissolved directly into the medium while hot to reach the desired concentration. The soft overlay was made by mixing 75 µl of the colloidal sulfur suspension and 75 µl of inoculum with 600 µl of hot (90°C) TAA medium at 0.25% (wt/vol) Phytigel containing uracil (50 µg/ml) and 5-FOA at the same concentration (800 to 1,200 µg/ml) as that of the second layer of supporting gel. All plating manipulations were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) under a 93% N₂-7% H₂ atmosphere. After inoculation, plates were incubated in home-made stainless steel anaerobic jars at 85°C under a 99% N₂-1% H₂S (100-kPa) atmosphere. Colonies were typically visible after 48 to 72 h. Uracil auxotrophs resistant to 5-FOA were further purified by three rounds of streaking on selective plates. Reversion frequencies were determined by plating serial dilutions onto TAA medium devoid of uracil.

Growth measurements. Growth in Hungate tube was monitored by measurement of the optical density at 600 nm with a Spectronic 20 spectrophotometer equipped to hold 18-mm-diameter tubes, with a calibration curve. Direct cell counts were performed with a Thoma counting chamber (0.01-mm depth; Weber, London, England) viewed under a phase-contrast microscope. Viable-cell counts were made in triplicate by plating serial dilutions as described above. Plating efficiency was determined by dividing the number of CFU by the direct cell count number and multiplying the result by 100.

Transformation procedures. *P. abyssi* uracil auxotrophs PA-13 and PA-20 were used as recipient strains for all transformation experiments with the pYS2 vector. Attempts were made to transform *P. abyssi* strains by a modified CaCl₂ procedure previously reported to be successful for transformation of both *P. furiosus* and *S. acidocaldarius* (3) but did not produce any transformants on plates. Electroporation of *P. abyssi* in high-resistance buffer was also tried, essentially as described for *Sulfolobus* spp. (39) except that both 0.05 M sucrose and 10% glycerol were tested as the electroporation buffer. The whole procedure was also tested under anaerobic conditions following the precautions described for electroporation of *Methanococcus* species (34). Several combinations were tested by varying the electrical parameters and the composition of the electroporation buffer under aerobic and anaerobic conditions. However, again none of these efforts were successful (Table 1). One possible explanation for the failure of both methods is that the transformation efficiency was below the detection threshold of our plating procedure (Table 1).

The first successful transformations were obtained with a whole-cell PEG-mediated transformation method similar to that described for the methanogenic archaeon *Methanococcus maripaludis* (43, 44). However, this method suffered from a very low transformation frequency and poor reproducibility (Table 1). Attempts to increase the transformation frequency by modifying the original protocol were unsuccessful. Therefore, transformation of spheroplasts rather than whole cells was investigated as a mean to improve the PEG-mediated transformation. We used a PEG-mediated transformation of spheroplast method similar to the one described for extremely halophilic *Archaea* (11). PEGs of different molecular weights (600, 1,000, 6,000, and 8,000) tested in this study were from Fluka, of the highest purity available (Molecular Biology grade), and used without further purification.

The mutants were grown at 90°C, shaken at 180 rpm, in 100-ml serum bottles containing 50 ml of TAA medium supplemented with uracil. When the cultures

reached an OD₆₀₀ between 0.12 and 0.15 (5 × 10⁸ to 10⁹ cells/ml by direct cell counting), which corresponds to late log phase to early stationary phase, they were removed from the incubator and allowed to cool for about 30 min. Residual sulfur present in the cultures was eliminated by simple decantation. Cells were harvested by centrifugation (5,000 × g for 15 min at 4°C) and from this step on were always kept on ice. Cells were gently washed in 0.2 volume of ice-cold SM 4× solution (SM 4× solution contained 80 g of NaCl, 0.8 g of KH₂PO₄, 0.2 g of NaBr, 0.04 g of SrCl₂ · 6H₂O, 0.08 g of H₂BO₃, 1 g of KCl, and 2 g of sodium citrate per liter) recovered by centrifugation (5,000 × g for 10 min at 4°C) and finally resuspended in 0.01 volume of spheroplasting buffer (1 M NaCl, 27 mM KCl, 50 mM Tris-HCl [pH 7.5], and 20% sucrose) to reach a final concentration of ≈5 × 10¹⁰ cells/ml.

Spheroplasts were obtained by adding EDTA to a final concentration of 10 mM. Conversion of cells to spheroplasts, verified by direct observation with a phase-contrast microscope, was usually observed after 5 to 10 min. Spheroplasts typically prepared from a 50-ml culture grown to late log phase provided a sufficient number of viable cells for about five assays. As previously reported in the case of halophiles (11), we found that younger cells were somewhat more resistant to spheroplast formation and that stationary-phase cells became increasingly susceptible to lysis, both effects resulting in poor transformation. We found that EDTA at a final concentration of 10 mM allowed the conversion of more than 90% of the cells into spheroplasts in 5 to 10 min. Higher concentrations increased the proportion of cell lysis. Spheroplasts seemed to be relatively stable for at least 30 min following addition of EDTA.

Transforming DNA (0.5 µg) was added per 100-µl aliquot of spheroplast suspension. After 5 min of incubation at 4°C, an equal volume of a solution containing 50% PEG-600 (Fluka) and 50% spheroplasting buffer was added and gently but thoroughly mixed with the spheroplasts, and the preparations were kept on ice for 30 min. PEG was eliminated by dilution of the suspension with 100 µl of spheroplast dilution solution (SM 4× solution plus 15% [wt/vol] sucrose) and centrifugation (5,000 × g for 5 min at 4°C). Spheroplasts were finally resuspended in 100 µl of dilution solution, transferred to 5 ml of reduced YP medium in Hungate tubes, and incubated at 85°C for 1 h to allow regeneration of the cell wall.

Selection of transformants was achieved by plating aliquots onto TAA without uracil with the overlay technique described above. To determine the influence of the transformation/spheroplasting procedure on the viability of *P. abyssi*, appropriate dilutions of cell samples that had gone through mock transformation (without DNA) were also plated on TAA supplemented with uracil. Only ≈10% of the total CFU (counted on the nontransformed control) survived the spheroplasting treatment and regenerated to form colonies on plates. Colonies of putative transformants were subcultured in liquid TAA medium. Chemically competent *E. coli* cells were prepared according to Inoue (20a) and transformed by the 5 min transformation protocol described by Pope and Kent (36).

Preparation of plasmidic and total DNA. Plasmids were isolated from *E. coli* by the alkaline lysis method (37). Large scale preparations of plasmid pYS2, to be used for transformation, were further purified by equilibrium centrifugation in a CsCl ethidium bromide gradient according to established protocols (37) or with Qiagen Tip 100 columns (Qiagen) following the manufacturer's recommendations.

Small-scale preparations of plasmid DNA from *P. abyssi* strains were typically made from 50-ml cultures, with either the guanidine isothiocyanate-phenol extraction method described previously (28) or the alkaline lysis protocol (37). Total DNA from *P. abyssi* was prepared as previously described (8). About 100

ng of such preparations was used as the template in PCR amplifications, and about 3 μ g was used for restriction enzyme analyses and subsequent Southern hybridizations.

Cloning and sequence analysis of *pyrE* and *pyrF* genes of *P. abyssi* mutants. The *pyrE* and *pyrF* genes were amplified by PCR from genomic DNA of the wild-type strain GE9, from the auxotrophic mutants, and from three independent transformants. Two sets of primers (oligonucleotides were from Genset S.A., Evry, France) were used. They were designed on the basis of the genome sequence of *P. abyssi* strain GE5 (Géoscope, Evry, France; <http://www.géoscope.cns.fr/Pab/>): the primers PAB-U1 (5'-CTCCCTCCAATCAACTTA-3') and PAB-D1 (5'-AGTCGTAATGCTTCTTGA-3') were used for *pyrE* amplification, and the primers PAB-U2 (5'-TCTAGCTTTGGACGTTTA-3') and PAB-D2 (5'-AACATTTGGGAGAGCTGT-3') were used for amplification of the *pyrF* gene. PCRs were performed with the Expand High Fidelity PCR kit (Roche Biochemicals), and the resulting 0.83-kb and 0.65-kb PCR products for *pyrE* and *pyrF*, respectively, were cloned into the pCR2.1-TOPO plasmid (TOPO TA cloning kit; Invitrogen). DNA sequences were determined from double-stranded templates by automated dye terminator sequencing with universal primers flanking the vector's cloning site.

Routine sequence analyses were done with the Lasergene software package (DNASTAR, Madison, Wis.). Homology searches were conducted on line with the Blast 2.0 program (2), and alignments were performed with the Clustal X program (42).

Construction of shuttle vectors. Plasmid pGT5 (3,444 bp) was linearized with *Nsp*II, and the resulting blunt-ended molecule was cloned into the unique *Stu*I site of *E. coli* plasmid vector pLitmus38 (New England Biolabs). The intact *pyrE* gene of *S. acidocaldarius* was previously amplified from genomic DNA as a 1.5-kb *Sac*I fragment by inverse PCR, and its nucleotide sequence and start point of transcription were determined (41). A 1.1-kb *Bam*HI fragment consisting of the 0.6-kb *pyrE* coding region and about 0.25 kb of the upstream and \approx 0.25 kb of the downstream sequences, was subcloned into the *Bam*HI cloning site of the pLitmus38/pGT5 plasmid, resulting in pYS1 (7.2 kb).

The shuttle vector pYS2 was obtained by deleting a \approx 1-kb *Nsi*I-*Pvu*II fragment of pYS1. The cohesive end generated by *Nsi*I was converted to a blunt end by removal of the overhanging nucleotides with T4 DNA polymerase (New England Biolabs) in the presence of deoxynucleoside triphosphates. Circularization was done with T4 DNA ligase (New England Biolabs). The resulting construct, pYS2 (6.4 kb), was analyzed by restriction fragment analysis after transformation and propagation in *E. coli* strain SURE.

DNA analyses of *P. abyssi* transformants. Total and plasmid DNAs, intact or digested with the appropriate restriction enzymes (New England Biolabs), were separated by electrophoresis on 0.8% agarose gels containing 0.5 μ g of ethidium bromide per ml and visualized under UV light with a charge-coupled device (Bio-Rad). DNA in the gel was transferred to a nylon membrane (Hybond N+; Amersham) and hybridized according to standard procedures (37). A pYS2-specific probe was prepared by recovery and purification (with the QiagexII kit; Qiagen) of the restriction fragments resulting from the double digestion of pYS2 with *Hind*III and *Bam*HI, separated on an agarose gel. All purified fragments except the 1.1-kb *Bam*HI fragment containing the *pyrE* gene of *S. acidocaldarius* were pooled and randomly prime labeled with fluorescein-11-dUTP with the ECF labeling and detection kit (Amersham). Hybridization signals were recorded on a Storm 860 scanner and analyzed with the ImageQuant Analysis software (Molecular Dynamics).

Colony hybridization analysis of *P. abyssi* transformants was performed by standard procedures (37) and the probe described above.

Measurement of OPRTase activity. Cell extracts were prepared from late-exponential-phase cultures of *P. abyssi* strain GE9 (wild type, mutants, and transformants). After elimination of sulfur residues by simple decantation, cells were collected by centrifugation at 4°C and 8,500 rpm in a GSA Sorvall rotor for 20 min, yielding about 2 g of cell paste per liter of culture. Cells were washed by resuspension in 20 ml of TNE and concentrated by centrifugation at 4°C and 7,000 \times g for 15 min. The pellets were stored at -20°C until processed. Frozen cells were thawed, resuspended in Tris-HCl buffer (0.05 M, pH 8.8), and broken by sonication for 10 min.

The OPRTase assay mix contained 10 μ l of 0.05 M Tris-HCl (pH 8.8), 10 μ l of 20 mM MgCl_2 , 10 μ l of 5 mM sodium orotate, 20 μ l of 6 mM phosphoribosylpyrophosphate, and 20 μ l of cell extract in a total volume of 100 μ l. The reaction was carried out at 70°C, and the products of the assay were analyzed by high-pressure liquid chromatography (HPLC) on Zorbax HPLC columns (MacMOD Analytical; detection at 254 nm) with Millennium software (Waters). Protein concentrations were determined according to the method of Bradford (5).

Assessment of plasmid stability and relative copy number. To determine the segregation stability of the vector pYS2 in *P. abyssi*, a late-log-phase culture of plasmid-carrying *P. abyssi* mutant PA9-13 grown in TAA medium was diluted 100-fold into 50 ml of TAA medium supplemented or not with uracil (50 μ g/ml) and incubated at 90°C till the stationary phase was reached ($t_0 + 15$ h). Aliquots of these cultures were then diluted 100-fold in TAA and TAA plus uracil, respectively. The rest of the cultures were harvested, and plasmid DNA was extracted, digested with *Hind*III and *Bam*HI, and analyzed by agarose gel electrophoresis. The whole procedure was repeated 10 times during 10 successive days (\approx 70 generations).

The relative copy number of pYS2 in PA9-13 and PA9-20 transformants was determined by comparison with that previously calculated for pGT5 in *P. abyssi* strain GE5 (25 ± 3 copies/chromosome) (8). Cultures of transformants carrying pYS2 and of strain GE5 containing pGT5 were grown in parallel in TAA medium and stopped at the same time in the exponential phase. The final cell density determined by direct cell counts were very similar for all cultures. Plasmid DNA was isolated as described earlier under exactly the same conditions for all samples. After digestion with *Hind*III and agarose gel electrophoresis, the relative copy number of pYS2 and pGT5 was calculated by comparing the intensity of the 0.9-kb *Hind*III band common to both plasmids and correcting for the variation in cell number used for the respective plasmid preparations. The intensities of the bands were integrated with the NIH Image program (<http://rbs.info.nih.gov/nih-image/download.html>).

Nucleotide sequence accession numbers. The DNA sequences of the *pyrE* gene from *S. acidocaldarius* and from *P. abyssi* strain GE9 have been deposited in GenBank under accession numbers AJ459777 and AY083796, respectively.

RESULTS

Isolation of uracil-auxotrophic mutants. *P. abyssi* strain GE9, previously characterized in our laboratory (31), was used as the wild type. This strain was chosen because it is naturally devoid of plasmid pGT5 (present in *P. abyssi* strain GE5), which we anticipated to be a potential source of vector instability in complementation experiments with a pGT5-based vector.

Spontaneous mutants resistant to 5-FOA were isolated by plating about 10^8 cells from late-exponential-phase cultures onto TAA plates containing 800 μ g (\approx 2.5-fold the determined MIC) of 5-FOA and 50 μ g of uracil per ml, as previously described (46). Plates of TAA medium containing the same concentrations of 5-FOA but no uracil were also inoculated with the same number of cells. No growth was observed in these control experiments. Appropriate dilutions of the cultures were plated without inhibitor in order to determine viable-cell counts and to calculate mutation frequencies per CFU. Resistant colonies appeared after 4 to 5 days of incubation at 85°C with a frequency of $2 \times 10^{-6} \pm 1 \times 10^{-6}$ per CFU, comparable to that previously determined for other *Thermococcales* (48).

Ten independently isolated 5-FOA-resistant mutants were subsequently purified by streaking onto selective medium and checked for auxotrophy in liquid medium. Whereas all drug-resistant strains grew as well as the wild type in the presence of uracil, 7 out of the 10 mutants tested did not grow at all in uracil-free medium. All these uracil auxotrophs were stable and exhibited a high level of resistance to 5-FOA ($>1,400$ μ g/ml). Their reversion frequency was determined by plating serial dilutions of cultures onto uracil-free medium.

Genetic characterization of uracil-auxotrophic mutants. As we expected the mutations responsible for the observed phenotype to occur in either the *pyrE* gene (encoding OPRTase) or the *pyrF* gene (encoding orotidine 5'-monophosphate decarboxylase), we determined the complete nucleotide se-

quences of these genes for all the auxotrophic strains and compared them to their wild-type counterparts. Two mutants were found to harbor a large deletion in the *pyrF* gene, and another had no mutation in either the *pyrE* or the *pyrF* gene. Because we were interested only in *pyrE* mutants, these mutants are not considered in this study. Among the four simple *pyrE* mutants, two were selected for their lower range of reversion frequency, PA9-13 ($3.5 \times 10^{-7} \pm 2 \times 10^{-7}$) and PA9-20 ($5 \times 10^{-7} \pm 1 \times 10^{-7}$) and are described below.

The *pyrE* nucleotide sequences of strains GE5 and GE9 showed 95% identity. Both sequences contained an ORF of 549 bp, corresponding to the *pyrE* coding region, preceded by a motif (TTTAAA) centered 52 bp upstream the ATG that fits the box A consensus of archaeal promoters. The deduced amino acid sequences from the *pyrE* gene of strains GE5 and GE9 exhibited 98% similarity. Comparison of the wild-type GE9 *pyrE* sequence with that of the two auxotrophs revealed the presence of a single mutation in the coding region (no mutation was detected in the 132 bp upstream of the ATG). Although they were obtained independently, PA9-13 and PA9-20 showed the same frameshift mutation by insertion of a single base pair (T340) resulting in a stop codon (TGA348) in the novel reading frame. The corresponding polypeptide was 71 amino acid residues shorter than the wild-type OPRase (182 amino acids). The deleterious effect of the mutations can be easily explained by the fact that the truncated polypeptide lacks most of region VLLVEDVTTTGG, highly conserved in *Archaea* and *Bacteria*. This corresponding sequence (VMLVD-DVITAGT) has been shown to be the phosphoribosylpyrophosphate-binding site of the *Salmonella enterica* serovar Typhimurium OPRase and to correspond to the active site of the enzyme (38).

Construction of a shuttle vector with a selectable genetic marker. The plasmid pGT5 from *P. abyssi* GE5 was used as a basis for the construction of an autonomously replicating shuttle vector. Functional analysis of this plasmid permitted us to identify the essential elements required for its propagation in *Pyrococcus* cells and therefore to establish a strategy for the construction of a cloning vector. pGT5 contains two major ORFs which together cover 85% of the genome (14). The larger one encodes a protein of 75 kDa, Rep75, involved in the initiation of plasmid replication via a rolling-circle mechanism (29, 30). Other key elements, typical of rolling-circle replicons, the double-strand origin (*dso*) and single-strand origin (*sso*) of replication, were also identified.

No clear function could be attributed to the second major ORF (ORF2), but by analogy with the modular organization of bacterial rolling-circle plasmids, we suggested that it could be involved in plasmid maintenance or copy number control (14). Since other small rolling-circle plasmids encode only a Rep protein, ORF2 might be dispensable for replication. We therefore predicted that the best site for introduction of foreign DNA would be between ORF1 and ORF2 or within ORF2. The unique *NspBII* site in the intergenic region downstream of ORF1 was chosen because of its location between the putative Rep75 transcriptional terminator (hairpin region RIII) and the hypothetical ORF2 promoter (14). Thus, insertion of foreign DNA in that site would apparently not disrupt any important sequence. The backbone of potential shuttle vectors was then obtained by cloning the complete pGT5 sequence linearized by

NspBII into the *E. coli* plasmid vector pLitmus38 (a pUC18 derivative). Preliminary transformation experiments have shown that such a shuttle vector could replicate in both *E. coli* and *Pyrococcus* hosts but was unstable in *E. coli* and lacked a selectable marker (1).

The uracil auxotrophs PA9-13 and PA9-20 described above carried the appropriate background for selection by complementation with a wild-type *pyrE* gene. A potential problem with the use of a native gene as a selectable marker is the possibility of recombination between the introduced gene and the resident genomic copy. To avoid this problem, we chose the *pyrE* gene of another hyperthermophilic archaeon, *S. acidocaldarius*, the sequence of which and its transcription start site had just been established (41). The amino acid sequence deduced from the *S. acidocaldarius pyrE* gene exhibited 60% similarity with its *P. abyssi* homologue, but the two genes did not show significant identity at the nucleotide level. Although these two archaea are phylogenetically distant, their transcriptional signals are sufficiently similar to be reciprocally recognized by the respective host transcription apparatus, as indicated by previous reports on heterologous expression of *Sulfolobus* genes in *Pyrococcus* (3) and vice versa (K. Stedman, E. Martusewitsch, C. Schleper, J. van der Oost, and W. Zillig, Abstr. Int. Cong. Extremophiles 2000, abstr. L49, 2000).

Therefore, a PCR product consisting of the *S. acidocaldarius pyrE* coding region and promoter sequence was introduced in the pGT5/Litmus construct, resulting in plasmid pYS1. As the size of the construct (7.2 kb, compared to 3.4 kb for pGT5) could be a potential factor of instability, we decided to reduce the pGT5 portion to the minimum required for replication in *Pyrococcus* spp. This was achieved by deleting most of ORF2, which we assumed to be dispensable for vector replication. The resulting construct, pYS2 (6.4 kb) (Fig. 1), was further used for transformation of the *P. abyssi pyrE* mutants derived from strain GE9.

PEG-mediated spheroplast transformation of *P. abyssi*. The spheroplast transformation method used (see Materials and Methods) was adapted from the protocol described for halophilic archaea (11). The use of spheroplasts instead of whole cells for PEG-mediated transformation resulted in a significant improvement in the transformation frequency for both the PA9-13 and PA9-20 mutants (Table 1). Although there was significant variability in the number of transformants obtained in each experiment (which we believe is due in part to the difficulties inherent to plating these organisms), we were reproducibly able to achieve at least 10^2 transformants per μg of pYS2 per 10^9 cells of *P. abyssi* auxotrophs. Control experiments in which PEG was omitted never gave any transformant. PEG of various molecular weights (600, 1,000, 6,000, or 8,000) did not significantly change either the survival rate or the transformation efficiency. Therefore, whereas the presence of PEG appeared to be absolutely required for efficient transformation, its average size (within the range tested) did not appear to be an important parameter.

We also examined the effect of varying the PEG 600 concentration and found that a 25% final concentration was the best compromise between survival frequency and transformation frequency. No transformants were obtained with less than 0.05 μg of pYS2, and the best transformation frequencies were obtained with 0.5 to 1 μg of DNA. We also examined the

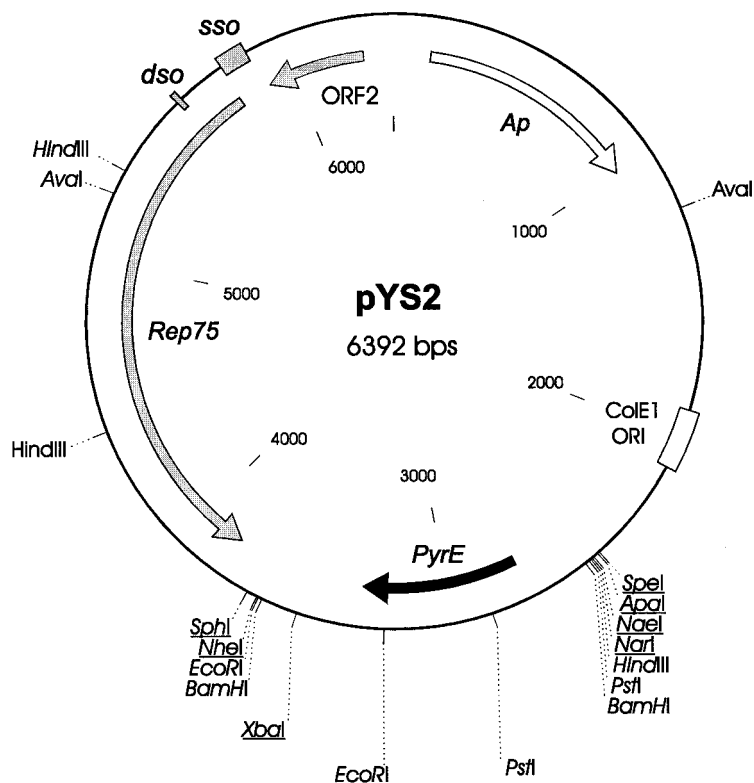


FIG. 1. Map of shuttle vector pYS2. Relevant restriction sites are shown; unique restriction sites are underlined. The white box and arrow show the *E. coli* pLitmus38 plasmid moiety. The gray boxes and arrows correspond to the pyrococcal pGT5 plasmid moiety (*dso* and *ssO*, double-stranded and single-stranded origin of replication, respectively; Rep75, replication initiator encoded by the major ORF of pGT5; ORF2, truncated portion of the second main ORF of pGT5). The solid arrow indicates the *S. acidocaldarius pyrE* gene under the control of its own promoter.

eventuality of DNA restriction upon entry into *P. abyssi* cells, but we did not observe significant differences in transformation efficiencies obtained for pYS2 prepared from a *P. abyssi* transformant versus pYS2 prepared from *E. coli* strain SURE.

Demonstration of functionality of *pyrE* as a selectable genetic marker. To demonstrate the ability of the *S. acidocaldarius pyrE* gene to complement *P. abyssi pyrE* mutants, we followed the growth of four independent transformants cultivated in TAA medium supplemented or not with uracil (50 $\mu\text{g/ml}$). The wild-type GE9 strain and the mutants PA9-13 and PA9-20 cultivated in the same conditions were used as controls. The four transformants tested grew equally well with or without uracil and exhibited growth rates comparable to those observed for the wild type. The maximal cell densities reached ($\approx 10^9$ cells/ml) for the transformants and the wild type were also similar. As expected, no growth was observed for the control cultures of the mutants in uracil-free medium. Moreover, growth of the transformants and the wild type was completely inhibited by 5-FOA at a concentration (600 $\mu\text{g/ml}$, ≈ 2 times the MIC), which did not affect the growth of the auxotrophs.

Accordingly, the OPRTase activities of the transformants and the wild type were comparable (Table 2), though consistently lower for the transformants (on average $\approx 30\%$ lower), while the OPRTase activities of the mutants were markedly decreased (on average 28-fold reduction). As expected, orotidine 5'-monophosphate decarboxylase activities were in the

same range for the transformants, the wild type, and the *pyrE* mutants (data not shown).

As reversions could possibly be responsible for the *Ura*⁺ phenotype of the transformants, we verified the sequences of their genomic *pyrE* alleles. For each of the four transformants analyzed, the *pyrE* sequence was identical to that of the original auxotrophic recipient; it contained the same base pair insertion (T340) that caused a frameshift and produced an inactive enzyme. These results indicate that the *pyrE* gene from *S. acidocaldarius* present on pYS2 encodes a functional enzyme capable of conferring uracil prototrophy on *pyrE* mutants of *P. abyssi*. Finally, these results demonstrate the utility of this system for the selection of *Pyrococcus* transformants.

Stability and relative copy number of shuttle vector pYS2. Plasmid DNAs from several independent *P. abyssi* transformants were extracted from subcultures grown in TAA medium. Aliquots of these preparations were used to transform *E. coli*, and the ampicillin-resistant clones obtained were picked and their plasmid DNA was prepared by alkaline lysis. Each DNA sample was analyzed by agarose gel electrophoresis. As illustrated in Fig. 2, plasmid DNA could be readily isolated from *P. abyssi* transformants in sufficient amounts to allow direct visualization on agarose gels. This result constitutes direct evidence that the vector replicates efficiently in *P. abyssi* cells. Moreover, the restriction endonuclease cleavage pattern of each DNA was identical to that of the original pYS2 DNA, indicating that no obvious deletions or rearrangements oc-

TABLE 2. Growth tests and OPRTase activity for wild-type *P. abyssi* GE9, mutants PA9-13 and PA-20, and two representative transformants harboring vector pYS2

Strain and genotype	Growth on TAA-based medium supplemented ^a with:				Avg OPRTase activity ^b ($\mu\text{M}/\text{h}/\text{mg}$ of protein) \pm SD
	No supplement	Uracil	5-FOA	Uracil + 5-FOA	
GE9	+	+	-	-	0.18 \pm 0.02
PA9-13 (<i>pyrE pyrF</i> ⁺)	-	+	-	+	<0.01
PA9-13-T02 (<i>pyrE pyrF</i> ⁺ [pYS2 <i>pyrE</i> ⁺] ^c)	+	+	-	-	0.13 \pm 0.01
PA9-20 (<i>pyrE pyrF</i> ⁺)	-	+	-	+	<0.01
PA9-20-T21 (<i>pyrE pyrF</i> ⁺ [pYS2 <i>pyrE</i> ⁺])	+	+	-	-	0.12 \pm 0.02

^a TAA medium supplements were 50 $\mu\text{g}/\text{ml}$ for uracil and 600 $\mu\text{g}/\text{ml}$ for 5-FOA, cultures were inoculated at an initial cell density of 1×10^6 to 5×10^6 cells/ml with an exponentially growing preculture. Cell densities, were scored after 48 h of incubation at 90°C (cultures that did not grow after 48 h did not grow after longer incubation); +, $\geq 10^8$ cells/ml; -, $< 10^7$ cells/ml.

^b OPRTase activity values averages of three measurements.

^c The *pyrE*⁺ gene carried on pYS2 corresponds to the *S. acidocaldarius* wild-type *pyrE* gene.

curred in pYS2 during its passage in *Pyrococcus* cells and back in *E. coli*.

As illustrated in Fig. 2 (other data not shown), by comparing the relative intensity of the 0.9-kb *Hind*III band of pGT5 prepared from strain GE5 (Fig. 2, lane 1) and that of the corresponding band from pYS2 prepared from the same number of cells of *P. abyssi* transformants (Fig. 2, lanes 2, 4, and 6), we estimated the vector copy number to be in the same range in transformed cells as that of pGT5 in GE5, between 20 and 30 copies/chromosome (13).

A long-term growth experiment was performed with transformed *P. abyssi* cells to assess vector stability. A single clone containing pYS2 was cultured in TAA medium until it reached the stationary phase (typically an overnight incubation) and then diluted 1:100 in the same medium supplemented or not with uracil. Samples were taken every day and used for plasmid DNA preparation and restriction digest analysis. No change in plasmid DNA was observed after 10 days (≈ 70 generations) of continuous growth (not shown). Only a slight decrease in the

relative amount of pYS2 was detected upon prolonged cultivation in nonselective conditions (Fig. 2, lane 6).

DISCUSSION

The development of genetic techniques for routine analysis of *Pyrococcus* spp. has been a long-time goal for researchers in that field. With the methods presented here, a crucial step towards this goal has been taken. For the first time, we have shown that DNA can be reproducibly introduced into *Pyrococcus* cells and that transformants can be directly selected on plates, a goal that had not been achieved previously (1, 3).

The shuttle vector reported here possesses a variety of features required for a cloning vector. It is very stable and replicates efficiently in both *E. coli* and *P. abyssi*. Vector pYS2 appears to be particularly stable in *P. abyssi*, as it could be easily recovered from transformants continuously cultured for more than 70 generations, even in the absence of selection pressure. The stability in *E. coli* may appear surprising, since we (Y. Zivanovic and S. Lucas, unpublished data) and others (3) previously observed that similar constructs based on the pyrococcal plasmid pGT5 combined with pUC derivatives suffered from important deletions and rearrangements in *E. coli*. The problem was partly solved by introducing the *rom/rop* gene from pBR322 in the pUC moiety in order to reduce the copy number in *E. coli* (3). This operation was not necessary for pYS2, as it was stable at high copy number not only in *E. coli* but also in *P. abyssi*.

Since pYS2 lacks most of the second major open reading frame (ORF2) of pGT5, it is clear that ORF2 is not required for replication in *P. abyssi*. It is also unlikely that ORF2 is involved in copy number control, because the vector copy number in transformants is similar to that of pGT5 in its natural host strain, *P. abyssi* GE5. More likely, this function is provided by the replication initiator Rep75 itself, which is able to autoregulate its activity (29). One can speculate that the deletion of ORF2 also contributed to the increased stability of the vector in *E. coli*. One obvious advantage of the high copy number is the facility to isolate the vector from transformant cultures in amounts sufficient to allow direct visualization on standard agarose gels. This has not been possible for other previously described plasmid-based vectors for *Pyrococcus* and *Sulfolobus* spp. (3, 6), which, because of their very low copy number, could only be detected in transformants by PCR or Southern blot and

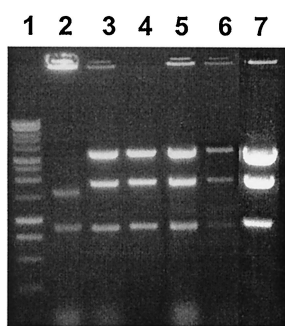


FIG. 2. Stability of shuttle vector pYS2. Plasmid DNA was isolated from *P. abyssi* strains and from *E. coli* cells as described in Materials and Methods, digested with *Hind*III, and electrophoresed on an agarose gel. Lane 1, 1-kb ladder (Promega). Lane 2, endogenous plasmid pGT5 isolated from *P. abyssi* GE5. Lane 3, plasmid DNA from a PA9-20 Ura⁺ clone obtained after transformation with vector pYS2. Lane 4, pYS2 isolated from an Amp^r clone of *E. coli* obtained after backtransformation with plasmid DNA from a *P. abyssi* transformant. Lane 5, plasmid DNA from a PA9-13 transformant after 16 h of growth in TAA medium. Lane 6, plasmid DNA from a PA9-13 transformant after 10 days of continuous growth under nonselective conditions (TAA medium plus 50 mg of uracil per ml). Lane 7, original pYS2 prepared from *E. coli* and used for transformation of *P. abyssi* mutants.

for which plasmid DNA could be recovered only after back-transformation in *E. coli*.

Because of its high stability and small size (6.39 kb), we assume that vector pYS2 could accommodate further insertions of foreign DNA, an additional marker and/or a candidate gene for expression in *Pyrococcus* spp. Their cloning will be greatly facilitated by the availability of several convenient unique restriction sites.

As an alternative to the use of antibiotic resistance markers, which are not suited for the selection of transformants in hyperthermophiles, we have demonstrated here that the *pyrE* gene encoding the OPRtase from *S. acidocaldarius* can be used for the positive selection of *P. abyssi* transformants on plates, by complementation of the appropriate uracil-auxotrophic recipients. The *pyrE* and *pyrF* genes are particularly useful genetic markers, as both mutant and wild-type alleles can be positively selected in most microorganisms, as they confer resistance to 5-fluoroorotic acid and bring about the return to prototrophy, respectively. Moreover, we have shown that the pYS2 vector, bearing the *S. acidocaldarius pyrE* gene, conferred on *P. abyssi* transformants the ability to grow on minimal medium as well as the wild-type strain does.

Accordingly, we found that the OPRtase activity measured in cell extracts of the transformants was comparable to that of the wild type. This was somewhat surprising since, in light of the high copy number of pYS2, we expected to measure a higher OPRtase activity. Possible explanations are that (i) although the transcriptional and translational signals of the *S. acidocaldarius pyrE* gene seem closely related to those generally found in *Pyrococcus*, they might allow only low-level expression of that gene in *P. abyssi*; (ii) this situation may reflect differences in the complex regulatory circuits (at the transcriptional and/or the enzymatic level) of this activity in two different cellular contexts; (iii) activity assays have shown that the optimal temperature for the *S. acidocaldarius* OPRtase is around 75 to 80°C (D. Charlier and D. Gigot, unpublished data). Therefore, it is possible that at 90 to 95°C, the normal temperatures for cultivation of *P. abyssi*, and in anaerobic conditions, the enzyme retains only partial activity. Further investigations are required to clarify this point.

Another important finding reported here is that PEG can induce genetic transformation in *Pyrococcus* spp. While efficient PEG-mediated transformations have been reported for many bacteria, yeasts, and fungi (20, 23), only a few reports concerned *Archaea*. A simple PEG-mediated transformation method proved efficient on whole cells of *Methanococcus maripaludis*, yielding over 10⁵ transformants μg^{-1} with an integrative vector (44). In contrast, using this method with *P. abyssi*, we obtained only a very few transformants per microgram of pYS2 (Table 1).

Because the use of proto/spheroplasts versus whole cells often gave higher transformation efficiencies for bacteria and archaea (11, 32, 34), we tested this solution for *P. abyssi*. With a protocol adapted from a previously described method for halophilic archaea (11), we have shown that more than 90% of *P. abyssi* cells could be converted to spheroplasts but that only 10% of them regenerated as colonies on plates. This is much below what has been reported for methanogens and halophiles (typically above 60%) (11, 32, 34). Nevertheless, the use of spheroplasts rather than whole cells improved the PEG-mediated

transformation efficiency of *P. abyssi* about 100-fold (Table 1). Still, this is about 3 to 4 orders of magnitude below what is currently obtained for mesophilic archaea (10, 32, 49). Among the reasons generally evoked to explain poor transformation efficiencies is the presence of a restriction/modification (R/M) system in the recipient host. Although we failed to detect restriction activity in *P. abyssi* GE9 and did not observe significant differences in the transformation efficiency with pYS2 vector prepared from a transformant, this hypothesis cannot be completely ruled out because such a type I R/M system has recently been identified in the genome sequence of *P. abyssi* strain GE5 (24).

It is difficult to predict how the copy number and stability of pYS2 derivatives will be influenced by the introduction of supplementary fragments. Even so, the construction of a selectable shuttle vector that replicates efficiently and is stably maintained at high copy number in *E. coli* and in *P. abyssi* certainly opens new perspectives for the elaboration of even more suitable cloning and expression vectors. Better vectors should contribute greatly to the development of additional genetic tools for the exploration of hyperthermophilic archaea and for the expression and purification of thermophilic enzymes produced in the correct cellular environment and at high temperature.

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