Extremophiles (1997) 1:183–191

ORIGINAL PAPER

Rajagopal N. Aravalli · Roger A. Garrett

Shuttle vectors for hyperthermophilic archaea

Received: January 12, 1997 / Accepted: May 29, 1997

Abstract Progress in understanding the basic molecular, biochemical, and physiological characteristics of archaeal hyperthermophiles has been limited by the lack of suitable expression vectors. Here, we report the construction of versatile shuttle vectors that can be maintained, and selected for, in both archaea and bacteria. The primary construct, pAG1, was produced by ligating portions of the archaeal cryptic plasmid pGT5 and the bacterial plasmid pUC19, both of which exhibit high copy numbers. A second vector construct, pAG2, was generated, with a reduced copy number in *Escherichia coli*, by introducing the Rom/Rop gene from pBR322 into pAG1. After transformation, both pAG1 and pAG2 were stably maintained and propagated in the euryarchaeote *Pyrococcus furiosus*, the crenarchaeote *Sulfolobus acidocaldarius,* and in *Escherichia coli*. An archaeal selective marker, the alcohol dehydrogenase gene from *Sulfolobus solfataricus*, was isolated by polymerase chain reaction (PCR) amplification and cloned into the two constructs. They were stably maintained and expressed in the two archaea and conferred resistance to butanol and benzyl alcohol. However, the vector pAG21, deriving from pAG2, proved the more stable in *E. coli* probably due to its lower copy number in the bacterium. Conditions are presented for the use of the vectors which, potentially, can be used for other hyperthermophilic archaea.

Key words Archaea · Hyperthermophile · *Pyrococcus* *Sulfolobus* · Shuttle vector · Alcohol dehydrogenase

Communicated by G. Antranikian

R.N. Aravalli · R.A. Garrett (✉)
Institute of Molecular Biology, University of Copenhagen, Sølvgade 83H, DK-1307 Copenhagen K, Denmark
Tel. +45-35-32-20-10; Fax +45-35-32-20-40
e-mail: garrett@mermaid.molbio.ku.dk

Introduction

The archaea constitute a domain of microorganisms many of which grow under extreme environmental conditions. In general, they can be classified into three main groups according to their natural habitats and nutritional requirements: the methanogens, the extreme halophiles, and the extreme thermophiles. However, phylogenetic analyses, based on 16S rRNA and other molecular sequences, reveal two primary archaeal kingdoms: the euryarchaeota and crenarchaeota (Woese et al. 1990) and, recently, a third kingdom, korarchaeota, has been proposed (Barns et al. 1996).

The archaeal hyperthermophiles that thrive at temperatures near the boiling point of water are of particular interest for evolutionary studies because they exhibit relatively slow rates of evolution and are located close to the putative branch point of the archaea and eucarya (Pace 1991; Stetter 1996). These organisms are also of interest as sources of thermostable enzymes and novel pharmaceutical products.

While many molecular, biochemical, and physiological features of bacteria and eukaryotes are well characterized and understood, only limited information is available on the archaea. With recent rapid developments in the genome sequencing of archaeal thermophiles (Bult et al. 1996; Charlebois et al. 1996; Garrett 1996; Sensen et al. 1996), thousands of gene sequences are becoming available, and many of their functions have been, and will continue to be, deduced from sequence similarity studies with known bacterial or eukaryotic genes. As a consequence of this development, it is especially important to express and explore the function of these genes in their natural hosts, a task that has not been possible previously due to the lack of suitable expression vectors.

Although progress has been made in developing such vector systems for the methanogens (Bertani and Baresi 1987; Worrel et al. 1988; Gernhardt et al. 1990; Metcalf et al. 1997) and extreme halophiles (Cline and Doolittle 1987; Lam and Doolittle 1989; Holmes and Dyall-Smith 1990),

only preliminary attempts have been reported on construction of shuttle vectors for archaeal hyperthermophiles based both on plasmids and a mobile intron (Aagaard et al. 1996). In these studies, the plasmid construct was partly unstable in *Escherichia coli*, after transfer from archaeal cells, and, moreover, the construct contained no selective marker. In order to overcome these problems, new shuttle vectors were constructed using the cryptic archaeal plasmid pGT5 from *Pyrococcus abyssi* and pUC19. The constructs pAG1 and pAG2 are high-copy-number and low-copy-number plasmids, respectively, in *E. coli*. Moreover, the alcohol dehydrogenase gene from *Sulfolobus solfataricus* was shown to be a suitable selectable marker for pAG2, since archaeal cultures are sensitive to alcohols. Here, we demonstrate the stability, propagation, and selection of these vectors in *S. acidocaldarius*, a crenarchaeote that is not closely related to *S. solfataricus*, and a euryarchaeote *Pyrococcus furiosus*, as well as in *E. coli*.

Materials and methods

Materials. *P. furiosus* (DSM 3638) and *S. acidocaldarius* (DSM 639) were purchased from the DSM culture collection (Darmstadt, Germany). *E. coli* JM109 (Yanisch-Perron et al. 1985) was used for cloning, amplification, and selection of plasmids. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, *Hae*III methylase, and Klenow fragment of DNA polymerase [Amersham (Buckinghamshire, UK), Boehringer Mannheim (Germany), and New England Biolabs (Beverly, MA, USA)] were used according to manufacturers' recommendations. Deoxynucleoside triphosphates (dNTPs) were from Pharmacia (Uppsala, Sweden). 1-Butanol and benzyl alcohol were from Merck (Darmstadt, Germany). Archaeal plasmid pGT5 (Erauso et al. 1996) was a gift from Drs. D. Prieur (Roscoff, France) and P. Forterre (Orsay, France).

Cell growth conditions. *E. coli* was grown aerobically on a rotary shaker at 250 rpm in LB medium containing 10 g Bacto-tryptone (Difco, Detroit, MI, USA), 5 g yeast extract (Difco), and 10 g NaCl per liter at 37°C, in liquid medium, and on solid medium that in addition contained 1.5% agar (Difco) (Sambrook et al. 1989). Ampicillin was included at a final concentration of 100 μg/μl. *S. acidocaldarius* cells were grown either aerobically in minimal medium supplemented with 2 g tryptone and 1 g yeast extract per liter at 70°C, with shaking at 300 rpm, or on solid medium with 1% Gelrite (Sigma, St Louis, MO, USA) as described previously (Grogan 1996). *P. furiosus* were grown at 95°C in DSM medium 283 lacking NaBr, $SrCl_2$, $NiCl_2$, and trace minerals (Aagaard et al. 1996). This medium was supplemented with 1 g yeast extract and 5 g peptone (Difco) per liter. Cell cultures were diluted every 2 days to maintain a steady A_{600} of 0.2.

Vector constructs. The bacterial vector pUC19 (Yanisch-Perron et al. 1985) was digested with *Ssp*I and *Eco*RI, and pGT5 was cut with *Nsi*I, and *Ava*II. The cohesive ends generated by *Eco*RI, *Nsi*I, and *Ava*II were converted into blunt ends with the Klenow fragment in the presence of dNTPs. The 2.1-kb pUC19 fragment and 2.6-kb pGT5 fragment were isolated from a 0.7% agarose gel by QIAquick gel extraction (Qiagen, Hilden, Germany). They were ligated together with T4 DNA ligase at 14°C overnight. This construct, pAG1, was analyzed by restriction fragment analysis after transforming and propagating in *E. coli* (see later). A second construct pAG2 was made by ligating the 1127-bp *Eco*47III-*Alw*NI fragment, containing the Rom/Rop gene from pBR322 (Bolivar et al. 1977), together with the large *Pvu*II-*Alw*NI fragment of pAG1.

Isolation of a selectable marker. The alcohol dehydrogenase (ADH) gene from *S. solfataricus* (DSM1617), together with its promoter and terminator (Ammendola et al. 1992), was isolated by polymerase chain reaction (PCR) amplification using the following primers: 5′-CTAGAATTCGTCAGTAATGCTATTACGTT-3′; 5′-TATGAATTCGAGAGAATTGT-CGATTACATC-3′; and genomic DNA as template. The reaction was performed with Robocycler (Stratagene, La Jolla, CA, USA) using *Pfu* polymerase (Stratagene). Samples were denatured initially at 95°C for 3 min followed by 30 cycles with the settings: denaturation at 95°C for 45 s, annealing at 48°C for 45 s, and elongation for 2 min at 72°C. The PCR product was purified using QIAquick (Qiagen) and phosphorylated with T4 polynucleotide kinase. It was ligated to pAG2 to form the vector pAG21.

Preparation of plasmid and total DNA. After transforming into *E. coli* JM109, mini-preps of the plasmids were made (Sambrook et al. 1989) from the colonies that grew on LB plates with ampicillin, using JetStar ion exchange columns (Genomed, Research Triangle Park, NC, USA). All vector constructs were methylated with *Hae*III methylase prior to transforming into the archaea to prevent degradation in vivo, and the DNA was deproteinized by treatment with phenol and chloroform, and precipitated with ethanol. Total archaeal DNA was prepared as follows. Cells were pelleted by centrifuging at 6000 rpm for 10 min and resuspending in 1/20th volume of TE buffer, 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). Proteinase K (Sigma) and sodium dodecyl sulfate (SDS) were added to final concentrations of 200 μg/ml and 1%, respectively. This mixture was incubated at 37°C for 30 min followed by 1 h at 50°C. DNA was extracted twice with phenol and twice with a phenol-chloroform-isoamyl alcohol (25:24:1) mixture. It was then precipitated with ethanol, washed once with 70% ethanol, dried, and redissolved in TE buffer (pH 8.0).

Transformation procedures. Competent cells of *E. coli* JM109 were prepared and transformed with plasmids as described (Sambrook et al. 1989). *S. acidocaldarius* cells were harvested in mid-log phase (A_{600} 0.4), washed three times with 1/10th volume of 20 mM sucrose solution and resuspended in 50 μl 20 mM sucrose. Methylated plasmid

DNA (0.5 μg) was then added to the cells and left on ice for 20 min before transformation. *S. acidocaldarius* cells were transformed by electroporating at room temperature in 0.1-cm cuvettes with the following settings of the Gene Pulser apparatus (Bio-rad, Hercules, CA, USA): 1.5 kV, 400 Ω, and 25 μF. Cells were left on ice for 20 min after electroporation, transferred to the growth medium, and incubated at 70°C. Competent cells of *P. furiosus* were prepared and transformed as described (Aagaard et al. 1996).

Copy number determination. Relative copy numbers of the vector constructs pAG1 and pAG2 in *E. coli* were determined by two methods. In the first, exponentially growing cells were transformed with either construct and were harvested during exponential growth at identical cell densities. Plasmid DNA was isolated as described earlier, and the yield was estimated from A_{260} measurements. In the second method, β-lactamase activity was assayed for *E. coli* cells transformed with each construct as follows. Cell culture (2 ml) was harvested, and cells were pelleted at 6000 rpm for 10 min and resuspended in 500 μl 0.1 M KPO_4, 0.5 mM EDTA (pH 7.5). They were treated in a Sorvall sonicator at room temperature for 3 × 40 s. The crude extract was then assayed for β-lactamase activity in 0.1 M KPO_4 using nitrocephin (Becton-Dickinson, Mountain View, CA, USA) as substrate (Hove-Jensen 1985). Reactions were performed at room temperature and the optical densities were measured at 482 nm.

Alcohol sensitivity of archaea. To determine the minimum inhibitory concentrations (MIC values) of butanol and benzyl alcohol that prevent growth of *P. furiosus* and *S. acidocaldarius*, increasing levels of alcohol, in the range 0–200 mM, were included in the cultures. Cell growth was monitored over a period of 14 days by collecting samples every two days and measuring the A_{600} values. For those experiments where the cells were transformed with shuttle vectors, 150 mM butanol and 40 mM benzyl alcohol were used for selection unless otherwise stated.

Detection of pAG21 in archaeal transformants. After transforming, archaeal cells were grown for 14 days with serial dilutions. Samples were taken every second day and total DNA was extracted and retransformed into *E. coli*. DNA minipreps of the ampicillin-resistant transformants were made as before, and the plasmid DNA was subjected to restriction fragment analysis. The presence of plasmid in the total DNA preparation was established by PCR using the primers 5′-CGGGTTCTCAAGTGATACGC-3′ and 5′-GCTCACCGAAATAGGACAGC-3′ that are specific for pGT5. The PCR reactions were performed with Taq polymerase. Samples were denatured initially at 95°C for 3 min followed by 30 cycles with the settings: denaturation at 95°C for 45 s, annealing at 48°C for 45 s, and elongation for 90 s at 72°C. The construct pAG21 was monitored using the alcohol dehydrogenase (ADH)-specific primers: 5′-ATGAGAGCAGTTAGATTAGT-3′ and 5′-TTATGGTATGAGTACTTGTC-3′.

Southern hybridizations were also performed with total DNA using standard procedures (Sambrook et al. 1989) as follows: 1 μg of total DNA, or 1 μg plasmid DNA, was cut with *Hind*III and the fragments were separated on a 0.6% agarose gel. They were transferred to nitrocellulose filter and probed with a purified PCR product, obtained using the pGT5-specific primers, that was labelled with [α-^{32}P]CTP. After washing the filters under stringent conditions, the bands were analyzed by autoradiography.

Results and discussion

Construction of shuttle vectors

The archaeal plasmid pGT5 and the bacterial plasmid pUC19 were chosen for constructing a vector. The former is a cryptic plasmid from *P. abyssi* GE5 that replicates via a rolling circle mechanism and contains two open reading frames, ORF1 and ORF2, coding for polypeptides of 75 and 46 kDa, respectively (Erauso et al. 1996). ORF1 shows sequence similarities to the Rep proteins and some conserved nucleotide sequence motifs of the pC194 superfamily of plasmids that replicate by a rolling circle mechanism, while ORF2 has no sequence similarity with known nucleic acid or protein sequences in the databases (Erauso et al. 1996). In an earlier construct, the promoter region of ORF2 was separated from the coding region, thereby inhibiting the expression of ORF2, and there was no disadvantage for the propagation of the plasmid in archaea (Aagaard et al. 1996). Therefore, most of the ORF2 region was deleted in the present construct.

The first construct, pAG1, was generated by digesting pUC19 with *Eco*RI and *Ssp*I, and pGT5 with *Ava*II and *Nsi*I. The 2.1-kb pUC19 fragment containing the β-lactamase gene, the origin of replication, and the multiple cloning site, was ligated to the 2.6-kb pGT5 fragment containing ORF1, the ORF2 promoter, and the single stranded and double stranded origins of replication, to form a 4.7-kb shuttle vector (Fig. 1). A second vector, pAG2 of 5.3 kb, was constructed in an attempt to reduce the copy number in *E. coli*, by introducing the Rom/Rop gene from pBR322 (Bolivar et al. 1977) into pAG1 (Fig. 1) – as described later.

Replication and maintenance of the shuttle vector constructs in archaea

P. furiosus and *S. acidocaldarius* cells were transformed with pAG1 and tested for the stability and propagation of the shuttle vector constructs. The earlier vector construct pCSV1 (Aagaard et al. 1996) was used as a positive control. Transformed cells were suspended in 30 ml growth medium and cultured continuously for 14 days with dilutions at regular intervals to maintain a steady cell density and to ensure exponential cell growth. Samples were taken every 2 days and total DNA was isolated. Two different approaches were then followed to test for the presence of plasmid

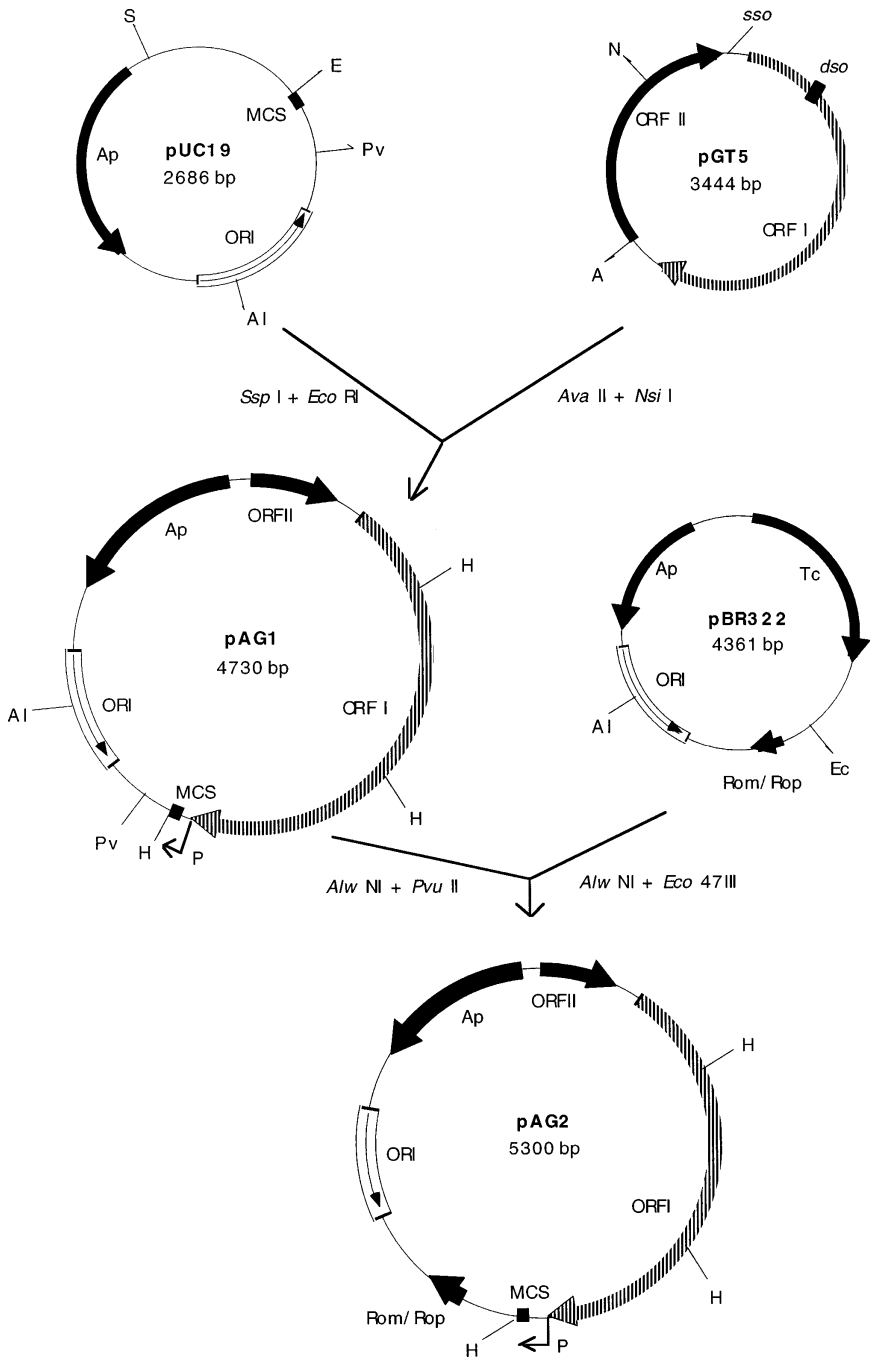

Fig. 1. Construction of the shuttle vectors pAG1 and pAG2 from the bacterial plasmid pUC19 and the archaeal plasmid pGT5. The large blunt-ended *Ssp*I-*Eco*RI fragment (*S–E*) of pUC19 was ligated to the large blunt-ended *Nsi*I-*Ava*II fragment (*N–A*) of pGT5 to form pAG1. The *Alw*NI-*Eco*47III fragment (*Al–Ec*) of pBR322 was ligated to the *Alw*NI-*Pvu*II fragment (*Al–Pv*) of pAG1 to form pAG2 *Af*, *Afl*III; *H*, *Hind*III, *Nd*, *Nde*I; *Ap*, ampicillin; *ORI*, contains the replication origin of pUC19; *sso* and *dso* indicate the starts of single and double stranded DNA synthesis, respectively, in pGT5; *Rom/Rop*, copy number control protein from pBR322; *MCS*, multiple cloning site; *ORF*, open reading frame. *Arrow* denoted by a *P* (archaeal promoter) precedes the transcription start site

DNA. In the first, total archaeal DNA was transformed into *E. coli*; plasmid DNA was then isolated from colonies that developed on agar plates containing ampicillin and was subjected to restriction fragment analysis. The number of transformants obtained with all samples of *P. furiosus* (Fig. 2a) and *S. acidocaldarius* (Fig. 2b) remained fairly constant with time, indicating that the plasmid was replicating in both archaea. Restriction fragment analysis of DNA minipreps from the *E. coli* transformants, showed that pAG1 was stably maintained in both archaea and in *E. coli* (Fig. 3, lane 1). In the second approach, a PCR assay was performed on the total archaeal DNA extract using primers specific for pGT5. A 643-bp product was produced when the shuttle vector was present (Fig. 3, lane 6). Neither deletions nor rearrangements were observed in pAG1 (Fig. 3, lane 1), whereas the earlier construct, pCSV1, was subject to deletions within the pGT5 component when the archaeal DNA was transformed into *E. coli* (Aagaard et al. 1996). Thus, both methods clearly showed that pAG1 (and pAG2 by the former method – Fig. 3, lane 2) was propagated and stably maintained in both archaea without selection. These results also confirm that the deletion of most of ORF2 from pGT5 did not impair replication or maintenance of the shuttle vector.

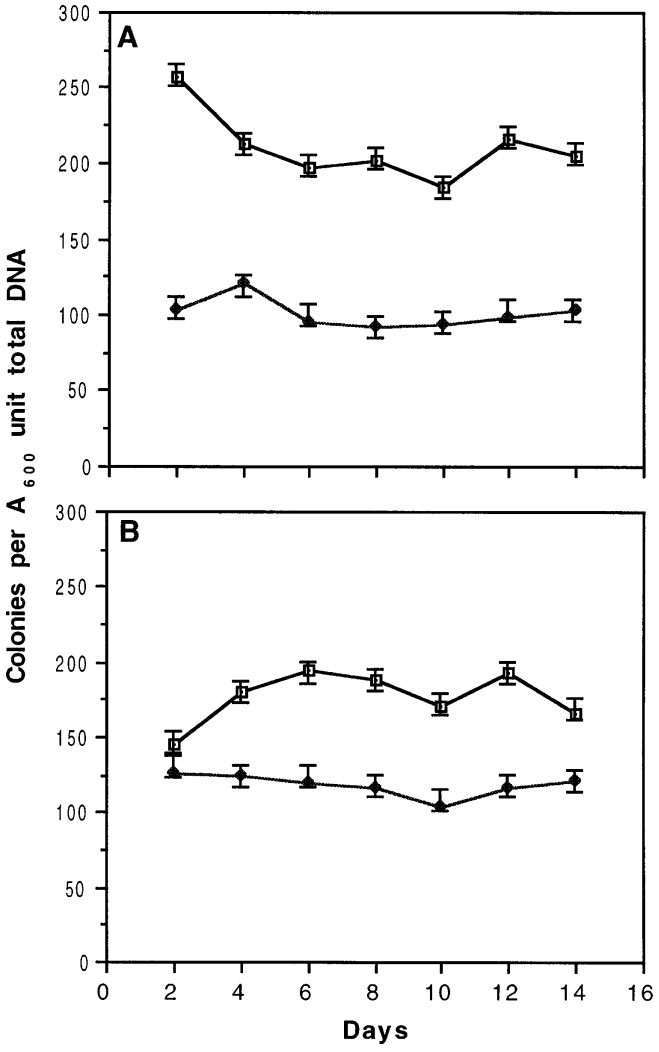

Fig. 2. Propagation of pAG1 in **A** *Pyrococcus furiosus* and **B** *Sulfolobus acidocaldarius*. After the amplification in archaea, pAG1 was isolated and retransformed into *Escherichia coli*. The numbers of colonies that developed on agar plates containing 100 μg/ml ampicillin are plotted against time. The results for pAG1 (*squares*) are compared with those from control samples transformed with pCSV1 (*diamonds*). *Error bars* denote variations for three independent determinations

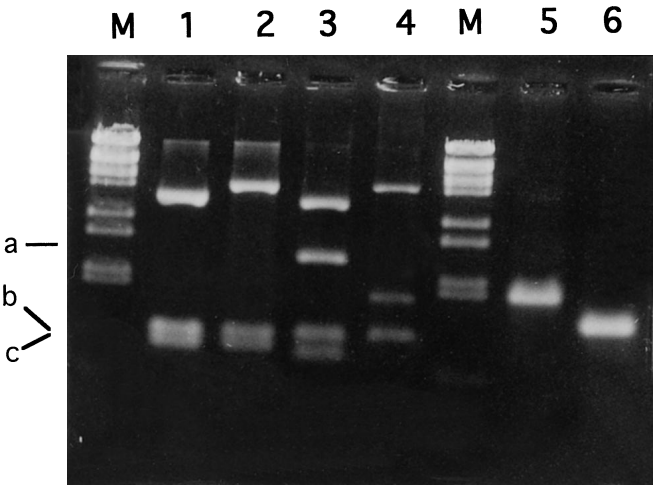

Fig. 3. Stability of the vector constructs. *Hind*III digests are shown for pAG1 (*lane 1*), pAG2 (*lane 2*), pAG1 + alcohol dehydrogenase (ADH) (*lane 3*), and pAG21 (*lane 4*). DNA fragments referred to in the text are indicated by lines: *a*, derives from a rearrangement in the pUC19 component of the vector after inserting the ADH gene into pAG1; *b* and *c*, derive from the pGT5 component of the vector. The bands in *lane 4* are of the expected sizes for pAG21. *Lanes 5 and 6* show PCR products amplified from total DNA isolated from archaeal cells transformed with pAG21, using primers specific for the ADH gene, including promoter and terminator (*lane 5*) and for the ORF alone (*lane 6*). *M*, phage λ DNA digested with *Bst*EII run as a size marker

Selectable marker

Butanol inhibits growth of *P. furiosus* and *S. acidocaldarius* completely at a concentration of 100 mM (Aagaard et al. 1995), and benzyl alcohol inhibits the growth of *S. solfataricus* at 25 mM concentration (R. Cannio, personal communication). Thus, induced resistance to alcohols could provide a mechanism for selection. Therefore, the minimum inhibitory concentrations (MICs) of butanol and benzyl alcohol were determined for *P. furiosus* and *S. acidocaldarius* over the alcohol concentration range 0–200 mM. For *P. furiosus*, MIC values were 58 mM for butanol and 55 mM for benzyl alcohol (Fig. 4a) and for *S. acidocaldarius* they were 127 mM for butanol and 40 mM for benzyl alcohol (Fig. 4b).

NAD-dependent alcohol dehydrogenases constitute one of three classes of alcohol dehydrogenases (ADH) that are present in all three domains and are essential enzymes in the fermentative pathway (Reid and Fewson 1994). *S. solfataricus* ADH has an associated aldehyde dehydrogenase activity and has a broad specificity for linear and branched primary alcohols, linear and cyclic secondary alcohols, linear and cyclic ketones, and anisaldehyde (Rella et al. 1987). Therefore, the ADH gene with its own promoter and terminator (Ammendola et al. 1992) was isolated by PCR amplification of *S. solfataricus* total DNA (Fig. 3, lane 5).

Effect of copy number on the stability of the vector constructs in *E. coli*

The unique *Pvu*II site of pAG1 was selected for inserting the ADH gene. The PCR product of the gene was phosphorylated with T4 polynucleotide kinase and ligated to the *Pvu*II linearized pAG1. After ligation and amplification in *E. coli*, recombinants were subjected to restriction fragment analyses and to PCR analyses using the primers specific both for the ADH gene, including the promoter and terminator, and for the ORF alone. Restriction fragment analyses indicated that the vector was unstable (Fig. 3, lane 3) and this was confirmed by the PCR analysis where the products of expected size (Fig. 3, lanes 5 and 6) were not observed (data not shown). Rearrangements occurred within the pUC19 section of the vector while the pGT5 component remained intact (Fig. 3, lane 3). Similar instability was observed when the ADH gene was cloned into the unique *Afl*III, *Hinc*II, or *Sma*I sites (data not shown). We inferred that the presence of the ADH gene caused

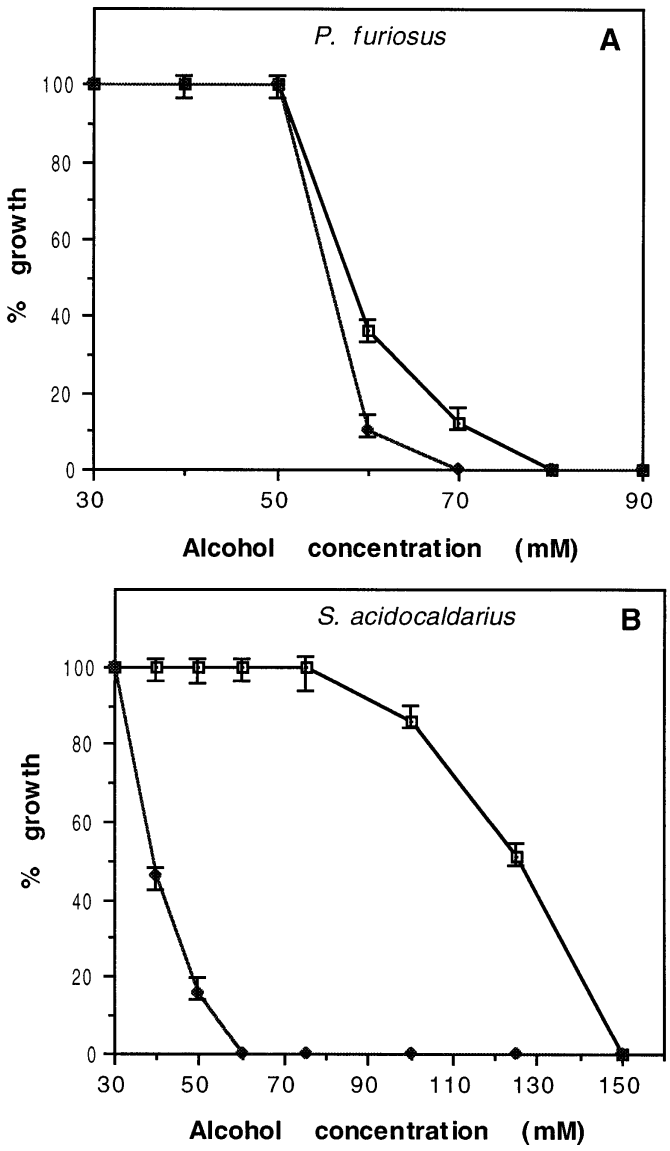

Fig. 4. Alcohol sensitivity of **A** *P. furiosus* and **B** *S. acidocaldarius* grown in the presence of increasing concentrations of benzyl alcohol (*diamonds*) and butanol (*squares*). The percentage growth was measured after 14 days and is normalized to cell growth in the absence of alcohol

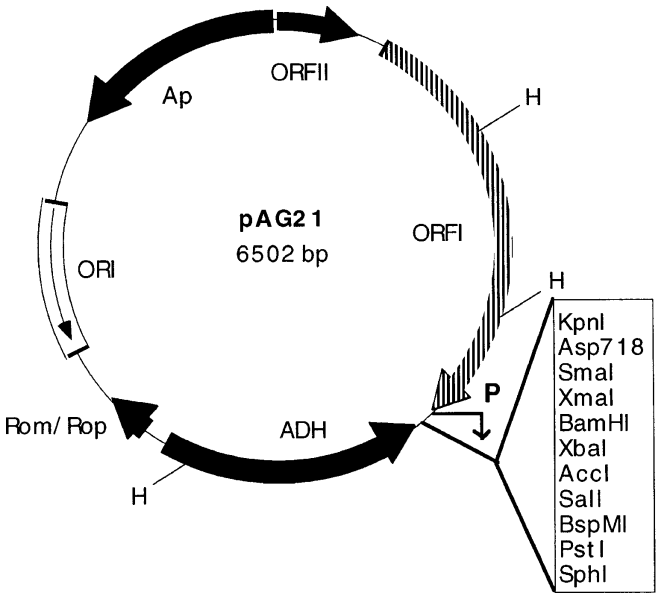

Fig. 5. Construction of pAG21. The 1.2-kb ADH gene from *S. solfataricus* was ligated to pAG2 to form pAG21. All the restriction sites shown in the multiple cloning site are unique. *H*, *Hind*III sites. The *arrow* denoted by *P* (archaeal promoter) precedes the transcription start site. Other symbols are defined in the legend to Fig. 1

the instability in *E. coli* although no such rearrangements were observed when the 23S rRNA gene of *P. furiosus*, carrying drug resistance mutations (Aagaard et al. 1996), was cloned into the pAG1 vector (data not shown).

An attempt was made to minimize the rearrangements observed in the pUC19 part of the shuttle vector by reducing its copy number in *E. coli*. The high copy number of the pUC19 vector is caused by the point mutation G112A in the Col E1 replication origin (Minton et al. 1988). This mutation is absent in pBR322, and can be suppressed by the Rom/Rop protein, resulting in a significantly lower copy number in *E. coli* (Lin-Chao et al. 1992). Therefore, the *Alw*NI-*Eco*47III fragment containing the Rom/Rop gene was isolated from pBR322 and ligated to the *Alw*NI-*Pvu*II fragment of pAG1 to produce the construct pAG2 (Fig. 1). The copy number of pAG2 was found to be 10–15-fold lower than pAG1 in *E. coli* (see Materials and methods) indicating that the Rom/Rop protein regulated the copy number of pAG2. pAG21 was then constructed by introducing the ADH gene into the unique *Sma*I site of pAG2 (Fig. 5). It was found that the reduced copy number of the vector in *E. coli* greatly increased the stability of vector carrying the ADH gene in *E. coli* (Fig. 3, lane 4).

Expression of the selectable marker gene in archaea

To test whether the ADH gene can be used as selectable marker in archaeal cells, *P. furiosus* and *S. acidocaldarius* were transformed with pAG21 and grown in the presence of the alcohols. Butanol was used at 60 mM, 70 mM, and 100 mM for *P. furiosus* and 100 mM, 125 mM, and 150 mM for *S. acidocaldarius*, and benzyl alcohol was tested at 60 mM, 75 mM, and 100 mM for *P. furiosus* and 40 mM, 50 mM, and 60 mM for *S. acidocaldarius*. Cells not transformed with pAG21 were used as a negative control, adding the same concentrations of alcohol as for the transformed cells. A positive control contained cells that were transformed with pAG21 but not exposed to alcohols.

Cell growth was monitored continuously, in the presence of alcohols, for a period of 14 days after transformation, by measuring A_{600} values every 2 days. Samples (5 ml) were

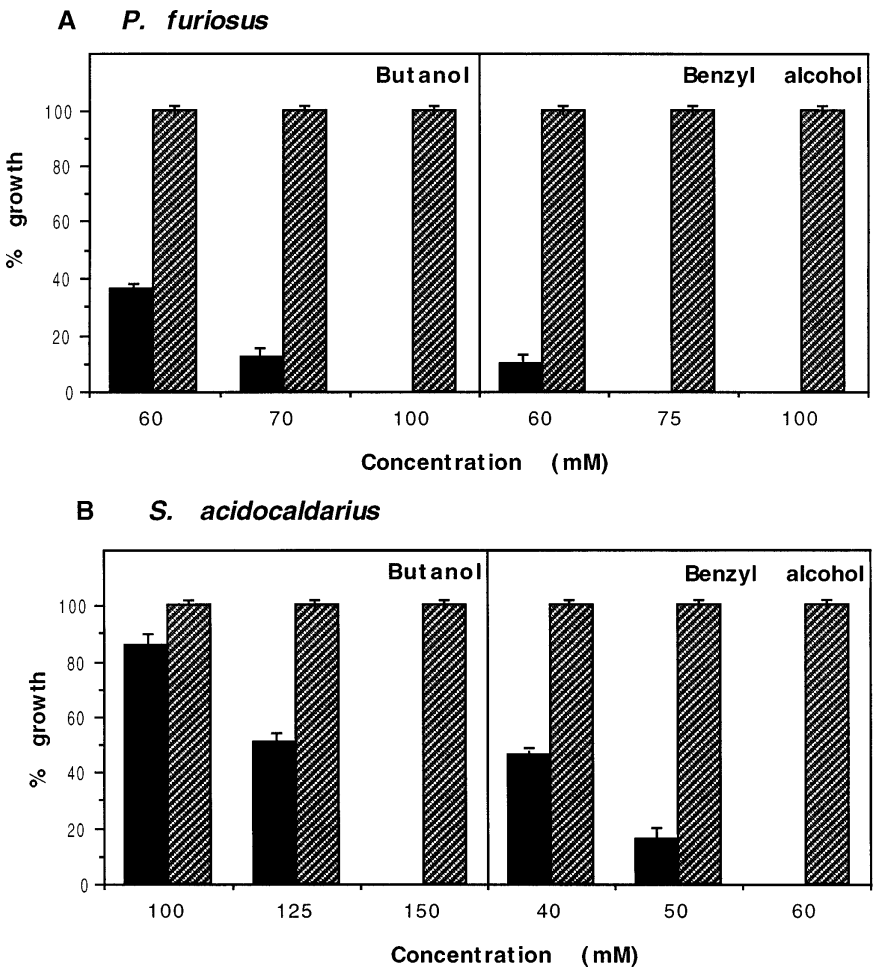

Fig. 6. Growth of **A** *P. furiosus* cells and **B** *S. acidocaldarius* cells in the presence of inhibitory concentrations of benzyl alcohol or butanol (see Fig. 4). The *histograms* indicate cell growth for untransformed cells (*filled*) and for cells transformed with pAG21 (*hatched*). Percentage growth values are normalized relative to control cells treated under similar conditions and grown without alcohol. *Error bars* denote the variation from three independent experiments

taken and DNA was extracted and retransformed into *E. coli*. The state of the pAG21 DNA isolated from *E. coli* cells was then shown to be stable by restriction fragment analysis (Fig. 3, lane 4). Moreover, PCR products amplified from the ADH gene were of the expected size (Fig. 3, lanes 5 and 6). Cell growth was normalized with respect to growth in control samples, treated under similar conditions but lacking alcohol, and the results (Fig. 6) show that the vector-borne ADH gene confers alcohol resistance on both *P. furiosus* and *S. acidocaldarius* cells.

Southern hybridizations were also performed on the total DNA isolated from *S. acidocaldarius* cells grown in the presence of benzyl alcohol. The results demonstrate an enhanced increase in the yield of pAG21, for cells grown over an 8-day period at the MIC value for benzyl alcohol of 40 mM (Fig. 4b), consistent with the shuttle vector replicating and being selected for (Fig. 7). All of these data indicate that pAG21 is stably maintained in both archaea and, moreover, that the ADH gene is active during selection.

Comparison of ADH gene sequences from organisms belonging to the three domains reveals relatively low sequence similarity between any two ADH genes (Reid and Fewson 1994). Moreover, the observed similarity is confined to small regions spread along the gene sequence. This limited similarity renders it unlikely that the vector-borne ADH gene from one organism will recombine into the host genome of another organism. The stability of shuttle vector pAG21 in the archaea tested is consistent with this inference.

The first product in the degradation pathway of alcohol is the corresponding aldehyde, which is more toxic to the cells than the alcohol itself. However, since the ADH gene also carries aldehyde dehydrogenase activity (Rella et al. 1987), the aldehydes that are formed during the first step of degradation are immediately converted to alkanoates or acids, depending on the alcohol used, thus relieving cells of toxic by-products of alcohol. Growth of the transformed archaea at inhibitory concentrations of alcohol clearly indicates that *S. solfataricus* ADH is expressed in *P. furiosus* and *S. acidocaldarius*. Thus, the shuttle vector pAG21 is likely to function as a general vector for many other archaeal hyperthermophiles which are also sensitive to alcohol (unpublished data).

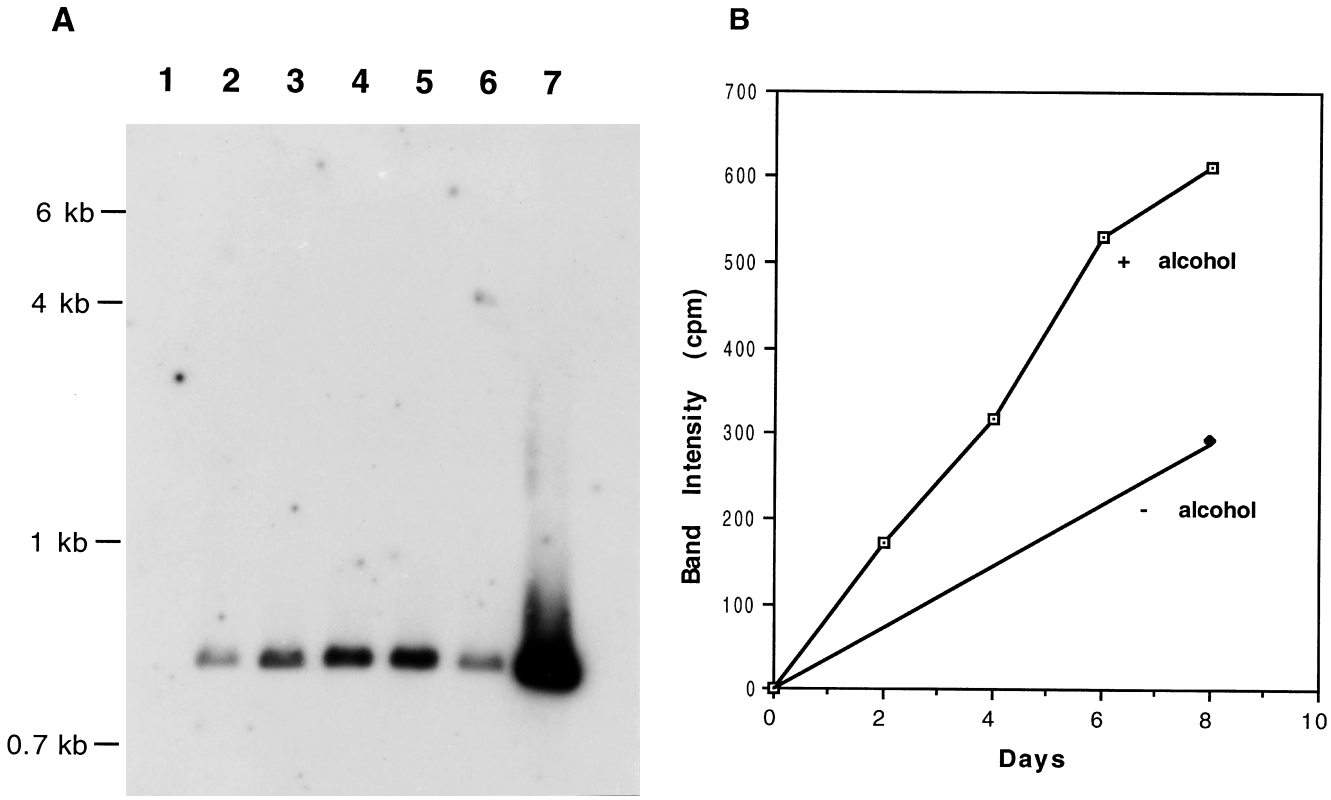

Fig. 7. A. Southern blot of DNA isolated from *S. acidocaldarius* cells, transformed with pAG21, and grown in the presence of 40 mM benzyl alcohol [corresponding to the minimum inhibitory concentration (MIC) value – see Fig. 2B]. Total DNA (1 μg) prepared from cell cultures transformed with pAG21 was digested with *Hind*III for 4 h. *Lane 1*, DNA from cells that were not transformed with pAG21 grown in the presence of benzyl alcohol; *lanes 2 to 5*, DNA isolated after 2, 4, 6, and 8 days, respectively, of continuous culture in the presence of benzyl alcohol; *lane 6*, control DNA from cells transformed with pAG21 but grown in the absence of alcohol; and *lane 7*, 1 μg pure pAG21 DNA prepared from *E. coli* cells. The plasmid DNA was probed with [^{32}P]-labelled pGT5-specific probes. Size markers derive from phage λ DNA digested with *Bst*EII. **B** A plot of the counts in the plasmid probe against the number of days of cell culture for the pAG21-transformed cells (*squares*) and untransformed cells (*diamonds*)

Conclusions

P. furiosus, an anaerobe growing optimally at 95°C, and *S. acidocaldarius*, an aerobe growing at an optimum temperature of 70°C, are representatives of the euryarchaeotal and crenarchaeotal kingdoms of archaea, respectively (Woese et al. 1990). The demonstration that the shuttle vectors can be used to transform both these divergent organisms, and are selectable, renders them potentially valuable as general vectors for archaeal hyperthermophiles. Moreover, since the basic constructs pAG1 and pAG2 contain the minimum regions necessary for plasmid replication and maintenance in the archaea and in *E. coli*, they can be used for further development of shuttle vectors by inserting alternative marker genes and strong archaeal promoters. As an example, we have shown that the ADH gene was unstable in *E. coli* when inserted into pAG1 but was stable in the lower copy number pAG2 construct.

The expression of the ADH gene from *S. solfataricus* in *P. furiosus* and in *S. acidocaldarius* demonstrates that an archaeal gene can be expressed successfully in both an organism of another archaeal kingdom (Kjems et al. 1992) and in another organism of the same genus, although *S. solfataricus* and *S. acidocaldarius* are not very closely related (Kjems et al. 1992; Trevisanato et al. 1996). Thus, potentially, any protein of interest from the archaeal thermophiles can be expressed using these shuttle vector constructs. Genes can be cloned into the unique restriction sites *Kpn*I, *Sal*I, *Xba*I, *Asp*718, *Sma*I, *Xma*I, *Bam*HI, *Acc*I, *Bsp*MI, *Pst*I, and *Sph*I in the multiple cloning site and the upstream archaeal promoter will direct the expression of genes. Further tailoring of the vectors may be possible when the function of the ORF1-encoded protein of pGT5 becomes clearer. Moreover, both pAG1 and pAG2 can be used for expressing archaeal genes in bacteria if the archaeal promoter is replaced by a bacterial promoter or if a bacterial promoter is inserted adjacent to the multiple cloning site. Each vector can also be used for complementation analysis, strain construction, and DNA sequencing. Thus, these "first generation" vectors should be valuable for the further development of genetic tools for hyperthermophilic archaea.

Acknowledgments We thank Daniel Prieur and Patrick Forterre for the plasmid pGT5, and Qunxin She, Carsten Petersen, and Jan Neuhard for helpful discussions and critical reading of the manuscript. This work was supported by EU grant no. B102-CT93-0274.

References

Aagaard C, Phan HP, Trevisanato S, Garrett RA (1995) A spontaneous point mutation in the single 23S rRNA gene of the thermophilic archaeon *Sulfolobus acidocaldarius* confers multiple drug resistance. J Bacteriol 176:7744–7747

Aagaard C, Leviev I, Aravalli RN, Forterre P, Prieur D, Garrett RA (1996) General vectors for archaeal hyperthermophiles: strategies based on a mobile intron and a plasmid. FEMS Microbiol Rev 18:93–104

Ammendola S, Raia CA, Caruso C, Camardella L, D'Auria S, DeRosa M, Rossi M (1992) Thermostable NAD^+-dependent alcohol dehydrogenase: relationship to other alcohol dehydrogenases. Biochemistry 31:12514–12523

Barns SM, Delwiche CF, Palmer JD, Pace NR (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc Natl Acad Sci USA 93:9188–9193

Bertani G, Baresi L (1987) Genetic transformation in the methanogen *Methanococcus voltae* PS. J Bacteriol 169:2730–2738

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113

Bult CJ, White O, Olsen GJ, Zhou L, Fleischmann RD, Sutton GG, Blake JA, FitzGerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougherty BA, Tomb JF, Adams MD, Reich CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JL, Geoghagen NSM, Weidman JF, Fuhrmann JL, Venter JC et al. (1996) Complete genome sequence of the methanogenic archaeon *Methanococcus jannaschii.* Science 273:1058–1073

Charlebois RL, Gaasterland T, Ragan MA, Doolittle WF, Sensen CW (1996) The *Sulfolobus solfataricus* P2 genome project. FEBS Lett 388:88–91

Cline SW, Doolittle WF (1987) Efficient transfection of the archaebacterium *Halobacterium halobium*. J Bacteriol 169:1341–1344

Erauso G, Marsin S, Benbouzid-Rollet N, Baucher MF, Barbeyron T, Zivanovic Y, Prieur D, Forterre P (1996) Sequence of plasmid pGT5 from the archaeon *Pyrococcus abyssi*: evidence for rolling-circle replication in a hyperthermophile. J Bacteriol 178:3232–3237

Garrett RA (1996) *Methanococcus jannaschii* and the golden fleece. Curr Biol 6:1377–1380

Gernhardt P, Possot O, Foglino M, Sibold L, Klein A (1990) Construction of a integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. Mol Gen Genet 221:273–279

Grogan DW (1996) Isolation of *Sulfolobus acidocaldarius* mutants. In: Robb FT, Place AR, Sowers KR, Schreier HJ, DasSarma S, Fleishmann EM (eds) Archaea: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Holmes ML, Dyall-Smith ML (1990) A plasmid vector with a selectable marker for halophilic archaebacteria. J Bacteriol 172:756–761

Hove-Jensen B (1985) Cloning and characterization of the *prs* gene encoding phosphoribosylpyrophosphate synthetase of *Escherichia coli*. Mol Gen Genet 201:269–276

Kjems J, Larsen N, Dalgaard JZ, Garrett RA, Stetter KO (1992) Phylogenetic relationships amongst the hyperthermophilic archaea determined from partial 23S rRNA gene sequences. Syst Appl Microbiol 15:203–208

Lam WL, Doolittle WF (1989) Shuttle vectors for the archaebacterium *Halobacterium volcanii*. Proc Natl Acad Sci USA 86:5478–5482

Lin-Chao S, Chen WT, Wong TT (1992) High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. Mol Microbiol 6:3385–3393

Metcalf WW, Zhang JK, Apolinario E, Sowers KR, Wolfe RS (1997) A genetic system for Archaea of the genus *Methanosarcina*: liposome-mediated transformation and construction of shuttle vectors. Proc Natl Acad Sci USA 94:2626–2631

Minton NP, Chambers SP, Prior SE, Cole ST, Garnier T (1988) Copy number and mobilization properties of pUC plasmid. Focus 10:56

Pace NR (1991) Origin of life – facing up to the physical setting. Cell 65:531–533

Reid MF, Fewson CA (1994) Molecular characterization of microbial alcohol dehydrogenases. Crit Rev Microbiol 20:13–56

Rella R, Raia CA, Pensa M, Pisani FM, Gambacorta A, De Rosa M, Rossi M (1987) A novel archaebacterial NAD^+-dependent alcohol dehydrogenase. Eur J Biochem 167:475–479

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sensen CW, Klenk HP, Singh RK, Allard G, Chan CCY, Liu QY, Penny SL, Young F, Schenk ME, Gaasterland T, Doolittle WF, Ragan MA, Charlebois RL (1996) Organizational characteristics and information content of an archaeal genome: 156 kb of sequence from *Sulfolobus solfataricus* P2. Mol Microbiol 22:175–192

Stetter KO (1996) Hyperthermophilic procaryotes. FEMS Microbiol Rev 18:128–130

Trevisanato SI, Larsen N, Segerer A, Stetter KO, Garrett RA (1996) Phylogenetic analysis of the phylogenetic order of Sulfolobales based on sequences of 23S rRNA genes and 16S/23S rDNA spacers. Syst Appl Microbiol 19:61–65

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Worrel VE, Nagle DP, McCarthy D, Eisenbraun A (1988) Genetic transformation system in the archaebacterium *Methanobacterium thermoautotrophicum*. J Bacteriol 170:653–656

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119