Small multicopy, non-integrative shuttle vectors based on the plasmid pRN1 for Sulfolobus acidocaldarius and Sulfolobus solfataricus, model organisms of the (cren-)archaea

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ABSTRACT

The extreme thermoacidophiles of the genus Sulfolobus are among the best-studied archaea but have lacked small, reliable plasmid vectors, which have proven extremely useful for manipulating and analyzing genes in other microorganisms. Here we report the successful construction of a series of Sulfolobus–Escherichia coli shuttle vectors based on the small multicopy plasmid pRN1 from Sulfolobus islandicus. Selection in suitable uracil auxotrophs is provided through inclusion of pyrEF genes in the plasmid. The shuttle vectors do not integrate into the genome and do not rearrange. The plasmids allow functional overexpression of genes, as could be demonstrated for the β-glycosidase (lacS) gene of S. solfataricus. In addition, we demonstrate that this β-glycosidase gene could function as selectable marker in S. solfataricus. The shuttle plasmids differ in their interruption sites within pRN1 and allowed us to delineate functionally important regions of pRN1. The orf56/orf904 operon appears to be essential for pRN1 replication, in contrast interruption of the highly conserved orf80/plrA gene is tolerated. The new vector system promises to facilitate genetic studies of Sulfolobus and to have biotechnological uses, such as the overexpression or optimization of thermophilic enzymes that are not readily performed in mesophilic hosts.

INTRODUCTION

Sulfolobus, a genus of thermoacidophilic crenarchaeotes, has provided much of the information currently available on the physiology and molecular biology of archaea from geothermal environments. Seminal studies of Sulfolobus spp. have addressed, for example, chromatin-binding proteins (1,2), replication (3), cell cycle (4), repair (5), transcription (6), translation (7,8) as well as metabolism (9). The genome sequences of three species, Sulfolobus solfataricus, Sulfolobus tokodaii and Sulfolobus acidocaldarius, have been published (10–12). Microarrays for these organisms are commercially available and proteomic studies have been undertaken (13,14).

On a practical level, these advances reflect the relative ease with which Sulfolobus spp. are manipulated in the laboratory. Sulfolobus cells can be grown aerobically and heterotrophically on a variety of complex and defined carbon sources, either in liquid media or on plates, with doubling times as short as a few hours. Since Sulfolobus spp. are hyperthermophiles with optimal growth temperatures around 80°C, their proteins are intrinsically stable and resistant to proteolysis. As a result, Sulfolobus enzymes expressed in mesophilic hosts can often be purified with the aid of a heat step, which removes most proteins of the host. The structural rigidity of thermophilic proteins also appears to be an advantage for crystallization, which is a prerequisite for X-ray analysis of 3D structure.

Ultimately, however, the comprehensive study of molecular phenomena in any organism requires genetic analysis and manipulation in vivo. Although numerous plasmids and viruses have been reported in Sulfolobus spp., the development of these natural genetic elements into experimentally useful tools for Sulfolobus has lagged behind the corresponding progress made with methanogenic and halophilic archaea (15). Although different plasmid and virus-based vectors have been constructed (16–21), to our knowledge only SSV1-based viral vectors (20–22) have been successfully applied by other groups to analyze genes in vivo.
We used the plasmid pRN1 as starting point to construct shuttle vectors for *Sulfolobus*. This plasmid is notable for its relatively small size (5.4 kb), copy numbers ranging from 10 to 20 in mid-log phase, and the three genes (*orf56, orf80* and *orf904*) that are conserved in other *Sulfolobus* plasmids. In previous work, we have analyzed the three conserved proteins (representing two DNA-binding proteins and the replication protein) and the transcriptional activity of the plasmid (23–25). Those studies revealed that ORF56 binds upstream of its own gene and down-regulates the expression of the co-transcript *orf56/orf904* (26). It therefore appears that ORF56 could be involved in regulating the copy number of the plasmid. Similarly, ORF80 is a sequence-specific DNA-binding protein, but the physiological function of this protein has remained unclear. Orf904 encodes the third conserved protein, a multifunctional 110-kDa replication enzyme that appears to play a central role in replication. However, neither the exact molecular mechanism of replication nor the replication origin is known for pRN1.

These questions would be open to experimental study in vivo by the availability of successful *Escherichia coli–Sulfolobus* shuttle vectors, but the lack of this information also makes it difficult to predict which features of pRN1 must be preserved in constructing such vectors. We therefore took an empirical approach, in which an artificial transposon was inserted at many locations around pRN1. The resulting plasmids provided a series of potential shuttle vectors differing only in the relative location and orientation of inserted genes, from which the best-performing constructs were identified. The series also provided a way to reveal experimentally which regions of pRN1 may be important for successful propagation in *Sulfolobus* hosts.

**MATERIALS AND METHODS**

**Strains and growth conditions**

This study used *S. solfataricus* strains PH1-16 (27) and PBL2025 (28), *S. islandicus* strains REN1H1 (29) R1, R20, S1, R1S1 and HVE10/4 H1 (this study), and *S. acidocaldarius* MR31 (30). Liquid cultures were grown in Brock's basal salts medium at pH 3.5 (31) or the mineral medium MR31 (30). Liquid cultures were grown with D-(+)-xylose at 0.2%, and tryptically hydrolyzed casein, i.e. tryptone (BD Biosciences), hydrolyzed casein, i.e. NZAmine AS (Sigma), or enzymatically hydrolyzed casein, i.e. tryptone (BD Biosciences), were added to the medium. Plates were solidified by addition of 0.6% Gelrite (Sigma) and 10 mM CaCl₂. Plates and shake flask cultures were incubated at 75°C.

**Random insertion into pRN1**

The Tn5-derived transposon TnPA21 (33) was amplified by PCR using a primer (5'-CTGTCCTCTATACAC ATCT) complementary to the mosaic end sequence, the terminal inverted repeat sequence found on both ends of the transposon. Native pRN1 (accession number NC 001771) was isolated from a culture of *S. islandicus* REN1H1 containing only the pRN1 plasmid (34) using the Nucleo Spin plasmid extraction Kit (Macherey Nagel). Plasmid preparations from ~100 ml of culture were combined and ethanol-precipitated to obtain 0.1 µg of pRN1 for the transposition reaction. The transposition reaction was carried out in vitro using the EZ-Tn5 transposase (Epicentre) according to the instructions of the manufacturer. Transposon and plasmid were mixed at a molar ratio of 1:1. The transposition reaction products were transformed into *E. coli* EC100 pir' (Epicentre). The resulting transformants were screened for correctly inserted transposons by restriction digestion with SacI and NotI. Twenty percent of the screened colonies (a total of 80 plasmids) showed two restriction bands with a combined length of 7.2 kb and were kept for further analysis.

**Construction of pA–pN and pJlacS**

Thirteen of the 80 constructs were chosen after more precise mapping of the insertion sites by restriction digestion. From these, the *E. coli* replicon (R6Kγ origin of replication and *cat* gene) introduced by TnPA21 was excised using the NotI and PspOMI sites present on the ends of the transposon sequence. The resulting pRN1 fragments interrupted at different sites were then cloned into the NotI linearized vector delta2pyrEF. The plasmid delta2pyrEF is a derivative of pBluescript with the *lacZ* and f1 origin regions deleted. Specifically, pBluescriptSKII(+) was cut with SspI and KpnI, re-ligated, cut with SacI and SapI and re-ligated. The *pyrEF* genes from *S. solfataricus* P2 (plasmid pBSKP-pyrEF, generously provided by Christa Schleper) were cloned into the Sall and PstI sites. For constructs pA to pN the transitional region from the delta2pyrEF part to the pRN1 part was sequenced to determine the exact insertion site and the direction of the transposition insertion and the direction of the cloning into the NotI site of delta2pyrEF. In construct pG, by restriction analysis using HindIII an additional HindIII site was found to be present and confirmed by sequencing. As the pRN1 part was not PCR-amplified, but stems from the native plasmid, we conclude that pRN1 had this mutation already when isolated, or that this mutation was introduced in *E. coli* during propagation of pG. Except for this point mutation, pG corresponds to the expected sequence.

To construct pJlacS we cloned the tf55ΔlacS expression cassette (20) into the unique SacII restriction site of pJ.

**Methylation of plasmids**

For transformation into *S. acidocaldarius* shuttle constructs were methylated at the N4-position of the inner cytosine residues of GGCC recognition sequences to circumvent restriction by the SuaI restriction enzyme (35). Plasmids were methylated in vivo as previously described (36) by transforming the shuttle constructs into *E. coli* ER1821 (New England Biolabs) bearing the
additional plasmid pM.EsaBC41 (New England Biolabs). Complete methylation was confirmed by the absence of any cutting after incubation with 5 U HaeIII for 1–4 h.

Electroporation
Constructs were electroporated either using a Gene Pulser I or Gene Pulser II (BioRad) following the protocol of Schleper et al. (1992) or using a Gene Pulser Xcell (BioRad) with a constant time protocol with input parameters 1500 V, 10.2 ms, 2 mm cuvettes or using the protocol described by Kurosawa and Grogan (36) (1250 V, 1000 Ω, 25 μF, 1 mm cuvettes). For *S. acidocaldarius*, regeneration was done for 30–40 min in tryptophan/xylose medium, water or recovery solution before plating on tryptophan/xylose plates or NZAmne/xylose plates. Best results were obtained with recovery solution. Recovery solution was prepared as a 2× concentrated solution (=1% sucrose, 20 mM β-alanine/1.5 mM malate buffer, pH 4.5, 10 mM MgSO₄). Directly after electroporation the 50 μl cell suspension was mixed in the cuvette with 50 μl of 2× recovery solution (room temperature), transferred into a 1.5-ml tube and incubated for 30 min at 75°C in a benchtop shaker at 600 r.p.m. before plating the cells. For lactose utilization in *S. solfataricus*, plating after electroporation was not feasible. Instead, electroporated cells were regenerated for 10 min in 1 ml of Millipore water (pre-warmed) at 75°C and then directly transferred into pre-heated lactose medium and cultivated in 50 ml flasks.

Retransformation
One microliter of genomic DNA preparation or 1–5 μl of plasmid prepared from *Sulfobolus* by alkaline lysis were transformed into RbCl-competent *E. coli* XL1-Blue cells or into the merBC deficient *E. coli* strain ER2267 (New England Biolabs).

Plasmid copy number determination
Copy numbers of the different shuttle constructs were determined as already described (26) by qPCR and cell number determination through plating, respectively.

Southern blots
Genomic DNA was prepared from 1 ml of culture using the Chemagic DNA Bacteria Kit (Chemagen, Baesweller, Germany) according to the instructions of the manufacturer. After digestion with either HindIII for constructs PA–pN or SacI for pJlacS restriction fragments were resolved in 1% agarose gels, transferred to a Hybond N membrane (Amersham) by capillary transfer, fixed by UV irradiation for 5 min on a UV transilluminator and hybridized to digoxigenin-labeled probes complementary to the pyrE gene (position 9–320 from the start of the pyrE gene from *S. solfataricus* P2) and pRN1 (position 4892–5048 in pRN1) for PA to pN or lacS (position 1124–1438 from the start of the lacS gene from *S. islandicus* REN1H1) and pRN1 for pJlacS. Labeling, hybridization (50% formamide, 42°C), washing (0.5× SSC, 60°C) and detection was done using the PCR DIG Probe Synthesis Kit and the Digoxigenin Labeling and Detection Kit (Roche).

Colony hybridization
Colonies from plates were transferred to Hybond N membranes and subsequently incubated for 10 min on a filter paper soaked with 0.5 M NaOH, 1.5 M NaCl then for 10 min on a filter paper soaked with 1 M Tris–HCl (pH 7.5), 1.5 M NaCl, then for 5 min on a filter paper soaked with 10× SSC. Membranes were cross-linked for 5 min on the 10× SSC filter paper using a transilluminator. Hybridization and detection were done as described for Southern blots with pRN1-specific probes.

β-Galactosidase assay
For convenience, the broad-specificity β-d-glycosidase (37,38) encoded by the *S. solfataricus* lacS gene was assayed as β-galactosidase activity. Crude extracts were prepared by a freeze–thaw method (20) in which cells were re-suspended in 50 mM Na-phosphate buffer, pH 7, and subjected to five freeze–thaw cycles (−196°C/+50°C). After centrifugation for 30 min at 13000 r.p.m. the supernatant was stored at −20°C, or assayed directly. All β-galactosidase assays were conducted in triplicate in a 75°C bench top shaker. The reaction mixture consisted of 1 μl of crude extract (or water for blanks), 92 μl of 50 mM Na-phosphate buffer, pH 7 and the assay was started by addition of 7 μl of 12 mg/ml−1 ortho-nitrophenyl-β-d-galactopyranoside (ONPG) solution. Incubation was continued for 5 min before the tubes were rapidly cooled on ice and 100 μl of 1 M Na₂CO₃ solution was added to stop the reaction. Concentration of ONPG was subsequently determined in a 96-well plate in a plate reader at 410 nm using a standard curve generated with ONPG. Protein concentration of the crude extracts was determined by the method of Ehresmann (39).

X-gal staining
A qualitative β-galactosidase assay was based on hydrolysis of 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal). For liquid cultures, 200 μl of culture were mixed with 20 μl of substrate solution (20 mg/ml in dimethylformamide) and incubated at 75°C until color development was observed. To score colonies, plates were sprayed with the same X-gal solution and incubated at 75°C.

Stability measurements
Small cultures (0.2-ml each) were produced under selective conditions by transferring colonies from selective plates to xylose/tryptophan medium without uracil. After 2 days of incubation, the cultures (n = 2 to 4 per construct) were sampled, diluted in sterile buffer and plated on plates with and without uracil supplementation; from the resulting colony counts, the numbers of Pyr⁺ and Pyr⁻ cells in the population was determined. The process was repeated after two cycles of transfer to uracil-supplemented liquid medium (3% inoculum), each involving growth to a final density of ~4 × 10⁸ CFU/ml. This resulted in a total of three measurements per population.
and an overall numerical expansion under non-selective conditions of \( \sim 10^3 \).

**RESULTS**

**Construction of shuttle vectors**

In principle, shuttle vectors can be constructed from two plasmids that replicate in different hosts simply by fusing them at two points that preserve all the important functions of each plasmid. However, in the case of pRN1, it was not clear which ORFs or intergenic regions may be important for successful replication in *Sulfolobus* hosts. We therefore used transposition to generate pRN1 constructs interrupted at a number of different sites without regard to the location or its sequence context. From the initial transposition mixture, 13 distinct insertion points were chosen for further development, which included addition of the *pyrEF* genes of *S. solfataricus* as selectable marker (Figure 1, Table 1). In addition to providing more chances for a successful construct, this unbiased approach allowed us to evaluate possible differences in the performance of the vector constructs in *Sulfolobus*. This would provide some of the first functional data regarding which of the conserved open reading frames are important for plasmid replication and maintenance.

As we expected the replication operon *orf56/orf904* to be essential, only one construct interrupted within this region was chosen for analysis. The other constructs were chosen to have the interruption sites distributed as evenly as possible over the remaining part of pRN1. The open reading frames *orf80, orf90a, orf72* and *orf90b* are also interrupted in at least one construct. We have already shown that *orf90a, orf72* and *orf90b* are very unlikely to play a role in plasmid replication or maintenance in view of their very low levels of expression (26).

**Recipient strains**

The plasmids pA–pN were electroporated into *Sulfolobus* strains representing different species. As the *S. solfataricus* *pyrEF* genes provide the selectable marker, stable uracil auxotrophs were needed as recipient strains. Table 2 gives an overview of the *Sulfolobus* *pyrEF* mutants tested as recipients for the various pRN1 constructs.

For *S. solfataricus* PH1-16 and the different *S. islandicus* mutants, the vectors seemed to be unstable, as only very low amounts of shuttle vector could be detected in some experiments. We did observe growth under selective conditions and positive PCR reactions with pRN1-specific primer pairs, but never observed positive results in Southern blots. Thus, the transformed cells seemed to lose the vector rapidly, and the continued growth observed on uracil-free medium may have been due to reversion, or recombinational conversion of the *pyrEF* mutations to the wild-type sequence.

For *S. acidocaldarius* MR31, electroporation yielded distinct, rapidly growing colonies on uracil-deficient plates, and growth of these primary transformants was maintained in uracil free-liquid medium (tryptone/xylose...
or NZAmine/xylose). Sequencing of the chromosomal pyrE locus revealed that no reversion of the mutated pyrE sequence had occurred in these clones, consistent with the nature of this mutation (an 18-bp deletion). Only one shuttle construct, pM (disrupted replication operon orf56/orf904), consistently failed to yield Pyr⁺ transformants in MR31.

Table 2. Sulfolobus species and strains tested as recipient strains for the shuttle constructs pA–pN

| Sulfolobus species and strain | Mutant name | Gene | Type of mutation | Reference | Successful with |
|------------------------------|-------------|------|------------------|-----------|-----------------|
| *S. acidocaldarius*           | MR31       | pyrE | Deletion         | 18-bp deleted | pA–pN, pMacS     |
| *S. islandicus* REN1H1        | R1         | pyrE | Point mutations  |           | S. Berkner and G. Lipps, unpublished results |
| *S. islandicus* REN1H1        | R20        | pyrEF| Insertion sequence | SMN1 in promoter region | (43) |
| *S. islandicus* REN1H1        | S1R1       | pyrEF/lacS | Point mutations/ frame shift | S. Berkner and G. Lipps, unpublished results |
| *S. islandicus* HVE10/4       | H1         | pyrEF | Point mutations  |           |                 |
| *S. solfataricus* P1          | PH1-16     | pyrF/lacS | Insertion sequences | ISC1359 ISC1217 SSO3004-SSO3050 deleted |
| *S. solfataricus* 98/2        | PBL2025    | lacS | Deletion         |           | S. Berkner and G. Lipps, unpublished results |

Shuttle constructs are stable and do not integrate or rearrange

Using Southern blots we examined whether the shuttle constructs pA–pN were present in the *S. acidocaldarius* clones selected after electroporation (Figure 2A). This analysis confirmed that the vector constructs had the correct size and did not integrate into the host genome, as
no bands in addition to the expected ones for the episomal form of the vectors were observed. In addition, none of the vector constructs were observed to undergo large rearrangements in S. acidocaldarius, with the exception of pB. For this construct, an additional band of ~6 kb was observed in the Southern blot, which indicated a rearrangement occurring in the *Sulfolobus* host. The Southern blot was repeated with a second enzyme (SacI) and again we did not have any indication for rearrangement of the plasmid constructs except pB or for integration into the host genome (data not shown).

Next, we tested by retransformation experiments if the original shuttle plasmids could be recovered intact from *S. acidocaldarius* transformants. As shown in Figure 2D, 2 to 30 transformants per construct were checked by restriction analysis and only the correct restriction pattern was observed. In the case of pA, pC, pD and pE, the shuttle plasmids were also isolated directly from transformed *Sulfolobus* cultures and analyzed by restriction digestion (Figure 2E).

From the initial set of constructs, plasmids pC and pE were chosen to evaluate long-term stability under selective conditions. Cultures of pC and pE transformants were cultivated continuously for ~200 generations without uracil supplementation. Then retransformation experiments and Southern blots were repeated (Figure 2B), with the same results.

The direction of the insertion in a given region does not influence performance of the vector. The vectors pF, pI and pG, for example, have insertion sites within 15 nt of each other. In pG, the *pyrEF* genes are oriented clockwise, in pI and pF counter clockwise, without detectable effects on plasmid stability or growth (Figure 3). In addition, the growth phenotype of transformed cells is comparable to that of the untransformed recipient strain when supplemented with uracil.

To test if the selection for uracil prototrophy ensures that every cell contains a shuttle vector, cells were plated on selective NZAmine/xylose medium and on non-selective tryptone/xylose medium supplemented with uracil. In eight different experiments comparable colony numbers were obtained on selective and non-selective plates showing that no cells escaped the selection. To prove that the vast majority of cells contained a shuttle vector, cells transformed with constructs pC and pE were plated on non-selective plates and examined by colony hybridizations with pRN1 specific probes (Figure 2C).

**Vector retention and copy number**

The facile generation of Pyr+ colonies by electroporation and direct plating on selective medium indicated that all the constructs tested, except for construct pM, could replicate in *S. acidocaldarius* under appropriate selection. In order to provide a more stringent and quantitative comparison of these constructs, we monitored their retention in populations growing in non-selective, uracil-supplemented liquid medium. Specifically, the fraction of Pyr+ cells in the population at three different times was determined, by dilution and plating on uracil-supplemented and unsupplemented plates.

Figure 3C shows the retention of 13 constructs over ~10 generations, corresponding to ~1000-fold numerical expansions of the host cell populations. Most constructs showed measurable loss under these conditions, resulting in ~10% Pyr+ cells in the cultures. In a few cases, however, plasmid retention was much lower. The most severe instability was seen in construct pH, in which the *orf80* gene is interrupted. This result provided evidence that the small DNA-binding protein encoded by *orf80* has an important role in the stable maintenance of pRN1 and related plasmids. Intermediate instability was observed for construct pJlacS (described below). We suspect that this construct with the very strong *tf55* promoter is a burden for the cell. Both pJlacS and pH yielded small or heterogeneous colonies when streaked on selective plates, consistent with the observed instability under non-selective conditions.

According to qPCR results, all constructs showed copy numbers within the range of 2–8 copies per cell, except for pB that showed low copy numbers around one. For pC and pE, the time course of the copy number during batch fermentation was also determined (Figure 4). The copy number increased in early and mid-log phase and decreased in stationary/death phase. This behavior has also been observed for the wild-type pRN1 plasmid (26). The copy number of the wild-type plasmid in its original host strain (together with pRN2) is higher, reaching 20 copies per cell when grown on rich media containing yeast extract (26) and 10 copies per cell when grown on tryptone media. When pRN1 alone is present in its original host (34) the copy number is only about two. The shuttle vectors therefore maintain a similar copy number in *S. acidocaldarius* as the native plasmid pRN1 in the original host strain *S. islandicus* REN1H1.

**Suitability for protein expression or reporter gene tests**

To test whether the vector tolerates the insertion of sequences containing expressed *Sulfolobus* genes we cloned the rather strong *tf55* promoter (20) together with the *lacS* gene into shuttle construct pJ generating the vector pJlacS. The stability of this construct was tested by retransformation into *E. coli* and Southern blotting (Figure 5A and B). The construct turned out to be stably replicated in *S. acidocaldarius*. Staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) revealed that the β-galactosidase was expressed under the control of the heat shock promoter (Figure 5C). This test could be done without prior isolation of a lacS mutant of strain MR31 because the endogenous β-galactosidase activity is very low, i.e. ~0.01 U/mg protein, in *S. acidocaldarius* (37). The enzyme activity was also measured quantitatively (as β-galactosidase) at different ODs of a MR31 culture transformed with pJlacS. Copy numbers were determined simultaneously and it was found that measured β-galactosidase activities correlated well with vector copy numbers (Figure 5E), as previously observed (16,20). It should be noted that the β-galactosidase activity
of MR31 transformed with pJlacS (2–11 U/mg) is much higher than the wild-type β-galactosidase activity of *S. solfataricus* (0.2 U/mg) (26) and is comparable to the β-galactosidase activity in a viral overexpression system (1.5–5 U/mg) (20).

**Replication in *S. solfataricus***

Demonstration of lacS expression in *S. acidocaldarius* pJlacS transformants suggested the possibility of a selection by conferring, or restoring, the ability to catabolize lactose or other β-glycosides. The *S. solfataricus* 98/2 lacS deletion mutant PBL2025 (40) was therefore tested for complementation by plasmid pJlacS. After three rounds of selection in liquid medium (0.4% lactose), >95% of all cells contained a shuttle vector, as shown by plating on selective lactose versus non-selective tryptone plates, and X-gal staining of colonies. On both plates equal numbers of colonies were observed, on the non-selective plate in addition to ~300 colonies also seven white colonies were observed (Figure 6). Southern blot, retransformation, growth and copy number determinations for pJlacS in *S. solfataricus* are summarized in

![Figure 3. Growth of transformants. (A) Growth curves for MR31 transformed with pA to pN. (B) Growth curves for the recipient strain MR31 without addition of uracil (U), with the addition of uracil and transformed with pC and pE. (C) Retention of the shuttle vectors under non-selective conditions.](image-url)
Figure 6 and indicate that pJlacS is stably replicated in _S. solfataricus_.

**Transformation efficiencies**

For _S. acidocaldarius_ direct determination of transformation efficiencies is possible, because plating of the primary electroporation mixture can be done after only 30 min of regeneration. Considering all transformations performed in the current study (n = 150), the efficiencies range from $1 \times 10^2$ to $6 \times 10^4$ transformants per microgram plasmid DNA. The batch of electrocompetent cells, electroporation protocol and regeneration procedure have an influence on the transformation efficiency, as already described (36).

On tryptone/xylose plates, the formation of very small colonies—that did not contain a shuttle vector—was observed in addition to the colonies of normal size that were able to grow in selective liquid medium. Controls without electroporation or without addition of shuttle vector also yielded small colonies, which were not able to grow when re-streaked on selective plates or cultivated in liquid medium. Based on their phenotype and frequency in strain MR31, we hypothesize that these ‘pseudotransformants’ contain spontaneous mutations elsewhere in the _S. acidocaldarius_ chromosome that partially suppress the pyrE phenotype.

Finally, we confirmed that complete methylation of the shuttle vectors is essential for efficient transformation of _S. acidocaldarius_. None of the constructs pA–pN yielded transformants when unmethylated or partly protected plasmids were electroporated.

**DISCUSSION**

**Selection**

Based on our results with various constructs and recipient strains, we conclude that the primary obstacle to establishing stably replicating shuttle vectors derived from _Sulfolobus_ plasmid pRN1 is not preservation of critical plasmid functions or identification of a required host species, but creation of a suitably reliable selection. For example, point and transposon mutants of _S. islandicus_ or _S. solfataricus_ that showed low reversion frequencies in small scale fluctuation tests ($15 \times 10^{-9}$ – $<6 \times 10^{-5}$ reversions per cell division, unpublished data) displayed for unknown reasons higher reversion frequencies after electroporation with a shuttle construct. These problems could be avoided by the use of a pyrE deletion mutant of _S. acidocaldarius_. In contrast to _S. solfataricus_ and _S. islandicus_, background growth on selective plates was not observed with _S. acidocaldarius_. Although the basis of this difference has not been established, _S. acidocaldarius_ lacks homologs of the cytosine/uracil/thiamine/allantoin permeases (SSO1905, SSO2042) present in _S. solfataricus_ and _S. tokodaii_ (ST1564) that might facilitate growth on medium with very low uracil concentrations.

**Essential regions of pRN1**

Under selective conditions, the vectors could be faithfully propagated in _Sulfolobus_. Rearrangements occurred in only two cases, pB and pJlacS, and only after many generations. We do not know why pB behaves differently in this respect, although it is the only construct interrupted in between orf90b and orf56. This region of pRN1 contains several repeats and other remarkable features like a stretch of 17 consecutive C residues (41). An interruption in this region is obviously not as well tolerated as in other regions. The only interruption site that abolished shuttle vector replication completely was that of construct pM, and is situated within the co-transcribed replication operon orf56/orf904. For pM no viable transformants could be isolated. The other conserved open reading frame, orf80, also called _plrA_
that is present on almost all sequenced genetic elements of *Sulfolobus* (42) is interrupted in pH. Interestingly, pH shows growth comparable to the other constructs and yields the same transformation efficiencies. Therefore *orf80* seems not to be essential for replication of pRN1, at least not when selective pressure is applied. However, under non-selective conditions, construct pH was lost at a much faster rate than any other construct that could be successfully established in *S. acidocaldarius*. The relative instability of this construct provides the first experimental evidence that the DNA-binding protein ORF80 has an important role in stable maintenance A of pRN1. The instability of this construct may also have practical uses. For example, it may facilitate transfer of *pyrEF*-marked genes to the host chromosome, by allowing such genes to be first established on an episome, and then stabilized in the population by recombinational integration at the homologous locus.

### Stability in *E. coli*

Many shuttle constructs developed so far for hyperthermophilic archaea have been observed to rearrange in *E. coli* (18), which hampers the use of these systems. Some of these problems may, in principle, be circumvented by the use of *E. coli* strains designed specially for dealing with unstable constructs. Additionally reducing the growth temperature to 30°C and using only 50 μg ml⁻¹

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**Figure 5.** Replication of pJlacS in *S. acidocaldarius* (A) Southern blot (SacI) of plasmid pJlacS from *E. coli*, MR31 transformed with pJlacS and untransformed MR31 (−c). The pRN1 specific probe detects the 3.7-kb restriction fragment, the lacS probe detects the 7.3-kb fragment. (B) Retransformation of pJlacS (SacI), +c: pJlacS from *E. coli*, 1,2,3: retransformants. (C) X-gal test with untransformed MR31 and MR31 transformed with pJlacS (10 min at 75°C). (D) Growth curves for MR31 transformed with pJ (control) and with pJlacS. (E) Reporter gene experiment showing copy numbers of pJlacS per cell and the corresponding β-galactosidase activities.
of ampicillin is necessary to prevent rearrangements in the pMJ vector system (22). We observed rearrangements for the construct pJ\textit{lacS} in one out of 10 preparations of this plasmid in \textit{E. coli} XL1-Blue cells at 37°C and 100 μg ml\(^{-1}\) of ampicillin. In general, however, the constructs pA to pN seemed to be fully stable in \textit{E. coli}. In particular, we never detected rearrangements in retransformed plasmids. In Southern blots, faint traces of plasmid rearrangements were visible for some preparations from \textit{E. coli} but did not interfere with successful transformation of \textit{Sulfolobus}.

**CONCLUSIONS**

We have developed multicopy, non-integrative, plasmid-based \textit{Sulfolobus–E. coli} shuttle vectors that are very stable in both hosts, and are suitable for the use in protein expression and reporter gene studies. Transformation is rapid and simple, involving electroporation of stable \textit{pyrE} mutants and plating on uracil-deficient media. The constructs are small, enabling direct cloning into unique SacII/XmaI and NotI restriction sites. The host range so far comprises \textit{S. acidocaldarius} and \textit{S. solfataricus}, the two most widely used and best-studied species of \textit{Sulfolobus} for which genome sequence information is available. The presence of the shuttle constructs in the cells does not cause significant growth retardation and there is limited risk of accidentally contaminating cultures because the vectors are not infectious. It should be emphasized that performance of these shuttle constructs has now been confirmed independently in three different laboratories using slightly different electroporation and cultivation protocols.

In addition, the use of \textit{S. acidocaldarius} as recipient strain has certain practical advantages which somewhat mitigate the inconvenience of requiring specific DNA methylation. \textit{Sulfolobus acidocaldarius} does not contain any integrated copies of pRN1 or genes homologous to pRN1 genes. This enables detailed experiments on essential regions and proteins for pRN1 replication and maintenance without interference from plasmid-gene homologs located on the host chromosome. Because of the low sequence similarity between \textit{S. acidocaldarius} and \textit{S. solfataricus} there is also minimal risk of undesired homologous recombination when cloning genes of \textit{S. solfataricus} into the shuttle vector, e.g. for protein
expression. *Sulfolobus acidocaldarius* is the only *Sulfolobus* species so far that does not contain active insertion sequences and seems to be genetically stable (12). In addition, it is the *Sulfolobus* species showing the highest growth rate with doubling times of around 3–4 h during exponential growth, and exhibits efficient homologous recombination (36). In this context, the series of pRN1 shuttle vectors we have constructed promises to add detailed genetic analyses to the already advanced biochemical characterization of various *Sulfolobus* gene products.

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