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METHOD PAPER

Unmarked gene deletion and host–vector system for the hyperthermophilic crenarchaeon *Sulfolobus islandicus*

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Abstract *Sulfolobus islandicus* is being used as a model for studying archaeal biology, geo-biology and evolution. However, no genetic system is available for this organism. To produce an *S. islandicus* mutant suitable for genetic analyses, we screened for colonies with a spontaneous *pyrEF* mutation. One mutant was obtained containing only 233 bp of the original *pyrE* sequence in the mutant allele and it was used as a host to delete the *β*-glycosidase (*lacS*) gene. Two unmarked gene deletion methods were employed, namely plasmid integration and segregation, and marker replacement and looping out, and unmarked *lacS* mutants were obtained by each method. A new alternative recombination mechanism, i.e., marker circularization and integration, was shown to operate in the latter method, which did not yield the designed deletion mutation. Subsequently, *Sulfolobus–E. coli* plasmid shuttle vectors were constructed, which genetically complemented Δ*pyrEF*Δ*lacS* mutation after transformation. Thus, a complete set of genetic tools was established for *S. islandicus* with *pyrEF* and *lacS* as genetic markers.

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Introduction

Since the concept of three domains of life became widely accepted (Woese et al. 1990), archaeal research has progressed rapidly; first through sequencing and bioinformatic analyses of archaeal genomes and, subsequently, via biochemical characterization of prominent proteins including those involved in informational processes (Cavicchioli 2007; Garrett and Klenk 2007). Several important concepts have developed from these studies, such as that archaea carry simpler forms of the eukaryotic informational machineries (Bell et al. 1998; Grabowski and Kelman 2003; Duggin and Bell 2006). Moreover, major efforts have been made in developing archaeal genetic systems in order to investigate how these eukaryotic protein homologs function in concert to execute molecular processes in vivo. As a result, several methodologies have been established and used for constructing gene knockout mutants in the euryarchaea, including haloarchaea (Peck et al. 2000; Bitan-Banin et al. 2003; Allers et al. 2004; Wang et al. 2004), methanogens (Pritchett et al. 2004) and *Thermococcus kodakaraesis* (Sato et al. 2003, 2005).

In crenarchaea, a gene knockout method and a few shuttle vectors were developed for *Sulfolobus solfataricus* using the *lacS* gene encoding *β*-glycosidase as selectable marker, in which transformants were obtained on a lactose minimal medium (Worthington et al. 2003; Aucelli et al. 2006; Berkner et al. 2007). Using this method, several gene knockout mutants have been constructed (Schelert et al. 2006; Szabo et al. 2006, 2007a; Zolghadr et al. 2007).

However, there is a pitfall with this system: genetic manipulation using the *lacS* marker is a very tedious process because it relies on utilizing a lactose minimal medium supplemented with an inorganic nitrogen compound as the sole nitrogen source. In this medium, all known *Sulfolobus* species grow very slowly. In contrast, complementing a uracil auxotroph by expression of *pyrEF* is selectable with a uracil-free rich medium (uracil dropout selection). Moreover, the absence of *pyrEF* can be selected for with 5-fluoroorotic acid (*pyrEF* counter-selection), which further facilitates the construction of unmarked gene knockouts. By using this selectable marker, virus shuttle vectors were reported for *S. solfataricus* (Jonuscheit et al. 2003) and plasmid shuttle vectors were developed for *S. acidocaldarius* (Berkner et al. 2007). More recently, a gene knockout scheme has also been described for *S. acidocaldarius* (Wagner et al. 2009).

While very few, or no, genetic elements have been reported for *Sulfolobus solfataricus* or *Sulfolobus acidocaldarius*, many genetic elements have been isolated from *Sulfolobus islandicus* including SIRV rudiviruses and SSV fuselloviruses and the archaeal helper and satellite viruses SSV2 and pSSVx (reviewed by Prangishvili et al. 2001). Most cryptic and conjugative plasmids are also carried by an *S. islandicus* strain, including pRN1, pRN2, pHEN7, pINGs, pKEF9, pHVE14, pARNs and pSOGs (reviewed by Lipps 2006). These genetic elements provide simple systems for studying archaeal molecular biology in *S. islandicus*. Furthermore, *S. islandicus* strains occur in acidic hot springs worldwide, including those located in Iceland, USA and Russia (Zillig et al. 1994; Whitaker et al. 2003, 2005). Thus, this organism is also a model for geo-biology and evolution studies. Recently genomes of nine *S. islandicus* strains have been completely sequenced: seven were done at USA-DOE Joint Genome Institute (www.jgi.doe.gov/genome-projects/), while two were completed by the joint efforts of the Danish and Chinese laboratories (unpublished results). We worked on developing genetic tools for genetics and reverse genetics of *S. islandicus*. Here, we report the constructed mutant hosts and genetic tools, which have been developed.

Materials and methods

Archaeal and bacterial strains, media and growth conditions

Sulfolobus islandicus strain REY15A was isolated and characterized previously (Contursi et al. 2006). *S. islandicus* E233 (Δ*pyrEF*), a spontaneous uracil-auxotrophic mutant, was isolated from the REY15A strain, and *S. islandicus* E233S1 and E233S2 (Δ*pyrEF* Δ*lacS*) were constructed by deleting the complete *lacS* gene from the E233 strain, using unmarked gene knockout methods (Table 1).

Sulfolobus media contained the same composition of mineral salts as described previously (Zillig et al. 1994). Supplementing of 0.1% tryptone, 0.05% yeast extract and 0.2% sucrose to the medium salts yielded the rich TYS medium, while adding 0.2% sucrose, 0.2% (w/v) vitamin-free casamino acids (Difco Vitamin Assay, BD) and a mixed vitamin solution (Zillig et al. 1994) yielded the selective medium SCV. The final pH value of each *Sulfolobus* medium was adjusted to about 3.5, using concentrated sulfuric acid.

Sulfolobus strains or colonies were inoculated into test tubes containing 6 ml TYS or SCV and grown up in an Innova 3100 oil-bath shaker (New Brunwich Scientific Corp) at 78°C, shaking at 150 rpm. Large-scale culturing was performed with Erlenmeyer flasks with long necks. *Sulfolobus* colonies were obtained on 0.7% Gelrite plates either by directly plating or by two-layer plating. In the latter the diluted liquid cultures were mixed with the same medium containing 0.2% Gelrite and plated as the top

Table 1 Strains and plasmids used in this study

Strain or plasmid	Genotype/features	Reference/sources
S. islandicus Rey15A	Wild type	Contursi et al. 2006
S. islandicus E233	Δ*pyrEF*, a spontaneous deletion mutant isolated from Rey15A	This work
S. islandicus E233S1	Δ*pyrEF* Δ*lacS*, *lacS* was deleted from E233 via unmarked gene deletion (via pKL1)	This work
S. islandicus E233S2	Δ*pyrEF* Δ*lacS*, *lacS* was deleted from E233 via unmarked gene deletion (via pKL2)	This work
S. solfataricus P2	Wild type, DSM1617	DSMZ
pHZ1	pGEM3Z carrying the *pyrEF* gene amplified from *S. solfataricus*, *Sulfolobus* basal vector	This work
pKL1	pHZ1 carrying L-arm and R-arm of the *lacS* gene of *S. islandicus*, gene knockout plasmid	This work
pKL2	L-arm was inserted into pKL1, yielding a gene knockout plasmid with two L-arms	This work
pHZ2	pRN2 was inserted into pHZ1, *Sulfolobus–E. coli* shuttle vector	This work
pHZ2lacS	*lacS* was amplified from *S. solfataricus* and inserted into pHZ2, shuttle vector	This work
pRN2	Cryptic vector isolated from *S. islandicus*, pRN family	Keeling et al. 1998

layer. Strains or transformants to be selected via uracil dropout selection were cultured in SCV, whereas the *pyrEF* mutants were cultured either in TYS or SCV supplemented with uracil (20 μg/ml). When 5′-fluoroorotic acid was included to give *pyrEF* counter-selection, it was added to 50 μg/ml.

Escherichia coli DH5α, pGEM3z and pGEM-T were used as host and cloning vectors, respectively, for conventional DNA cloning. *E. coli* transformants were cultured in Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin and cultured at 37°C.

General DNA manipulations

Standard methods of molecular cloning (Sambrook and Russell 2001) were used for general DNA manipulation. Restriction and modification enzymes were purchased from New England Biolabs, Fermentas or Takara Bio companies. Plasmid DNA was isolated from *E. coli* or *Sulfolobus* cells using QIAprep Spin Miniprep kit (QIAGen Westberg, Germany). Total DNA was prepared either by using QIAGen DNeasy kit or by extracting with phenol/chloroform after lysing *Sulfolobus* cells with SDS and proteinase K (Contursi et al. 2006).

DNA sequencing was performed using the dye-terminator chemistry and analyzed with MegaBACE 1000 sequencer (Molecular Dynamics/Amersham). DNA oligonucleotides used for polymerase chain reactions (PCR) were synthesized from TAG Copenhagen (Table 2).

Plasmid constructions

All plasmid constructions were based on an *E. coli* vector carrying a *pyrEF* marker called basal vector pHZ1 (Fig. 1). The marker cassette was amplified from *S. solfataricus* P2 (She et al. 2001) via PCR using MpyrEFfwd-*Kpn*I and MpyrEFrev-*Kpn*I primers (Table 2), containing the *pyrEF* genes and their putative promoter and terminator. The *Kpn*I restriction sites were incorporated into the primers to facilitate cloning. The high-fidelity enzyme Pfu DNA polymerase was used for PCR and the resultant PCR fragment was purified with the QIAGen PCR purification kit, treated with *Kpn*I and purified again. Then, the restricted *pyrEF* marker cassette was inserted into the *E. coli* cloning vector pGEM3z (Promega) at the *Kpn*I site to yield pHZ1 (Fig. 1).

The sequence of the *lacS* gene was identified from the draft genome sequence of *S. islandicus* HVE10/4 (provided by Prof. Roger Garrett) by searching the genome contig sequences with the *lacS* sequence of *S. solfataricus* P2, using the Sequencher program (Gene Codes). The flanking sequences of *lacS* (ca. 1.5 kb from each side) were retrieved from the corresponding contig and used to design the L-arm and R-arm, two homologous sequence

Table 2 Oligonucleotides used in this study

Primer	Sequence[a]
CheckEF-fwd	5′ ATAATGGTACCCCATCAAAC 3′
CheckEF-rev	5′ ATTGTGGTACCTACTGGCG 3′
MpyrEFfwd-*Kpn*I	5′ ATAAT*GGTACC*CCATCAAAC 3′
MpyrEFrev-*Kpn*I	5′ ATTGT*GGTACC*TACTGGCGT 3′
L-armfwd-*Sal*I	5′ GC*GTCGAC*GCCTTAATCACATCAGCAAC 3′
L-armrev-*Pst*I	5′ TGC*CTGCAG*CAGTATTATTTAAGCTTTGAGCG 3′
R-armfwd-*Pst*I	5′ TGC*CTGCAG*ACATTTTCA AGTCTCAGCACAC 3′
R-armrev-*Sph*I	5′ GC*GCATGC*GAGAAGGTGGGGATTACGGT 3′
L-armfwd2-*Aat*II	5′ GC*GACGTC*GCCTTAATCACATCAGCAAC 3′
L-armrev2-*Nde*I	5′ TGC*CATATG*CAGTATTATTTAAGCTTTGAGCG 3′
MlacSfwd-*Ava*I	5′ CG*CCCGGG*ACACCACTGAAGATACTCGCTC 3′
MlacSrev-*Sal*I	5′ GC*GTCGAC*TTAGTGCCTTAATGGCTTTACT 3′
CheckLacS-fwd	5′ GGTGTATACTCATAGCCCGAATCGT 3′
CheckLacS-rev	5′ GGCTAGGGAATACACTGCGAGATAT 3′
L-armProbe-fwd	5′ ATACCCTTTAACCTAACATTCC 3′
L-armProbe-rev	5′ TCATTACTCCGCAACCTACT 3′
qPCRAmp-fwd	5′ TCGTTGTCAGAAGTAAGTTGG 3′
qPCRAmp-rev	5′ GTCGCCGCATACACTATTC 3′
qPCRCdc-fwd	5′ ACGATTAACAACCTTGGCTCTTAG 3′
qPCRCdc-rev	5′ TCTTACCGCAGTGGCTTCTATG 3′

[a] Restriction sites are in italics

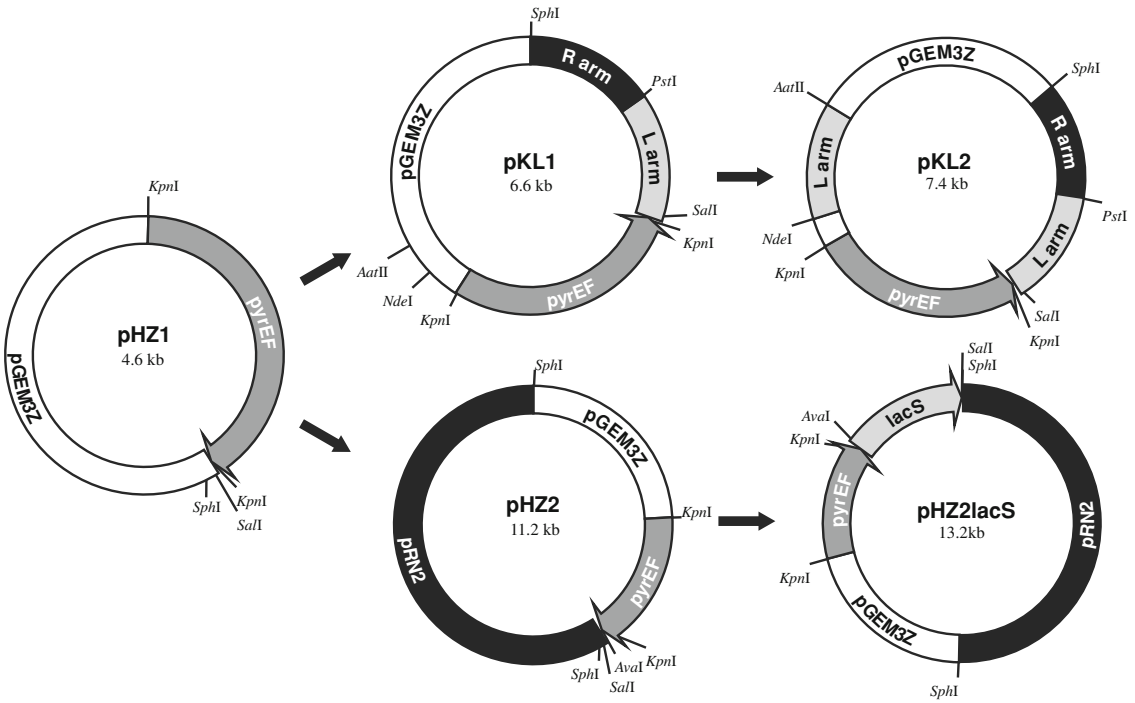

Fig. 1 Plasmid constructions. The basal vector pHZ1 was constructed by ligating the *Kpn*I-treated *pyrEF* marker cassette with the *E. coli* vector pGEM3z at the *Kpn*I site. Two homologous sequences, L-arm and R-arm, were inserted into pHZ1 at the *Sal*I and *Sph*I sites, yielding the knockout plasmid pKL1. Another L-arm (insertion arm) was cloned into pKL1 at the *Aat*II and *Nde*I sites, yielding the knockout vector pKL2. The shuttle vector pHZ2 was constructed by ligating pRN2 and pHZ1 after digesting each plasmid with *Sph*I. pHZ2lacS was constructed by inserting *lacS* marker into pHZ2 at *Aat*II and *Sal*I sites

arms to be used for gene knockout via homologous recombination. PCR primers for amplifying the L-arm and R-arm were designed with the Primer Primere program (Singh et al. 1998) and a *Sal*I, *Pst*I or *Sph*I site was included at the 5′-end in each primer (Table 2). PCR reaction and purification were carried out as described above, and the purified L-arm was treated with *Sal*I and *Pst*I, and the R-arm with *Pst*I and *Sph*I. Finally, both fragments were ligated onto the basal vector pHZ1 at the *Sal*I and *Sph*I sites in one-step ligation, yielding the knockout plasmid pKL1 (Fig. 1).

The knockout plasmid pKL2 was derived from pKL1 by cloning another copy of the L-arm onto it. The second L-arm was obtained as a PCR product with the primers containing *Aat*II and *Nde*I sites, respectively (Table 2). The resulting PCR product was cloned onto a pGEM-T vector and cleaved off again at the designed restriction sites before inserting into pKL1 (Fig. 1).

The *Sulfolobus* cryptic plasmid pRN2 (Keeling et al. 1998) and pHZ1 were used to construct *Sulfolobus–E. coli* plasmid shuttle vectors. The pRN2 plasmid was isolated from its original host *S. islandicus* REN1H1 (Zillig et al. 1998) and digested with *Sph*I. The resulting linear fragments were purified and ligated onto the *Sph*I-treated pHZ1, yielding the shuttle vector pHZ2 (Fig. 1). The *lacS* gene was amplified from *S. solfataricus* by PCR using MlacSfwd-*Ava*I and MlacSrev-*Sal*I primers (Table 2), and the resultant PCR product was restricted with *Ava*I and *Sal*I and inserted at the same sites on pHZ2, yielding pHZ2lacS (Fig. 1).

Isolation and characterization of spontaneous *pyrEF* deletion mutants

S. islandicus REY15A strain (Contursi et al. 2006) was grown up in TYS medium to an optical density of 0.5 (OD_{600}) and aliquots of 100 µl culture were spread onto TYS Gelrite plates containing uracil and 5-FOA. After incubation at 78°C for 5 days, 150 colonies were picked up, transferred into the test tubes containing 6 ml of the same medium and cultured at 78°C for 3–4 days. Then, total DNA was prepared from a 2 ml culture of each sample and their mutant *pyrEF* alleles were amplified as PCR products, using the CheckEF primer set (Table 2). A typical PCR reaction contained ca. 100 ng DNA template, 2.5 units Taq DNA polymerase, 1 µM of each primer and 2.5 mM of each dNTP. Amplification was carried out with ABI GeneAmp 9700 under the following conditions: 30 cycles of 30 s, 94°C; 45 s, 55°C and 2 min, 72°C, plus an initial denaturation (94°C) and a final elongation (72°C), each for 5 min. The sizes of the PCR products were estimated by agarose gel electrophoresis.

Transformation procedures

Electroporation transformation was used to transform *S. islandicus* strains according to the procedure described previously (Schleper et al. 1992) with the following modifications: (1) all manipulations were carried out at room temperature; (2) after electroporation, *Sulfolobus* cells were immediately transferred into 950 µl pre-warmed *Sulfolobus* medium (pH 5–6) and incubated at 75°C for 30 min without shaking. To plate for colonies of transformants, aliquots of 100 µl cell suspension were mixed with 10 ml of the corresponding medium supplemented with 0.2% Gelrite and plated onto pre-warmed SCV plates. After the top layer was set, these plates were put into a plastic box, tightly closed and incubated at 78°C. The two-layer cultivation method was adopted to plate *Sulfolobus* transformants since non-transformants also formed small colonies on selective plate upon direct plating. This is presumably because pyrimidine compounds released from the lysis of dead cells support the growth of non-transformants. Plating electroporated cells in the top Gelrite protects cells from lysis such that only true transformants form colonies on selective plates.

Typically, transformants appeared as single colonies after 5–7 days incubation when transforming with a shuttle plasmid, whereas *Sulfolobus* recombinants resulting from homologous recombination usually took 1–2 more days to form colonies of a similar size. Furthermore, since a much lower transformation rate was expected from a gene knockout experiment, ca. tenfold more plasmid DNA (up to 1 µg) was used for transformation.

X-gal assay of β-glycosidase

The β-glycosidase activity encoded by *lacS* (hereafter referred as LacS activity) in *Sulfolobus* colonies was detected with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining; 2 ml X-gal (2 mg/ml) was added onto a plate with colonies, rotated gently to distribute the chemical on the surface and incubated at 78°C. Blue color should be developed for $LacS^+$ within 2 h. To reveal LacS activity in a liquid culture, X-gal was added to the culture to 2 mg/ml (final concentration) and incubated at 78°C for 2 h before observing the color development.

β-galactosidase activity of LacS was quantified in cellular extracts by the ONPG (ρ-nitrophenyl-ß-D-galactopyranoside) method as described previously (Jonuscheit et al. 2003). Cell samples were taken from the cultures of *S. islandicus* E233, E233S and pHZ2lacS-transformed E233S at OD_{600} (optical densities at 600 nm) of 0.4. After centrifuging, cell pellets were resuspended in 10 mM Tris–HCl buffer (pH 8.0) and sonicated with Sonoplus (Bandelin) to disrupt cell walls. The cell debris was then removed by centrifuging at the maximum speed (13200 rpm) for 30 min, yielding the cellular extract. Then 10 µl cell extract was used for the assay with three biological repeats for each sample. The product (ρ-nitrophenol) was quantified by measuring optical density of the samples at 420 nm with a Nanodrop spectrophotometer (Thermo Scientific). Protein concentrations of the cellular extracts were determined with a micro BCA protein assay kit (Thermo Scientific), and one specific unit of β-galactosidase activity was defined as 1 µM ρ-nitrophenol produced by 1 mg protein per minute.

Characterization of transformants and gene deletion mutants

Colonies of transformants were picked up from plates by pipetting with a tip carrying a broad hole and released into SCV liquid medium (6 ml) in a test tube and incubated at 78°C for 3–7 days. When grown, samples were taken from the cultures and streaked onto plates to yield single colonies. These transformants were also screened for the mutant *lacS* alleles by PCR with CheckLacS primers. Once a desired mutant/strain was identified, it was purified by streaking for single colonies three successive times. Subsequently, chromosomal DNAs were isolated from the purified strains and used for PCR identification and for Southern analysis. If relatively large PCR products (>5 kb) were to be amplified, the LA Taq DNA polymerase (Takara Bio) was employed under the following conditions: 94°C 3 min; 30 cycles of 94°C 15 s, 53°C 30 s and 72°C 10 min (with a 5 s increasement in each cycle for the last 20 cycles), and a final extension at 72°C for 10 min.

Copy numbers of the shuttle vectors were determined using quantitative PCR in triplicate as described previously (Lee et al. 2006; Providenti et al. 2006). Two sets of primers (Table 2) were designed to determine absolute numbers of shuttle vector (β-lactamase gene) and chromosome (*orc/cdc6-1* gene) molecules in the total DNA preparations: qPCR of shuttle vector was carried out with qPCRampr primers and, that of the host chromosome, with qPCRcdc primer set. DyNAmo HS SYBR Green qPCR kit (New England Biolabs) was employed and PCR reaction was run on an iCycler (BioRad), with the signal values normalized with fluorescein calibration dye (Biorad). To maximize the PCR efficiency, template DNAs were linearized with *Hin*dIII and the purified DNA templates were quantified with Qubit Quantitation Platform kit (Invitrogen). The plasmid copy number was expressed as the ratio between the numbers of shuttle vector and chromosome for each sample.

Southern hybridization

Southern blot and hybridization were conducted according to a standard procedure (Sambrook and Russell 2001). Genomic DNA was prepared with the QIAGen tissue DNA kit from each recombinant or mutant to be analyzed and *ca.* 2–5 μg of total DNA of each sample was digested with *Ban*I, *Nde*I, *Pst*I or *Ava*I. The resulting DNA fragments were fractionated by agarose gel electrophoresis on a 0.8% agarose gel and transferred onto a Hybond N membrane (Amersham) via capillary transfer. The DNAs on the membrane were then cross-linked by UV irradiation for 4 min with the UVP 3UV transilluminator. Hybridization probes were labeled with Digoxigenin Labeling kit (Roche) and hybridization was performed at 60°C overnight. The hybridization signals were detected using the DIG detection kit with the CDP-Star (Roche) and the results recorded by exposing the membrane to an X-ray film.

Results

Isolation of *pyrEF* deletion mutants from *S. islandicus*

For most studied microorganisms, *pyrEF* mutants are readily isolated as 5-fluoroorotic acid (5-FOA)-resistant colonies since the enzymes encoded by *pyrE* and *pyrF* degrade this non-toxic analog into the toxic 5-fluoro-dUMP that kills cells having both activities. Using the same approach, we studied spontaneous mutations in *S. islandicus* REY15A and isolated uracil auxotrophs on 5-FOA plates. The apparent spontaneous mutation rate was estimated at *ca.* 10^{-6} for this organism. Analyzing *pyrEF* alleles in 150 FOA-resistant colonies (putative *pyrEF* mutants, *pyr*), by PCR with CheckpyrEF primers, revealed three mutants (*pyr*003, 118 and 128) harboring a mutated *pyrEF* allele that was apparently smaller than the corresponding wild type (1.9, 1.1 and 0.4 kb vs. 2.2 kb). Deletion points in these mutant *pyrEF* genes were determined by sequencing their PCR products, and they occurred at different positions (Fig. 2). A 292-bp sequence was deleted from *pyrE* (E197Δ*pyrE*) in *pyr*118, and an 1107-bp sequence was absent from the *pyrEF* locus in *pyr*003, including the partial *pyrE* and entire *pyrF* (denoted E233Δ*pyrEF*). The deletion in *pyr*128 occurred outside of *pyrEF* locus and included 152 bp *pyrB* and half of the downstream ORF (B153Δ*pyrBEF*).

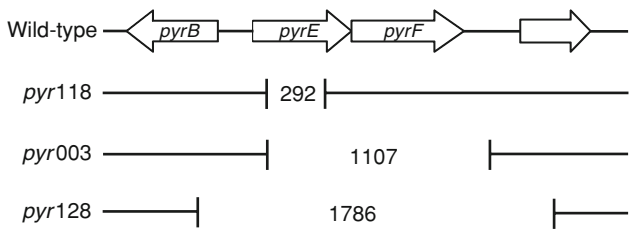

Fig. 2 Spontaneous *pyrEF* deletion mutants. Three large deletion mutants, *pyr*003, 118 and 128, were identified and their deletion points were determined by DNA sequencing. The deleted regions are shown as broken lines under the gene symbols and the sizes of the deletions are indicated

Only the former two mutants were suitable hosts for studying genetic complementation of uracil auxotrophs, by expressing the *pyrEF* gene, since complementing the auxotroph of *pyr*128 would require expressing *pyrB* in addition to *pyrEF* (Fig. 2). Furthermore, E233Δ*pyrEF* retained only the upstream part in the mutant allele, which minimizes the likelihood of homologous recombination occurring between the marker gene and the E233 mutant allele. Therefore, we chose E233 as the host for developing genetic tools for *S. islandicus*.

Unmarked gene deletion via knockout plasmid integration and partial diploid segregation

Two unmarked gene deletion methodologies, namely plasmid integration and segregation (PIS), and marker replacement and looping out (MRL), have been employed in archaeal genetic studies and they were tested in *S. islandicus* for constructing *lacS* deletion mutants with the E233 strain. The constructed knockout plasmids (pKL1 and pKL2), to be used for each methodology, are closely related: pKL1 contains two homologous arms placed adjacent to each other, whereas pKL2 carries another copy of a homologous arm inserted into the other side of the marker gene (Fig. 1).

When pKL1 was used to transform *S. islandicus* E233, 0–5 colonies of transformants/μg DNA were obtained. The *lacS* alleles were analyzed by PCR with CheckLacS primers in the cultures of six pKL1 transformants (Table 2), and three PCR products were obtained for each sample (Fig. 3b). The upper band was equivalent to the PCR product amplified from the cells containing the *lacS* partial diploid allele; the middle band was derived from the wild-type *lacS* locus, while the lowest was generated from the Δ*lacS* allele. The coexistence of these genotypes in the transformant cultures suggested that the integrated pKL1 was unstable; segregation of the partial diploid yielded either a gene deletion mutant or restored the wild-type host (Fig. 3a). Since the marker was removed from the chromosomes, both segregants should survive counter-selection and form colonies on 5-FOA plates, whereas all partial diploid cells would be killed under the same growth condition. Thus, X-gal staining could be used to distinguish the colonies of two segregants on 5-FOA plates where *lacS* deletion mutants would appear as white colonies, while the restored wild-type strain should remain blue.

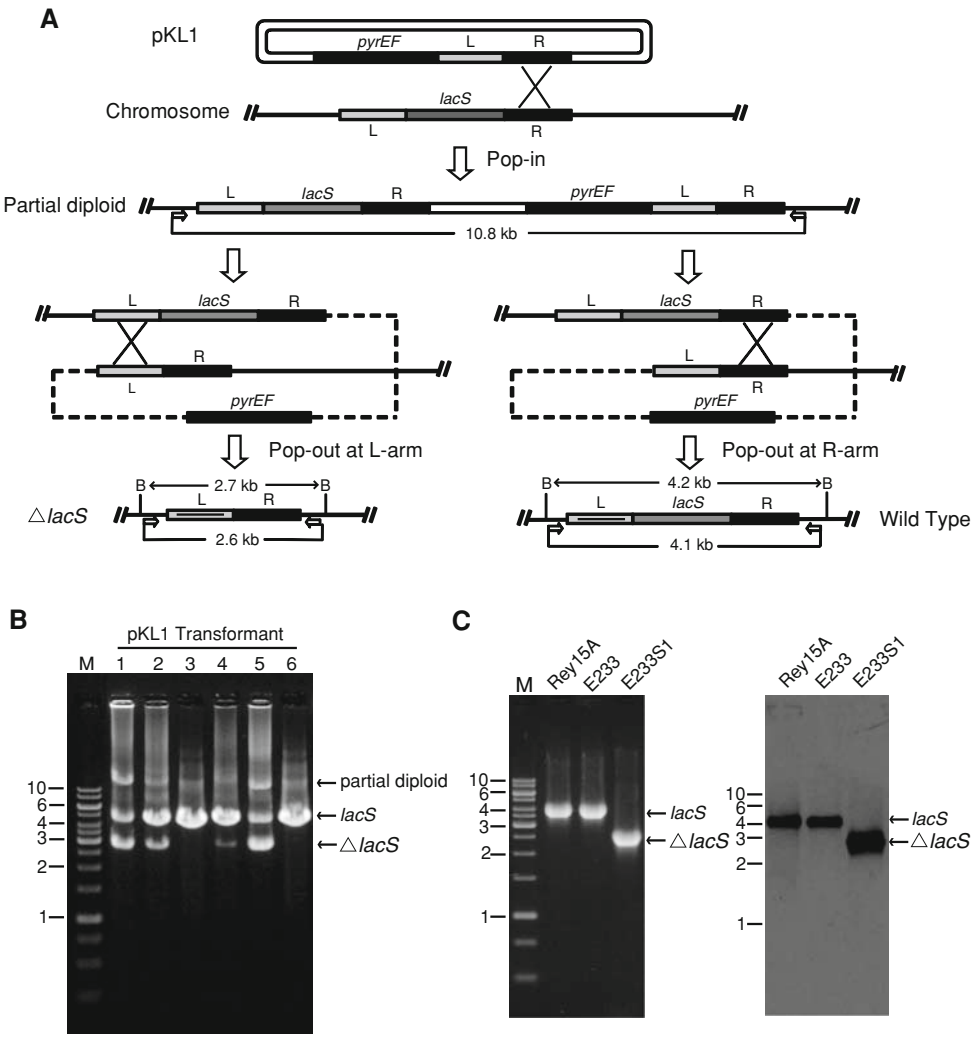

Fig. 3 Construction of *lacS* deletion mutant with gene knockout plasmid pKL1. **a** Illustrative scheme of mutant construction. 1. Integration at the R-arm. 2. Partial *lacS* diploid segregates at either the L-arm or R-arm, giving the knockout mutant or the original host, respectively. **b** Analysis of the *lacS* alleles of pKL1 transformants via PCR. Partial diploid (10.8 kb); *lacS* (4.1 kb); Δ*lacS* (2.6 kb). Six transformants (1 to 6) were analyzed, and the *lacS* alleles were amplified by PCR with CheckLacS-fwd and -rev primer set (*unfilled arrows*). **c** Identification of the unmarked *lacS* deletion mutant by PCR and Southern analysis. Rey15A—*Sulfolobus islandicus* Rey15A; E233—*S. islandicus* Δ*pyrEF* mutant. E233S1—*S. islandicus* Δ*pyrEF*Δ*lacS*. Total DNAs were prepared from the above strains and restricted with BanI (B). The sizes of the genomic fragments containing different *lacS* alleles were indicated. The probe used for hybridization was amplified from the L-arm (indicated as *solid bars* inside the L-arm symbols). *Ban*I fragments recognized by the probe are indicated with broken lines and their sizes shown in the middle

As expected, plating cultures of purified pKL1 transformants yielded both white and blue colonies on counter-selective plates containing X-gal. Several white colonies were then purified and characterized with PCR using CheckLacS primers and by Southern hybridization. As exemplified in Fig. 3c, only Δ*lacS* allele was detectable in the constructed mutant E233S1. The *lacS* deletion in this mutant was further studied by sequencing a PCR product of the Δ*lacS* allele. The *Pst*I site that was introduced into the junction of the L-arm and R-arm during cloning was found to be maintained at the Δ*lacS* allele in the E233S1 mutant (data not shown).

Unmarked gene deletion by replacement of the target gene with a marker cassette and subsequent marker looping out (MRL)

The MRL is another unmarked gene deletion procedure that was developed recently for *T. kodakaraensis* (Sato et al. 2003, 2005). To generate a *lacS* deletion mutant of *S. islandicus* using this method, the knockout plasmid pKL2 was linearized (Fig. 1) and used to transform *S. islandicus* E233. Higher transformation efficiency was obtained with linear pKL2 than with circular pKL1 (10–200 vs. 0–5 colonies/μg DNA). Interestingly, staining the transformants with X-gal revealed two types of transformants, $LacS^-$ colonies (white, denoted T1pKL2, 60–70%) and $LacS^+$ colonies (blue, 30–40%), although only $LacS^-$ colonies were predicted from the recombination scheme of *T. kodakaraensis* (Sato et al. 2005). Further analysis of $LacS^+$ colonies revealed that they fell into two categories: *ca.* 50% of them were the pseudo $LacS^+$ strains as they became $LacS^-$ after propagation in selective medium (T2pKL2), and the remaining half maintained the genuine $LacS^+$ phenotype (T3pKL2).

In order to obtain the unmarked *lacS* deletion mutant, T1pKL2 was grown in SCV and streaked on the SCV plates containing 5-FOA and uracil. Since only the unmarked deletion mutant could survive *pyrEF* counter-selection, colonies formed on these plates were directly

picked up, purified as described in "Materials and methods" and designated as E233S2.

Next, representatives from each group were studied by PCR with CheckLacS primers and by Southern hybridization with an L-arm probe. It was found that T1pKL2 and T2pKL2 carried identical *lacS* alleles since their cells contained either Δ*lacS*::*pyrEF* or Δ*lacS* (Fig. 4c). The genuine LacS$^+$ colonies (T3pKL2) must harbor additional DNA sequence in its *lacS* allele because it was apparently larger in size than Δ*lacS*::*pyrEF* (Fig. 4). This new *lacS* allele should have resulted from a recombination scheme alternative of MRL. Importantly, Δ*lacS* allele did not exist in cultures propagated from any genuine LacS$^+$ transformant, suggesting that the recombination any T3pKL2 strain could not lead to *lacS* deletion.

Southern analysis of T1pKL2 and T3pKL2 revealed that both carried a small *Ban*I fragment, which matched in size to that of the circular marker cassette (Cir-M) (Fig. 4a, d). To study whether Cir-M was present in T3pKL2, total DNA was prepared and restricted with *Nde*I, *Pst*I and *Ava*I, such that each cut the circular molecule once, and the resulting samples were analyzed by Southern hybridization. A weak fragment matching in size to the linearized Cir-M (*ca*. 3 kb) was present in all three restrictions (Fig. 5b), indicating that Cir-M persisted in T3pKL2. Further, several fragments of different sizes were heavily hybridized to the probe in the same experiment, and their sizes matched the predicted integrated Cir-M in the host chromosome (Fig. 5b). Thus, we reasoned that immediately after transformation, homologous recombination between the two copies of L-arm in pKL2 yielded Cir-M, which then integrated into the host chromosome via homologous recombination at the L-arm. Thereafter, integration, and dynamic interaction, between Cir-M and the host chromosome enabled these transformants to grow under the selection (Fig. 5a). Consequently, the resulting

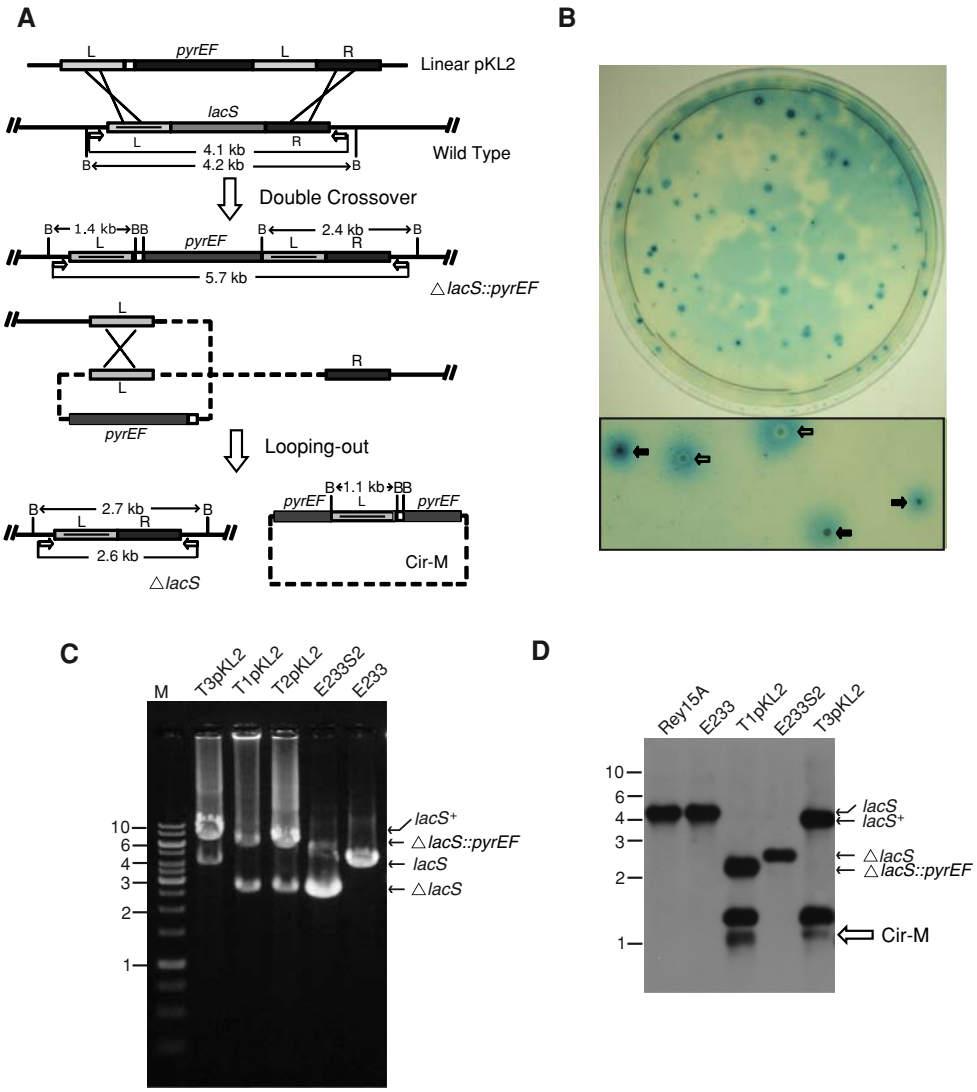

Fig. 4 Deleting *lacS* with linearized pKL2. **a** Flowchart for pKL2-mediated unmarked gene deletion. 1. *pyrEF* replaces *lacS* on the chromosome via a "double crossover" recombination event. 2. The marker is removed via looping out at the L-arm. **b** Colonies of *S. islandicus* transformants with the linear pKL2. *Unfilled arrows* indicate the yellowish colonies (expected recombinants); *solid arrows* indicate the blue colonies resulting from a new recombination mechanism of pKL2 (T3pKL2). **c** Characterization of pKL2 transformants via PCR. M—1 kb Fermentas Generuler marker. PCR products of the *lacS* alleles: Δ*lacS* (2.6 kb); *lacS* (4.1 kb); Δ*lacS*::*pyrEF* (5.7 kb); *lacS*$^+$ (7.3 kb, see Fig. 5a). **d** Southern analysis of the *lacS* alleles

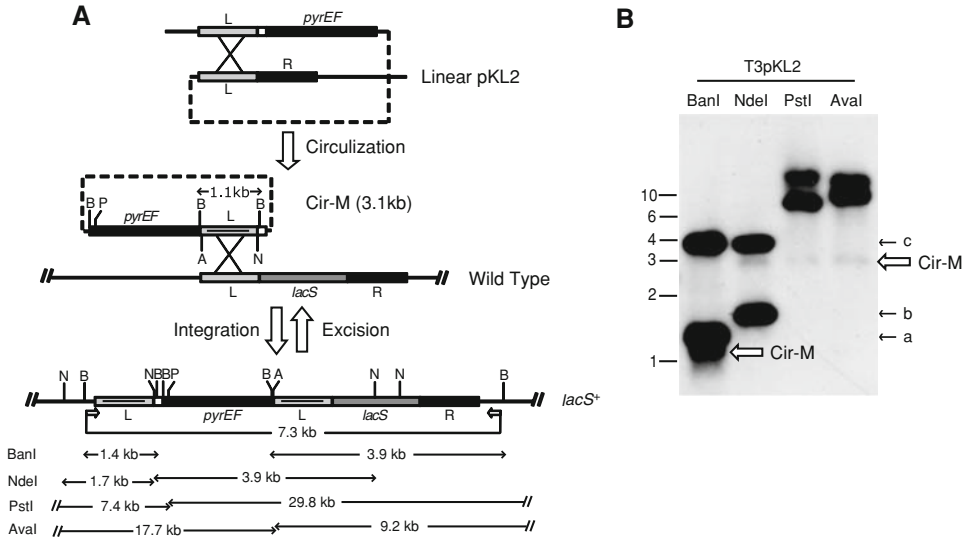

Fig. 5 Characterization of the pKL2-mediated marker circularization and integration. **a** Flowchart of the MCI recombination mechanism. 1. Formation of the circular cassette of L-arm and marker (Cir-M). 2. Dynamic interaction of Cir-M with host chromosomes. **b** Southern analysis of T3pKL2. The total DNA was digested with *Ban*I (B), *Nde*I (N), *Pst*I (P) or *Ava*I (A) and used for Southern analysis using the same L-arm probe. The hybridized fragments derived from Cir-M are indicated with *unfilled arrows*. The fragments derived from the integrated form are indicated (in kb): a—1.4; b—1.7; c—3.9. The integrated fragments of *Pst*I-cut DNA are 29.8 kb and 7.4 kb, *Ava*I-cut DNA are 17.7 kb and 9.2 kb

colonies maintained the LacS$^+$ phenotype because the intact *lacS* gene was maintained in their chromosomes.

Host–vector systems based on *S. islandicus* E233 and E233S mutants

E233 and E233S were also used as hosts for testing the *Sulfolobus* plasmid shuttle vectors. The shuttle vector pHZ2 was constructed with the cryptic plasmid pRN2 of *S. islandicus* REN1H1 (Keeling et al. 1998) as a *Sulfolobus* replicon and *pyrEF* as the marker. pHZ2lacS was produced by cloning *lacS* onto pHZ2 (Fig. 1). Transforming *S. islandicus* with these plasmid shuttle vectors yielded a high efficiency of transformation (10^4–10^6 colonies/μg DNA).

To test the stability of these shuttle plasmids in *Sulfolobus*, plasmid DNA was prepared directly from the cells of the *S. islandicus* transformants and analyzed. All checked transformants harbored pHZ2 (Fig. 6a). Furthermore, after the transformants were continually cultured in selective medium for *ca*. 35 generations, the plasmid identity was studied for 20 individual colonies and they all retained the same restriction patterns (Fig. 7a, b). Thus, pHZ2 and pHZ2lacS shuttle vectors are stably maintained under selection. Determining the copy number of the shuttle vectors by qPCR revealed 3–5 copies per chromosome in the studied transformant cultures (optical density $OD_{600} = 0.4$). However, in the absence of uracil dropout selection, shuttle plasmids were gradually lost from the cultures at an estimated *ca*. 15% per generation.

Expression of the *lacS* gene from the shuttle vector pHZ2lacS was studied both qualitatively and quantitatively. All pHZ2lacS transformation colonies on the selective plates were blue after staining with X-gal, indicating that they all carried the pHZ2lacS plasmid (Fig. 6b). Assaying for the *lacS* expression in liquid medium also revealed that the development of blue color was *lacS* gene-dependent since prolonged incubation of the cultures of E233S and the pHZ2 transformant showed no clear development of a blue color (Fig. 6c). The specific activity was studied in these strains by the ONPG method and while the LacS activity was at background level in the *lacS* deletion mutant E233S, in E233 and pHZ2lacS transformed E233S it was 1510 and 1076 (mU/mg protein), respectively. This suggested that *lacS* could be used as a reporter gene in *Sulfolobus islandicus*.

Discussion

We have developed genetic tools that enable loss of function and gain of function to be performed in the hyperthermophilic crenarchaeon *Sulfolobus islandicus*. In all of these studies, uracil dropout selection, i.e., genetic complementation of uracil auxotroph with *pyrEF* marker was employed to isolate transformants. The selection system allows the *Sulfolobus* transformation experiment to be conducted within 7 days. Moreover, the absence of *pyrEF* genes can be efficiently selected for with 5-FOA, yielding unmarked deletion mutants. This unmarked gene deletion

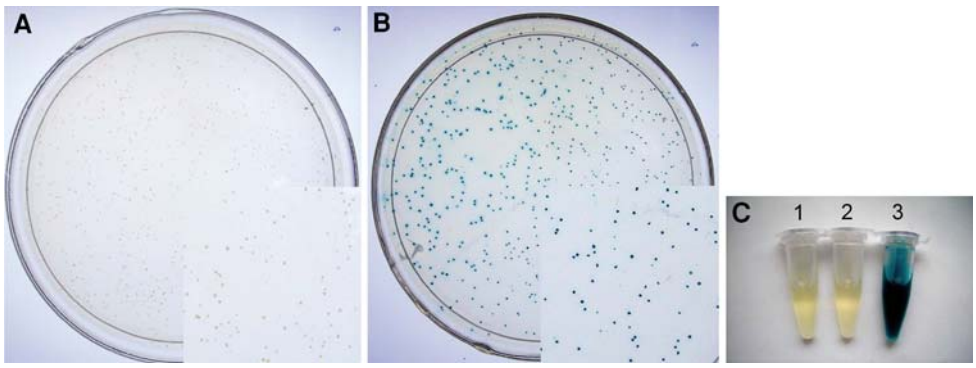

Fig. 6 Transformation of the double mutant E233S with the shuttle vectors pHZ2 and pHZ2lacS. *S. islandicus* E233S transformants of pHZ2 (**a**) and pHZ2lacS (**b**). The plates were stained with X-gal for 2 h. **c** Liquid cultures of E233S (1), E233S+pHZ2 (2), and E233S+pHZ2lacS (3) were stained with X-gal

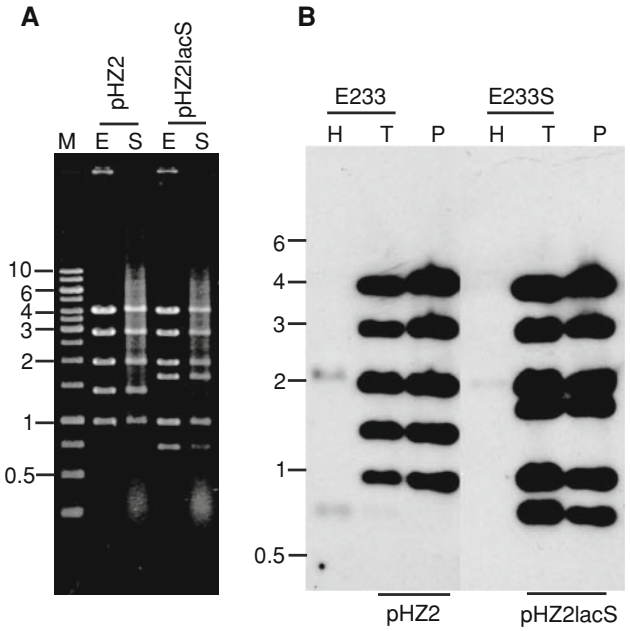

Fig. 7 Stability of the pRN2-based shuttle vectors in *S. islandicus* E233S. **a** Agarose gel electrophoresis of shuttle vector plasmids (pHZ2 and pHZ2lacS). The plasmids were prepared from *E. coli* (E) or *S. islandicus* (S) and digested with *Hin*dIII. **b** Southern analysis of pHZ2 and pHZ2lacS. pHZ2lacS was labeled and used as the probe for Southern hybridization. M—DNA marker; T—total DNA; P—plasmid; H—untransformed *S. islandicus* E233 or E233S

strategy can be exploited for carrying out multiple rounds of gene deletion with subsequent gene expression from a plasmid shuttle vector, using *pyrEF* as the sole marker.

Two unmarked gene deletion methods were tested in *S. islandicus*: namely plasmid integration and segregation (PIS), and marker replacement and looping out (MRL). The former is widely used in microbial genetics and has already been applied to archaeal species including methanogens, haloarchaea, *Sulfolobus solfataricus* (Schelert et al. 2004), and more recently *Sulfolobus acidocaldarius* (Wagner et al. 2009) and the strategy is also applicable to *S. islandicus* as shown here (reviewed by She et al. 2009). The MRL method was developed recently for *Thermococcus kodakaraensis* (Sato et al. 2005) and here we have shown that this method is also applicable to *S. islandicus*. Comparison of these methodologies has revealed some differences between them, including that the transformation rate is higher for MRL than for PIS. This may reflect the difference in recombination efficiencies between a linear versus a circular plasmid and the host chromosome in *S. islandicus*, as reported for *S. acidocaldarius* (Kurosawa and Grogan 2005).

Furthermore, transformation of *S. islandicus* with the linear pKL2 knockout plasmid not only follows the recombination scheme proposed for *T. kodakaraensis* (Sato et al. 2005), but also an alternative recombination scheme, namely MCI (marker circularization and integration) mechanism. The latter process must have started with circularization of the marker cassette during the first recombination event to yield Cir-M, which then integrates into the host chromosome (Fig. 5). The dynamic integration of Cir-M into, and excision from, the host chromosome give rise to the T3pKL2 transformants. Apparently, the MCI recombination scheme complicates the MRL unmarked gene deletion procedure, since the target gene can never be deleted in this recombination scheme. Therefore, MCI and MRL transformants should be distinguished and only the latter are to be utilized in the follow-up mutant construction.

An alternative means to improve the pKL2 gene deletion is to reduce strongly the formation of MCI transformants. Recently, it has been shown that homologous recombination can be facilitated for very short DNA segments in *S. acidocaldarius* (Grogan and Stengel 2008) and this raises the possibility of shortening the arms for marker gene looping out in MRL knockout plasmids. Since this modification does not change the arm lengths required for marker replacement, transformation efficiency should be maintained. If the arm length for looping out is to be reduced by 10–20 times (from 1 kb to 50–100 bp), the number of MCI transformants should be drastically decreased, presumably from 15–20% to below 1%.

The formation of MCI transformants can also be greatly reduced and eventually avoided by using different sequence segments for the two L-arms. Using the improved MRL method, we constructed a number of unmarked gene

deletion mutants of *S. islandicus*, including *dpo4*, encoding an error-prone DNA polymerase, and *orc/cdc6* genes encoding the putative archaeal initiators of chromosomal replication (Gökce et al., unpublished results).

S. islandicus E233S (Δ*pyrEF* Δ*lacS*) represents the first double deletion mutant of *pyrEF* and *lacS* genes that has been reported for a *Sulfolobus* strain. The *S. solfataricus pyrEF lacS* mutants described earlier are IS insertion mutants (Martusewitsch et al. 2000). One of these mutant hosts was useful for developing the first gene reporter system of *Sulfolobus* with a virus shuttle vector (Jonuscheit et al. 2003), but no other genetic tools have been reported for such a host. Two reports describe *lacS* deletion mutants isolated from *S. solfataricus* strains where the mutants carry a large deletion in the *lacS* locus (Bartolucci et al. 2003; Schelert et al. 2004). One of the mutants, *S. solfataricus* PBL2025, has been an important host for gene knockouts and for optimizing the gene deletion protocol (Albers and Driessen 2007) and several mutants have been constructed (Worthington et al. 2003; Schelert et al. 2004, 2006; Szabo et al. 2007a, b). But a more effective selection based on uracil dropout selection has not been reported for *S. solfataricus*.

Recently, uracil dropout selection was utilized for developing *S. acidocaldarius* plasmid shuttle vectors (Berkner et al. 2007). Genetic tools for gene knockout were also reported for this organism in which methylated plasmid DNA is used for transformation (Wagner et al. 2009). However, while these genetic tools enable genetic analyses to be performed in *S. acidocaldarius*, the important biology associated with the interactions between *Sulfolobus* hosts and their viruses/conjugative plasmids cannot be studied in this model organism because it is not a host for any known extra-chromosomal elements. In contrast, we found that *S. islandicus* REY15A serves as host for all tested fuselloviruses (SSV1, SSV2, pSSVx and pSSVi) and conjugative plasmids (pNOB8 and KEF9) (Contursi et al. 2006; unpublished data). Thus, the complete set of tools for genetic analysis in *S. islandicus* reported here and the complete genome sequence of the genetic host (to be published separately) will enable in vivo studies of archaeal molecular processes as well as molecular mechanisms of the archaeal host–virus/plasmid interactions, using *S. islandicus* as a model organism.

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