

Conditions for gene disruption by homologous recombination of exogenous DNA into the *Sulfolobus solfataricus* genome

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Summary The construction of directed gene deletion mutants is an essential tool in molecular biology that allows functional studies on the role of genes in their natural environment. For hyperthermophilic archaea, it has been difficult to obtain a reliable system to construct such mutants. However, during the past years, systems have been developed for *Thermococcus kodakarensis* and two *Sulfolobus* species, *S. acidocaldarius* and derivatives of *S. solfataricus* 98/2. Here we describe an optimization of the method for integration of exogenous DNA into *S. solfataricus* PBL 2025, an *S. solfataricus* 98/2 derivative, based on lactose auxotrophy that now allows for routine gene inactivation.

Keywords: lactose auxotrophy, selection.

Introduction

For many years, the functional analysis of genes and gene products of hyperthermophilic archaea was hampered by the lack of suitable generic genetic tools such as expression vectors, methods for the directed deletion of target genes and efficient gene transformation protocols that allow a high recovery of transformed cells. Recently, virus-based vector systems have been developed for *Sulfolobus* species (Stedman et al. 1999) and used in promoter studies (Jonuscheit et al. 2003), homologous and heterologous expression of proteins in *Sulfolobus* (Albers et al. 2006, Aucelli et al. 2006) and the complementation of deletion mutants (Bartolucci et al. 2003, Jonuscheit et al. 2003). A shuttle vector system has been successfully used for pyrococcal species (Lucas et al. 2002), whereas a targeted gene deletion system has been described for *Thermococcus kodakarensis* and *Sulfolobus solfataricus* (Worthington et al. 2003, Sato et al. 2005). Targeted gene disruption relies on the genomic integration of a selectable marker together with flanking regions of the gene of interest by recombination with circular, non-replicating DNA. In *S. acidocaldarius*, a form of conjugation has been described that generates prototrophic recombinants from pairs of auxo-

trophic mutations (Hansen et al. 2005). Moreover, extensive testing of different conditions revealed that even the electroporation of synthetic oligonucleotides resulted in their recombination into the chromosome (Kurosawa and Grogan 2005).

We set out to investigate different conditions for the effective recombination of exogenous DNA in the genome of *S. solfataricus* PBL2025, a derivative of *S. solfataricus* 98/2 (Schelert et al. 2004). Here, we describe an effective method that can be routinely used to generate gene disruption mutants in *S. solfataricus* PBL2025.

Materials and methods

Strains

Sulfolobus solfataricus PBL2025 (Schelert et al. 2004) was grown aerobically at 80 °C in the medium described by Brock et al. (1972), adjusted to pH 3 with sulfuric acid and supplemented with either 0.1% (w/v) tryptone or 0.4% (w/v) of different sugars as sole carbon and energy source unless otherwise indicated. Growth of cells was monitored by optical density at 600 nm (OD₆₀₀). *Escherichia coli* strain DH5 α was used for the propagation of plasmids.

Electroporation of *Sulfolobus solfataricus* cells

Electroporation conditions were essentially as described by Schleper et al. (1992). PBL2025 cells (50 ml) were grown overnight to an OD₆₀₀ ~ 0.1–0.3 in tryptone/sucrose medium. Cells were collected by centrifugation and resuspended in 50 ml of cold 20 mM sucrose. From this point on, cells were kept constantly on ice. The washing step was repeated with 10 ml of cold 20 mM sucrose. Finally, cells were resuspended in 20 mM sucrose at approximately 10¹⁰ cells ml⁻¹. An aliquot (50 μ l) of the cell suspension was used for each electroporation. Electroporation conditions were 1.5 kV, 25 μ F, 400 Ω with 2-mm cuvettes (Thermotron) and a Genepulser II (Bio-Rad) electroporator. The various modifications of the treatments of the cells after the electroporation step are listed in Table 1. The best post-electroporation procedure was to re-

suspend cells in 1 ml of demineralized water, leave them briefly (1–3 min) on ice and then incubate them for 10 min at 75 °C. The cells were then transferred to 50 ml of prewarmed lactose minimal medium.

Methylation of plasmid DNA

Plasmid DNA was methylated with HaeIII methylase according to the manufacturer's recommendations. After methylation, the DNA was purified with the GFX Purification Kit (GE Healthcare, The Netherlands).

Construction of the various plasmids

The up- and downstream flanking regions of *Sso0120* were amplified by PCR from genomic DNA isolated from *S. solfataricus* P2. The upstream flanking region was amplified with primers UP-F-*KpnI* and UP-R-*NcoI* (5'-CGGTACCGTGCGTATTATCTACGTTA-3' and 5'-CCCCCATGGCAGTGTTTATTTAAAGAA-3', respectively). The PCR product was digested with *KpnI* and *NcoI* and ligated into pET2268, which contains the *lacS* cassette with its own promoter and terminator region (Figure 1). The downstream flanking region was amplified with primers DWN-F-*BamHI* and DWN-R-*NofI* (5'-CCCGGATCCGGAGAATATTCATGATAC-3' and 5'-CCCCCCCCGCGGCCGCGAGTGCAAAGATACTTG-3', respectively), subsequently digested with *BamHI* and *NofI* and ligated into pET2268 already containing the upstream flanking region yielding pSVA37. The pSVA50 plasmid was constructed by inserting an *Sso3017*-containing PCR product into the *lacS* cassette of pET2268. The primers, 3017-F (5'-CCCCCATGGTCTCTTTAAACCAGACG-3') and 3017-R (5'-CCATAGAGGTAATGGCCAATGATACATG-3'), contained an *NcoI* and *MscI* restriction site, respectively, and were used to digest both the PCR product and pET2268 prior to ligation. pSVA63 was constructed by inserting the same up- and downstream flanking regions as for pSVA37 into pSVA50.

PCR analysis of deletion mutants

We performed PCR on the *Sso0120* deletion mutant with primers 120-F (5'-CACCAGTAGCCTTTACAG-3') and 120-R (5'-TTTGTTCGATACGGCGGTTG-3'), yielding a product of 2100 bp with the host strain PBL2025 and a 2900 bp prod-

uct upon successful integration of the *lacS* gene into the *Sso0120* locus (see Figure 2). *LacS* specific primers were LacS-F (5'-GGGGGCCATGGACTCATTTCCAAATAGCTTTAGG-3') and LacS-R (5'-GGGGGGATCCGTGCCTTAATGGCTTTACTGGAGGTACGC-3'). Primers for the detection of the successful integration of the knockout plasmid into the *Sso0120* gene locus were 3017-R and M3 (5'-CCCCCGATCCAATAGCTTTAGTTTCAACTTTATCACC-3'). For the analytical reactions, PCR Mastermix was employed (Promega) with 30 cycles of 30 s at 94 °C, 30 s at 52.5 °C and extension at 72 °C, where the duration depended on the specific PCR product. Genomic DNA for PCR analysis was isolated from 2 ml of *S. solfataricus* cells with the QuickPick SML gDNA Kit (Bionobile).

X-gal treatment of plates

The X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) was dissolved to 25 mg ml⁻¹ in dimethylformamide. Plates were sprayed with a solution containing 5 mg ml⁻¹ X-gal diluted in demineralized water, and subsequently incubated for 30 minutes at 78 °C. Finally, the blue colonies were scored.

Results

Selection of gene disruption strains after electroporation of plasmid DNA

PBL2025 has been described as a suitable host for the directed deletion of target genes in *Sulfolobus solfataricus* (Schelert et al. 2004). The strain is a natural deletion mutant lacking a chromosomal region of about 50 genes (*Sso3004*–*Sso3050*). The missing genes include *lacS* that encodes a β -galactosidase that is essential for growth of *S. solfataricus* on lactose minimal medium and therefore can be used as a selective marker.

Exogenous DNA is introduced into *S. solfataricus* by means of electroporation, but to maximize the yield of integrants, the post-electroporation conditions must be optimized to prevent the accumulation of false positives that slowly grow on the selective media. For this optimization, genes *Sso2684*–*Sso2681* were targeted. These genes encode putative pilin proteins

Table 1. Post-electroporation conditions.

Condition	Time after transfer to fresh lactose medium before plating (days)	Blue colonies produced (by carbon source)	
		Lactose	Tryptone
Eight h regeneration in 0.1% tryptone, 1 ml transferred directly to lactose medium	10	0	0
Resuspended in 20 mM sucrose, 10 min at 75 °C, transfer to lactose medium	7	13	21
Resuspended in H ₂ O, 10 min at 75 °C, transfer to lactose medium	8	141	171
Resuspended in Brock medium, pH 4.7, 10 min at 75 °C, transfer to lactose medium	14	0	0

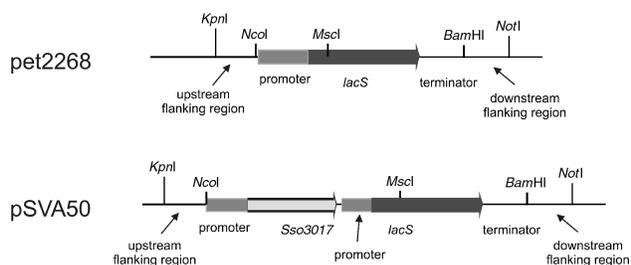


Figure 1. Schematic overview of the plasmids pET2268 and pSVA50 used for the isolation of gene deletion mutants in PBL2025. The *KpnI/NcoI* and *BamHI/NotI* restriction sites are used to clone the upstream and downstream flanking regions of the target gene(s), respectively.

(Zolghadr et al. 2007). To test different post-electroporational conditions, PBL2025 cells were prepared as described in the Materials and methods and electroporated with 300 ng of pSVA78 plasmid DNA (Table 2). This plasmid harbors the *lacS* gene with flanking regions of the *Sso02684* and *Sso2681* genes (upstream flanking region was 733 bp, downstream flanking region was 756 bp). Upon transformation of the cells, the deletion cassette will integrate into the genome resulting in an insertion of the *lacS* gene into the *Sso2684–Sso2681* structural genes. Cells with a successful integration grow with lactose as the sole energy and carbon source, exhibit β -galactosidase activity and show, by PCR analysis, an inserted *lacS* at the targeted gene locus. After the 10-min incubation at 75 °C, 100 μ l of cells was directly spread on lactose minimal medium plates and incubated at 78 °C for 7 days, but no individual colonies were retrieved. A sample of 900 μ l of the electroporated cells was transferred to 50 ml of liquid lactose minimal medium, including the control cells that were electroporated without plasmid DNA. After about twelve days, cells of all conditions (Table 1) reached an OD_{600nm} of 0.1 whereupon 3 ml of each culture was used to inoculate 50 ml of fresh lactose minimal medium. From this stage, no growth could be detected with the control cells, whereas plasmid electroporated cells reached an OD_{600nm} of about 0.4 after 7 to 14 days. Subsequently, cells were spread on lactose and tryptone plates and sprayed with 5 mg ml⁻¹ X-gal after 7 days of growth. As shown in Table 1, in only two of the tested conditions were blue colonies obtained, indicating the presence of *lacS* in these cells on either lactose or tryptone plates. These blue single colonies were inoculated in tryptone medium.

Genomic DNA of the cells growing on tryptone medium was isolated and subjected to PCR analysis. The PCR analysis with the *lacS* primers generally yielded the fragment of the expected size when performed with cells derived from the blue colonies. The PCR analysis with primers specific for the 3'- or 5'-end of *lacS* and the flanking region of the targeted gene yielded products of the expected size, indicating that recombination had occurred at the correct site. However, a PCR analysis with the gene specific primers mostly indicated that the target gene was still present in the analyzed strains. An example is shown in Figure 2, where PCR yielded the expected 2100 bp product for the undisrupted gene and the larger 2900 bp prod-

Table 2. Strains and plasmids.

	Genotype or sequence	Reference
<i>Strain</i>		
PBL2025	Δ (<i>Sso3004–3050</i>)	Schelert et al. 2004
<i>Plasmid</i>		
pET2268	<i>lacS</i> cassette containing its own promoter and terminator region	Szabo et al. in press
pSVA37	<i>Sso120::lacS</i>	This study
pSVA50	pET2268 containing <i>Sso3017</i> and <i>lacS</i>	This study
pSVA63	<i>Sso120::lacS/Sso3017</i>	This study
pSVA78	<i>Sso2681, Sso2683, Sso2684::lacS</i>	Zolghadr et al. in press

uct including the *lacS* gene for the disruption mutant (Figure 2). This indicates that either the cultures are mixtures of the wild type and gene disruption strain even though single blue colonies were picked from the plates, or the cells contain more than one chromosome. To single out the deletion strain, cells were again plated on tryptone plates and single blue colonies were selected, and this was repeated until the PCR analysis no longer indicated the presence of the wild type allele. Usually two to three rounds of single colony picking and replating was necessary to isolate the deletion mutant strain.

Optimization of growth and electroporation conditions for strain selection

For growth of *S. solfataricus* cells to occur on sugar minimal medium, it is necessary to adapt the cells to the sugar of choice by pre-growth on the respective sugar in the presence of tryptone or casamino acids before transfer of the cells to sugar minimal medium. After electroporation, successful recombinants are selected on lactose minimal media, therefore it was assumed that it would be beneficial to grow the cells before electroporation on tryptone medium with a sugar that induces lactose metabolism. Although the presence of sugar was necessary, no difference in growth after electroporation was observed when either sucrose or lactose was added to the tryptone medium.

Previously, methylated DNA has been used for transformation in *S. solfataricus* (Worthington et al. 2003). We compared the yield of blue colonies after selection on lactose minimal medium using *S. solfataricus* cells electroporated with either methylated or non-methylated plasmid DNA. However, this did not affect blue colony yield, whether the amount of plasmid DNA used was 100, 300 or 500 ng. Cells electroporated with 300 ng of plasmid DNA started to grow first (after about 8 days), whereas electroporation with 500 and 100 ng resulted in slower growth, which extended the selection protocol by 2 and 5 days, respectively.

As it has been reported that *S. acidocaldarius* can be effectively transformed with oligonucleotides, we tested whether variations in the length of flanking regions of the targeted gene

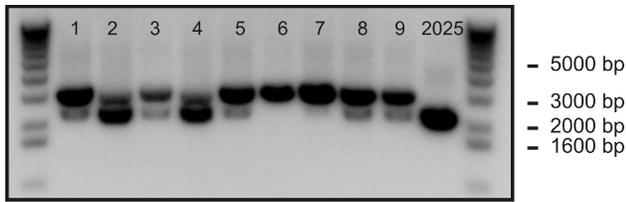


Figure 2. Analysis of genomic DNA of *Solfolobus solfataricus* cells derived from selected blue colonies from cells electroporated with pSVA37 on tryptone plates. The PCRs were performed with genomic DNA derived from nine individual cultures and from PBL2025 control cells. The primer set used is directed toward the targeted gene flanking regions and resulted in a 2100 bp product in the wild type cells corresponding to the undisrupted gene, and a 2900 bp band for the mutant cells corresponding to the disrupted gene with an insertion of the *lacS* selection marker.

affect the recovery of integrants. Gene fragments from 500 to 1200 bp were used, but no differences in the yield of blue colonies were observed.

Co-transformation of the lactose transporter in recombinant strain selection

For *S. solfataricus* strain MT4, a natural mutant has been described that, like PBL2025, lacks a 13 kb fragment of genomic DNA, which includes *lacS* and the gene upstream that is homologous with *Sso3017* (Bartolucci et al. 2003). The latter represents a lactose transporter which, together with *lacS*, is needed to restore growth of the natural selected mutant on lactose selective medium (Bartolucci et al. 2003). To determine whether the co-introduction of the transport gene *Sso3017* together with the *lacS* gene improves the recombination yield in PBL2025, plasmid pSVA50 was constructed containing both genes under the control of their natural promoters. Based on this plasmid, gene disruption plasmids were prepared for gene *Sso0120*, which encodes a secretion ATPase (Albers and Driessen 2005), that contained either only the *lacS* gene (pSVA37) or both the transporter and the *lacS* gene (pSVA63) (Figure 1). After transformation of both plasmids into PBL2025 cells, no significant advantage of the presence of the transporter was noticed with the recovery of transformed cells on lactose minimal medium and subsequent blue-white screening with the indicator X-gal on tryptone plates. However, blue colonies picked from cells containing pSVA63 were able to grow in 3 days on liquid lactose minimal medium, whereas only slow growth was observed in cells containing the deletion plasmid with only *lacS* as a marker. The PCR analysis showed that the blue colonies of pSVA37 transformed cells contained both *lacS* and a PCR product showing the insertion of *lacS* at the site of the target gene (Figure 3). However, mixed cultures were also obtained (Figure 2). All cultures from colonies containing pSVA63 showed a product for *lacS* and the target gene, but were negative for the PCR product of the flanking region indicating correct insertion (see Figure 3). This indicated that in, all these cases, the *lacS*-transporter cassette integrated at another site in the genome. Therefore the co-introduction of the transporter did not improve the selec-

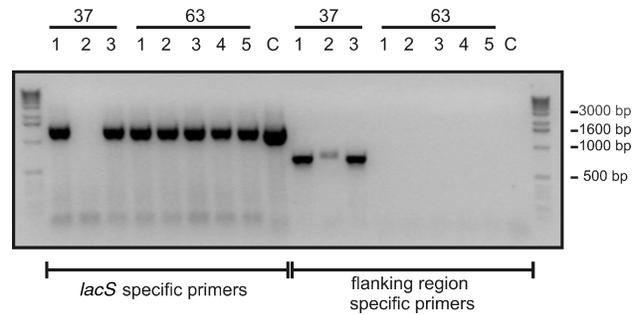


Figure 3. Analysis of genomic DNA of individual cultures obtained from selected blue colonies from cells electroporated with either pSVA37 or pSVA6. The left panel shows the presence of the marker gene using *lacS* specific primers. The right panel shows the positive identification of successful integrations using primers specific for an internal part of *lacS* and the 5' flanking region of the targeted *Sso0120* gene. As a positive control (C) for the *lacS* PCR *Solfolobus solfataricus* P2 genomic DNA was used, while for the PCR of the flanking region, genomic DNA of PBL2025 was used as a negative control. The DNA marker sizes are indicated.

tion procedure for deletion mutants on lactose minimal medium.

Discussion

We have presented an optimized and effective protocol for the insertion of exogenous DNA into the genome of *S. solfataricus* PBL2025, an *Sulfolobus solfataricus* 98/2 derivative strain, and for the selection of gene disruption mutants. *Sulfolobus solfataricus* strain PBL2025 contains a deletion of nearly 50 genes, including *lacS*, the gene encoding a β -galactosidase which has been shown to be essential for growth of *S. solfataricus* on lactose (Schelert et al. 2004). The lactose auxotrophy has been used before as a marker for targeted gene deletion mutant selection (Schelert et al. 2004). In *S. acidocaldarius*, uracil auxotrophy has been shown to be a useful marker in conjugative DNA transfer and genomic integration of exogenous DNA (Kurosawa and Grogan 2005, Hansen et al. 2005). Unfortunately, in *S. solfataricus*, this marker has not so far been used for the construction of deletion mutants. As for *S. acidocaldarius*, after electroporation, cells can be directly selected on plates containing the toxic uracil homolog 5-fluoroorotic acid, whereas *S. solfataricus* cells first need to be accommodated to growth in liquid minimal lactose medium for selection before they can be transferred to plates. At least 7–12 days growth in liquid medium is required, which prolongs the selection procedure (Lubelska et al. 2006). The lag time of growth could not be shortened by pre-growth of the cells on tryptone/lactose medium before electroporation. Therefore, it would be useful to isolate a stable uracil auxotroph of *S. solfataricus*, possibly by a targeted disruption of the *pyrEF* genes, because then a switch of carbon source would be unnecessary and thereby the delay of growth due to adaptation to the new carbon source could be avoided.

Our data show that the post-electroporational treatment is

critical for gene disruption strain selection in *S. solfataricus* (Table 1). In particular, a 10-min treatment of the cells in water at 75 °C before transfer to liquid medium was critical, as was described for *S. acidocaldarius* (Kurosawa and Grogan 2005). After the first selection round, blue colonies represented mixtures of wild type and deletion mutant cells (Figure 2). How can this be explained? It has been shown that *Sulfolobus* tightly controls its chromosome number, oscillating between one and two chromosomal copies in the G1 and G2 phase of the cell cycle, respectively (Bernander and Poplawski 1997). Moreover, recent studies indicate that *Sulfolobus* contains hemicatenane sister chromatid junctions at all three replication initiation sites of the chromosome and that such structures are also present at other genomic regions. These junctions of both chromosomes were detected in both replicating and post-replicating cells (Robinson et al. 2007). This might indicate that the gene disruption plasmid is integrated by homologous recombination into only one of the chromosomes, but as the tight junctions of the chromosome are present, both the wild type and the deletion situation are propagated through the culture.

In conclusion, we have examined and optimized a protocol to obtain targeted gene deletion mutants of *S. solfataricus* PBL2025 employing lactose auxotrophy as a selective marker. In the future, this method will benefit from the isolation of more stringent selective markers, but the current protocol has yielded several gene deletion mutants (Zolghadr et al. 2007) in which the functionality can be studied.

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