Expanding and understanding the genetic toolbox of the hyperthermophilic genus *Sulfolobus*

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Abstract

Although *Sulfolobus* species are among the best studied archaeal micro-organisms, the development and availability of genetic tools has lagged behind. In the present paper, we discuss the latest progress in understanding recombination events of exogenous DNA into the chromosomes of *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* and their application in the construction of targeted-deletion mutant strains.

Background

Manipulation of the genetic information of organisms is a vital tool to investigate the role and function of genes. In model organisms from all three domains of life, methods have been developed to integrate engineered DNA site-specifically into the genome. These methods rely mainly on homologous recombination and allow new genes to be introduced into a genome, parts of a genome to be deleted or specific mutations to be established in the genome. After changing the genotype, it is possible to analyse the phenotype (reverse genetics). For eukarya and bacteria, these methods are very well established and continue to be extremely powerful for the elucidation of the functions of genes. However, archaea are lagging behind [1]. The main problem was and is the availability and development of efficient selectable markers. Most bacterial antibiotics are ineffective in archaea, although some examples have been used successfully. Novobiocin, an inhibitor of the DNA gyrase, has been used to develop a vector for halophilic archaea [2,3], and puromycin is the most widely used antibiotic for methanogens [4]. Owing to the elevated temperatures of the habitats of hyperthermophilic archaea, it has proven even more difficult to find suitable marker systems. However, for *Thermococcus kodakaraensis*, a genetic system has been established successfully recently using uracil and tryptophane auxotrophic mutants [5].

The thermoacidophilic *Sulfolobus* species belong to the most studied archaea, but, so far, studies have been greatly hampered by the lack of genetic tools. However, recently, considerable progress has been made in the improvement of plasmid systems and also protocols for the generation of deletion mutants have been developed for different *Sulfolobus* species [6–10]. The plasmid systems available, including self-spreadable virus vector systems and other *Escherichia coli*–*Sulfolobus* shuttle vectors, have been reviewed recently by Berkner and Lipps [7] in detail and will not be discussed further here.

The lack of suitable selectable markers again posed a major problem in the progress of the development of methods for directed gene deletion in *Sulfolobus* species. Although auxotrophic strains for uracil biosynthesis of *Sulfolobus acidocaldarius* [11] and *Sulfolobus solfataricus* [12] have been isolated, these have not been used as selectable markers. One problem is that Gelrite, the polymer used for solid media in hyperthermophiles, contains traces of uracil, so that the background after selection can be quite high. A more severe problem is that the widely used *S. solfataricus* strains P1 and P2 do not recombine foreign plasmid DNA into their chromosome [10]. However, an *S. solfataricus* strain PBL2025, originating from *S. solfataricus* strain 98/2, has been shown to be capable of homologous recombination and is used for the generation of deletion mutants [6]. This strain lacks the gene that encodes a β-galactosidase, lacS, that is essential for growth of *S. solfataricus* on lactose minimal medium and can therefore be used as a selective marker. Details on the progress of constructing directed gene-deletion mutants in *S. solfataricus* PBL2025 and *S. acidocaldarius* are described below.

Recombination by single- and double-cross-over events into the chromosome of *S. solfataricus*

The first successful targeted-deletion mutant in *S. solfataricus* PBL2025 was constructed by Schelert et al. in 2004 [6]. For integration of foreign DNA into the chromosome, a plasmid was used containing the up- and down-stream flanking region of the target gene and a marker cassette encompassing the lacS gene with its original promoter and terminator region. Positive clones are first selected by growth on lactose minimal medium. After plating, blue colonies can be analysed for correct integration of the construct. We optimized the post-electroporation conditions and found that a 10 min
incubation step in demineralized water yielded the largest amount of positive clones after selection on lactose minimal medium [9]. Moreover, methylation of the plasmid DNA, which is necessary for electroporation of plasmids in *S. acidocaldarius*, is obsolete for *S. solfataricus* PBL2025 [9].

By using linear DNA for recombination, double-cross-over events can be obtained. Therefore either linearized plasmid DNA or a PCR product covering the flanking region and the *lacS* cassette of the gene-targeting construct were transformed into PBL2025. After selection, single blue colonies were obtained that were shown to contain the expected deletion-mutant genotype by PCR and Southern blot analysis. In three cases tested, recombination of the linear DNA fragment occurred in 90% of the colonies tested at the correct position. This showed that linear DNA fragments containing the up- and down-stream flanking regions of a GOI (gene of interest) and the *lacS* cassette can be used efficiently for the deletion of genes in *S. solfataricus* PBL2025.

Using plasmid DNA for electroporation resulted in blue colonies that exhibited the expected deletion-mutant product as well as the wild-type gene product after PCR analysis of their genomic DNA (Figure 1B). Only after a
Figure 2 | Tandem insertion of plasmid DNA into the chromosome of *S. solfataricus*

(A) Southern blot analysis of genomic DNA of four different semi-knockout strains of SSOGOI digested with HpaI and using a lacS probe for detection. M, markers. As shown in the cartoon of tandem insertion (B), a third band is only present if more than one copy of the gene-targeting construct is inserted.

few more selection rounds on solid media were true deletion strains isolated [9,13]. PCR and Southern blot analysis were performed on strains exhibiting both the wild-type and deletion-mutant PCR products. Primers directed against the ampicillin cassette of the gene-targeting constructs were used for PCR analysis of integration strains (strains containing both the wild-type and gene-deletion PCR products) and correct deletion mutants. As shown in Figure 1(A), the integration-strain SSOGOI (*S. solfataricus* GOI) gave the same specific band as found in the plasmid control pET401, which is the backbone plasmid for gene-targeting constructs. However, this band was absent from the correct knockout strain ΔSSOGOI, as it was from the PBL2025 wild-type strain. This indicated that the recombination of plasmid DNA occurred via single-cross-over events and resulted in strains in which the whole gene-targeting plasmid was integrated into the chromosome (Figure 1B). Southern blot analysis confirmed further the presence of the plasmid DNA in the semi-knockout chromosome (results not shown).

Further Southern blot analysis showed that the integration strains in many cases did not contain only one copy of the gene-targeting construct, but tandem insertion occurred as has also been observed in *Methanococcus voltae* [14] (Figure 2). However, the number of tandem insertions has yet to be determined.

**Recombination by single- and double-cross-over events into the chromosome of *S. acidocaldarius***

The introduction of foreign DNA into genomic DNA by recombination has already been studied in quite some detail in *S. acidocaldarius* by Dennis Grogan and co-workers, and they could demonstrate that even oligonucleotides of 14 bp in length can be incorporated efficiently by OMT (oligonucleotide-mediated transformation) [15,16]. However, a genetic system had not yet been established for the construction of targeted-deletion mutants.

In contrast with *S. solfataricus* strains, uracil selection is more efficient in *S. acidocaldarius*. Uracil auxotrophic strains contain mutations in the pyrEF gene sequences, encoding orotate phosphoribosyltransferase and orotidine-5′-monophosphate decarboxylase respectively. PyrEF catalyse the last two steps of the *de novo* uridine monophosphate synthesis pathway [17]. Using 5-FOA (5-fluoro-orotic acid), which is converted into toxic 5-fluorouracil in wild-type cells, several uracil auxotrophic strains of *S. acidocaldarius*...
Figure 3 | Scheme of the construction of a markerless deletion mutant in *S. acidocaldarius* using the pΔ2pyrEF construct

After integration into the chromosome by single cross-over, a second recombination step can lead to either a wild-type genotype (A) or the desired deletion-mutant genotype (B). *amp*<sup>R</sup>, ampicillin cassette; *pyrEF*, *pyrEF* cassette of *S. solfataricus*.

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were isolated [11,18]. We used the *S. acidocaldarius* strain MR31 that has an 18 bp deletion in the *pyrE* gene as a host for gene-targeting constructs. The gene-targeting plasmid pΔ2pyrEF was constructed using pBluescript as a backbone for replication in *E. coli* and the *pyrEF* cassette from *S. solfataricus* was cloned into this vector. The *pyrEF* cassette of *S. solfataricus* was used to avoid homologous recombination with the endogenous *S. acidocaldarius* *pyrEF* cassette. This vector was used to construct plasmids for single- and double-cross-over recombination events into the *S. acidocaldarius* chromosome. For double-cross-over recombination events, the up- and down-stream flanking regions of the target gene were cloned up- and down-stream of the *pyrEF* cassette in the gene-targeting vector pΔ2pyrEF. Because of the presence of the restriction endonuclease SuaI in *S. acidocaldarius*, the plasmid, when transformed unmethylated, is restricted after DNA uptake and yields linear DNA fragments which can serve as substrates for a double-cross-over integration event. The *E. coli* part of the shuttle vector contained numerous recognition sites for SuaI, whereas the remainder of the construct has no restriction site for SuaI. After transformation, cells were selected directly on plates or first selected for two to three rounds in liquid medium and then on plates which did not contain uracil. All colonies tested by PCR and/or Southern blot showed correct replacement of the GOI. In this manner, we constructed two deletion mutants of *S. acidocaldarius*, a tryptophane auxotroph mutant lacking the *trpA* gene and a ΔSaci_1494 strain.

To recombine plasmid DNA into the chromosome via single-cross-over events leading to a markerless deletion strain, the up- and down-stream flanking regions were cloned consecutively upstream of the *pyrEF* cassette (Figure 3). Integrants were obtained by uracil selection on plates and integration of the gene-targeting plasmid was confirmed by Southern blot analysis. To remove the GOI from the chromosome, the plasmid has to be removed by a second recombination step, leading either to the wild-type situation or to the deletion-mutant genotype. This process was not observed spontaneously, but occurred very efficiently after counter-selection for uracil auxotrophs on 5-FOA-containing plates.

Concluding remarks

In our work, we have shown that exogenous DNA recombines into the chromosomes of *S. solfataricus* and in the genetically more stable *S. acidocaldarius* via single- and double-cross-over events. In *S. solfataricus*, tandem insertion of plasmid DNA was observed after recombination via single cross-over. We demonstrated that directed deletion mutants can be constructed in both *Sulfolobus* strains, which will give a starting point for the development of more elaborate and efficient genetic tools for these species.

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**References**


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