Versatile genetic tool box for the crenarchaeote *Sulfolobus acidocaldarius*

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**INTRODUCTION**

In contrast to the bacterial domain, research on Archaea has long been hampered by the absence of genetic tools to study gene functions *in vivo*. One major reason for this was that archaea are resistant to most commonly used antibiotics that are used in microbial genetics as selectable markers (Leigh et al., 2011). These compounds very often target the peptidoglycan synthesis which is absent in most archaea (Albers and Meyer, 2011) or the antibiotics are readily degraded at the conditions at which various archaea live.

Genetic systems were described for haloarchaea and methanogens only in the late 1990s, respectively, and have constantly improved since then (Leigh et al., 2011). For another euryarchaeon *Thermococcus kodakariaensis* an effective gene deletion system was established by Sato et al. (2003, 2005) for which also a complementary expression vector has been optimized (Santangelo et al., 2008). The most recent system was developed for *Pyrococcus furiosus*. Hence, nowadays genetic toolboxes exist for a variety of euryarchaeota (Waeger et al., 2010).

In the kingdom crenarchaeota, only for organisms of the order Sulfolobales genetic systems have been developed. Sulfolobales are thermoacidophilic microorganisms that grow optimally at temperatures around 80°C and pH values between 2.5 and 3.5. Since the first description of *Sulfolobus acidocaldarius* by Brock et al. (1972), members of the Sulfolobales have developed into model organisms for studying DNA transcription, replication, translation, DNA repair, RNA processing, and cell division. Moreover, most Sulfolobales possess the non-phosphorylated Entner–Doudoroff pathway and its regulation upon temperature shifts was the topic of a systems biology approach (Albers et al., 2009). This initiative has led to the development of standard operating procedures for omics approaches in Sulfolobales and should facilitate the exchange and comparability of obtained data (Zaparty et al., 2009).

The first shuttle vectors and a gene deletion method in *S. solfataricus* were based on the β-galactosidase, LacS, as a selection marker (Worthington et al., 2003; Aucelli et al., 2006; Berkner et al., 2007) and this method was successfully employed in a number of studies (Schelert et al., 2004, 2006; Szabo et al., 2007; Zolghadr et al., 2007; Frols et al., 2008; Maaty et al., 2009). However, as the selection is based on minimal media containing only lactose as a carbon source, this method is quite tedious as *Sulfolobus* species do not grow well on sugar minimal media. In some archaeal genetic systems the complementation of uracil auxotrophic mutants by the pyrEF genes is used on uracil free media to obtain gene deletion mutants. This marker also enables for the use of 5-fluoroorotic acid (5-FOA), which can be employed as counter selection marker for constructing markerless deletion mutants. For *S. islandicus* a system for obtaining unmarked deletion mutants relying on the pyrEF selection including a shuttle vector has been established (Deng et al., 2009; Peng et al., 2009) and successfully used in different studies (Zhang et al., 2010; Gudbergsdottir et al., 2011).

Other Sulfolobus shuttle vectors that rely on pyrEF selection were constructed based on the virus SSV1 and the plasmid pRN1 (Jonascheit et al., 2003; Berkner et al., 2007). For *S. islandicus* it has furthermore been shown recently that the drug simvastatin can be used to select for the presence of plasmids in a host that overexpresses the *hmg* gene, encoding the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA; Zheng et al., 2012).

Recently, we employed pyrEF as a selectable marker to obtain insertional deletion mutants in *S. acidocaldarius* and moreover for persistence of a shuttle vector with an inducible promoter.
for expression (Wagner et al., 2009; Berkner et al., 2010). It was earlier demonstrated that S. acidocaldarius can efficiently recombine primers down to a length of 14 bp into its genome (Grogan and Stengel, 2008). Taking advantage of this ability Grogan and colleagues showed very recently that short flanking regions of only 40–50 bp incorporated into the S’ end of primers led to site-specific integration of the obtained PCR products containing pyrE as a selectable marker into the S. acidocaldarius genome (Sakofsky et al., 2011). This method has been used in high-throughput put screens especially in Saccharomyces cerevisiae and opens these possibilities now for S. acidocaldarius. However, these methods lead to the consumption of the only available marker and therefore abolish the possibility of complementation in trans and also the construction of double or triple gene mutants.

Therefore we established different efficient methods to obtain unmarked deletion mutants in S. acidocaldarius relying on uracil auxotrophy as a selectable marker using the pop in/pop out method. These methods were employed for genomically tagging of genes enabling pull down experiments for studies on protein complexes. In addition, an ABC transporter of S. solfataricus was ectopically inserted into the upsE locus of S. acidocaldarius. Moreover, we present the use of a copper inducible promoter for homologous and heterologous production of proteins. Summarizing, we present here a versatile and complete genetic toolbox for S. acidocaldarius.

RESULTS
CONSTRUCTION OF MW001
Several different auxotrophic mutants of S. acidocaldarius were published before. However, quite a few of these were obtained by UV mutagenesis (Grogan, 1991). Therefore the possibility existed that these mutants would also contain secondary mutations in their genomes. For that reason a new directed S. acidocaldarius pyrE deletion mutant was constructed. This mutant, dubbed MW001, was obtained by transforming the S. acidocaldarius wild type strain DSM639 with a PCR product that contained 930–1500 bp of the up- and downstream flanking regions of the pyrE gene (saci1597), which would delete the full length pyrE gene by homologous recombination. After transformation the cells were streaked on gelrite plates containing uracil and 5-FOA. Surprisingly, none of the obtained colonies exhibited the expected complete deletion of the pyrE gene, but only a deletion of 322 bp (91–412 bp). This phenomenon happened repeatedly and this particular deletion has also been isolated before (Grogan and Hansen, 2003). The deletion of the 322 bp in pyrE in MW001 was confirmed by PCR (Figure 1B) and sequencing. Growth of MW001 in medium containing NZ-amine medium was only possible upon the addition of uracil and was completely restored at a concentration of 10 μg/ml uracil (Figure 1A).

IN-FRAME UNMARKED DELETION MUTANTS
We employed three different methods to obtain in-frame unmarked deletion mutants in S. acidocaldarius MW001. The first method is based on the classical “pop in/pop out” scheme using a single crossover recombination step (see Figure 2A). We designed pSVAA406 for this purpose, which contained the pyrEF cassette of S. solfataricus (pyrEFSSO) and a multiple cloning site upstream of it, which was used to insert the approximately 500 bp long up- and downstream flanking regions of the gene of interest. The obtained deletion mutant plasmid was methylated and 100 ng plasmid DNA was electroporated into MW001. Cells were streaked on first selection plates that contained no uracil to select for the cells that would integrate the plasmid into their genome via a single crossover (Figure 2A). To enforce the “pop out” by a second single crossover recombination event, cells were then streaked on second selection plates that contained 5-FOA as only pyrEF cells are resistant to 5-FOA. This second single crossover recombination step will either produce the wild type situation or the expected deletion mutant (Figure 2A). The method is here illustrated by deleting upsE (saci1494). In the last screening step we obtained in 50% of the colonies the wild type situation and 50% of analysed colonies we got the expected in-frame deletion mutants (Figure 3A). As upsE is part of the UV inducible pili operon in S. acidocaldarius which encodes proteins that after UV induction mediate cellular aggregation (Ajon et al., 2011), we confirmed that the obtained ΔupsE strain could indeed not aggregate anymore upon UV treatment (Figure 3B). The correctness of the obtained deletion mutants was confirmed by sequencing of PCR products that were achieved by using primers that were at least 200 bp up and downstream located to the primers used to construct the flanking regions for the deletion plasmid. This deletion method has been successfully used to produce single gene deletion mutants (Ellen et al., 2011; Meyer et al., 2011), and also double and triple deletion mutants as the marker cassette can be reused as often as wanted (Henche et al., 2012; Lassak et al., 2012). These mutants have also been successfully complemented by expression vectors.

To improve the deletion mutant procedure we introduced the reporter gene lacS that encodes a β-galactosidase from S.
solfataricus into pSVA406 yielding the deletion mutant plasmid pSVA407 (Figure 2B). Cells expressing lacS turn blue when sprayed with substrates like X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside), therefore integrants could be easily detected on the first selection plate. Figure 3 shows an example of such a plate, also demonstrating that most of the growing colonies were lacS positives.

Furthermore, we developed a third method which yields markerless deletion mutants via double crossover events. The deletion mutant plasmid pSVA431 consists of the pyrEFSSO marker cassette together with lacS reporter cassette that are flanked by two different multiple cloning sites. The first multiple cloning site harbors an approximately 500 bp part of the gene of interest and in the second multiple cloning site the up and downstream flanking regions were placed consecutively (Figure 2C). This cassette was transformed as a linear fragment into MW001. As the absence of uracil on the first selection plates selects for colonies containing the pyrEFSSO cassette, cells will only be able to form colonies when they integrate the linear fragment with double crossover via the matching part of the gene and the downstream flanking part of the gene (Figure 2C). Therefore all obtained colonies on first selection plates by means of this method turned blue after X-gal spraying (data not shown). When these strains were streaked on second selection plates containing 5-FOA and uracil, only the cells that loop the pyrEFSSO cassette and the lacS gene via a single crossover of the upstream flanking region out, can form colonies. Consequently, all white colonies that grow on second selection plates are deletion mutants (Figure 3D, right half of the gel). One further advantage of this method is that the genotype of integrants still harbors the functional full length gene of interest. If via this technique no colonies can be obtained on second selection plates or if the obtained colonies stay blue after X-gal spraying (which would mean that point mutations in the pyrEFSSO cassette occurred), the target gene can be considered essential for growth under these conditions.

**ECTOPIC INTEGRATION OF FOREIGN DNA INTO THE S. ACIDOCALDARIUS MUTANT**

To demonstrate that we can introduce ectopically DNA sequences into the S. acidocaldarius genome, we introduced the glucose transporter of *S. solfataricus* (so2847-so2850; glcS, T, U, and V, respectively, see Figure 4A; Albers et al., 1999) into the upsE locus (saci1494) of MW001. To this end plasmid pSVA445 was constructed containing the whole glucose transporter under the control of the maltose inducible promoter of *malE* (saci1665; Berkner et al., 2010) and the flanking regions that matched the sequence of *upsE*. pSVA445 was methylated and transformed linearized into MW001. Cells were plated on first selection plates. Colonies that had integrated pSVA445 were streaked on second selection plates. Obtained colonies were screened for positive clones that would have integrated the glucose transporter cassette of 4700 bp (glc transporter with promoter; 4450 bp only transporter; 6220 bp glc gene transporter together with pyrEF marker; data not shown) under the control of the maltose promoter. Successful production of the glucose transporter from the *S. acidocaldarius* genome was tested by analyzing wild type cells and the insertion mutant by western blot analysis using antibodies against GlcV, encoded by the last gene of the glucose transporter operon. As shown in Figure 4B,
GlcV was detected in the membrane fraction of the insertion mutant, showing that the mal promoter upstream of glcS was sufficient to drive expression of the whole ABC transporter operon.

**GENOMICALLY TAGGING OF PROTEINS**

To enable tag based affinity purification of homologously produced proteins and identification of native protein complexes, we used the “pSVA406” method (Figure 2A) to add tags to genes of interest in the genomic context that encoded for, e.g., His- or One-Strep-tags. Two examples will be discussed here, the purification of AglB (SacI_1274), a multiple spanning membrane protein, and of SacI_1210, a cytosolic protein. AglB was tagged in the genome by constructing plasmid pSVA1252 that contained the C-terminal part of aglB in which the stop codon was exchanged by a One-Strep-tag coding sequence and the downstream part of aglB. Using the pop in/pop out method the tag was inserted into the genome of MW001 and correct insertion was verified by sequencing MW001 sacI1274::sacI1274-One-Strep. This strain was grown and cells processed as described in Section “Materials and Methods.” Solubilized membrane proteins were subjected to Strep-tag affinity chromatography and the samples were analyzed by SDS-PAGE and immunoblotted with Streptactin antibodies (Figures 5A,B).

The purified AglB-One-Strep appeared fuzzy, most probably as it is glycosylated, but its identity was confirmed by mass spectrometry. Two other proteins were co-purified that were identified as SacI_0260 and SacI_0262, subunits of the pyruvate carboxylase. These two proteins seem to be biotinylated proteins that bind to the chromatography material. The same proteins have been observed during Strep affinity purifications from cell extracts from *S. solfataricus* (Albers et al., 2006). Binding of these two proteins can be avoided by the addition of avidin to the cytoplasmic fraction.

In contrast to AglB, SacI_1210 was tagged with a tandem tag containing a Strep and a 6xHis-tag. This enabled us to test purification by either Strep- or His affinity chromatography using the same strain. Strep affinity chromatography of SacI_1210-Strep/His resulted in the same two contamination bands seen also in the AglB purification and no detectable SacI_1210 (data not shown). His-tag affinity chromatography resulted in highly pure SacI_1210-Strep/His in one step (Figures 5C,D). However, in contrast to *S. solfataricus* *S. acidocaldarius* exhibits a 14.7 kDa hypothetical protein, SacI_0386, that contains a natural stretch of six histidine residues at the C-terminus and therefore this protein co-purifies during His affinity chromatography (not shown in this blot). Concluding, His-tag affinity chromatography seems to be better
suited for isolation of proteins from *S. acidocaldarius* as Strep-tag isolation leads to the co-purification of two very prominent contaminating proteins of around 50 kD.

**INDUCIBLE PROMOTERS**

Several promoters have already been tested for efficient production of proteins in *S. acidocaldarius* of which the maltose inducible promoter of the maltose binding protein (*saci1165*) turned out to be the most reliable one (Berkner et al., 2010; Meyer et al., 2011; Henche et al., 2012; Lassak et al., 2012). Still this promoter exhibits quite some basic expression even under non-induced conditions. As a possible alternative we examined the copper inducible promoter of the copper inducible copper resistance cassette (*saci0874–saci0872*) that encodes CopT, the regulator of the operon, CopM, a metallochaperon, and CopA, a P-type copper ATPase (Ettema et al., 2006; Figure 6A). On the one hand CopT was described to be a negative regulator (Ettema et al., 2006) and on the other hand in a recent study (Villafane et al., 2011) CopT (called CopR in that publication) was described to be an activator of the *cop* operon. Therefore, the *mal* promoter of pCmalLacS was replaced...
Figure 6 | Comparison of copper inducible promoter and maltose inducible promoter for expression in MW001. (A) Operon structure of the copper resistance cluster of \textit{S. acidocaldarius}. Black line indicates the part of the operon that was included into pSVA1673. (B) Schematic representation of the plasmids pCMallacS and pSVA1673 used in (C,D). (C) Direct comparison of LacS expression under control of Pmal with the \textit{copMA} promoter region together with \textit{copT} (highlighted in Figure 6A by a gray line, plasmids are schematically shown in Figure 6B). Hence, LacS expression would be driven by the \textit{copMA} promoter (Figure 6A). The regulatory gene \textit{copT} was included to ensure correct repression/activation of the \textit{copMA} promoter.

The pSVA1673 was transformed into MW001 which was grown in medium supplemented with a minimal trace element solution to ensure very low levels of copper. The basic levels of expression were half of that obtained by the \textit{mal} promoter under comparable conditions (Figure 6C). However, the induction of the promoter was only 1.4 fold after 4 h at a copper concentration of 1 mM whereas the maltose promoter was induced up to 1.8 fold. The copper promoter could be induced gradually by varying the amount of copper present in the medium (Figure 6D). Copper concentrations higher than 2 mM were not suitable because of cell lysis.

**DISCUSSION**

Detailed \textit{in vivo} studies were long hampered in archaea as the development of genetic systems was very often obstructed by the absence of plasmids or selection markers. However, in the last few years enormous progress has been made and for most of the archaeal model systems genetic tools now exist (Leigh et al., 2011). Especially in the Sulfolobales genetic systems have been developed. For \textit{S. solfataricus} and \textit{S. islandicus}, respectively (Worthington et al., 2003; Albers and Driessen, 2008; She et al., 2009) these are proven to be successful as work based on them has been published.

Here we present a complete and versatile genetic system for \textit{S. acidocaldarius}. Our system advances the system developed by Grogan that is based on marker insertion into the target gene (Sakofsky et al., 2011), to a system in which the selection marker can be reused. This therefore enables us to construct multiple allele mutants (single, double, and triple), specifically mutate genes in the genomic background or tag proteins genomically and complement obtained mutants by using an expression vector. In this manuscript we showed exemplary three different methods that can be employed to obtain markerless deletion mutants. We have used the same method for different projects in which we deleted target genes, constructed single, double, and triple mutants and complemented these using the pCmal based vector system (Berkner et al., 2011).
We have also experienced that some genes could not be deleted by the pop in/pop out method, but we were able to obtain a deletion mutant by the PCR based insertion method (Sakofsky et al., 2011). This might be due to regulatory elements that might be disturbed by the insertion method. Therefore both methods can and should be used complementary.

We demonstrated that we can genomically tag genes in situ which will facilitate protein complex isolation and testing protein truncates or point mutations in their original genomic neighborhood. For affinity purification of such tagged proteins, His-tag affinity chromatography in our experience yielded better purification results, which could of course be due to a better accessible tag as the His-tag was located at the outmost C-terminus of the protein whereas the Strept-tag might have been occluded. However, His-tag affinity purification only yields one co purifying impurity. In the future other tags as, e.g., FLAG and HA tag will be tested to expand the genetic tool box.

Moreover, we introduced a ~5 kb ABC transporter operon from *S. solfataricus* into the *S. acidocaldarius* genome and could demonstrate that the last gene was transcribed and translated correctly. This opens the option of integrating large sequences into the *S. acidocaldarius* genome and therefore streamlines its application in synthetic biology approaches.

For the expression in *S. acidocaldarius* we also tested the copper inducible *copMA* promoter, which showed decreased basic expression levels in comparison to the maltose inducible promoter. Therefore the *copMA* promoter might be useful for production of toxic proteins in *S. acidocaldarius*, especially regarding that the Strept-tag might have been occluded. However, His-tag affinity purification only yields one co purifying impurity. In the future other tags as, e.g., FLAG and HA tag will be tested to expand the genetic tool box.

Concluding, we present here a very robust and versatile genetic tool box for *S. acidocaldarius* that has already proven to be widely applicable and very useful in delineating gene functions in this organism. We are currently working on the development of additional selective markers and the optimization of the expression vector.

**MATERIALS AND METHODS**

**STRAINS AND GROWTH CONDITIONS**

*Sulfolobus acidocaldarius* DSM 639, MW001, and all constructed deletion mutants were aerobically grown in Brock media (Brock et al., 1972) with a pH of 3 at 76°C. The media were supplemented with 0.1% (w/v) tryptone or with 0.1% (w/v) N-Z-Amine and 0.2% dextrine. The growth of the cells was monitored by measurement of the optical density at 600 nm.

For pouring *Sulfolobus* plates a two times concentrated Brock media was supplemented with 6 mM CaCl₂ and 20 mM MgCl₂. For first selection plates 0.2% N-Z-Amine (Fluka) and 0.4% dextrin, for second selection plates 0.2% tryptone, 0.4% dextrin, 200 μg/ml 5-FOA, and 20 μg/ml uracil was added to the two times concentrated solution and prewarmed to 75°C. This solution was mixed with an equal volume of fresh boiling 1.4% Gelrite solution (Carl Roth, Karlsruhe, Germany) and poured in 40 ml portions into petri dishes (150 × 20 mm, Sarstedt, Nümbrecht, Germany).

**CONSTRUCTION OF DELETION MUTANT PLASMIDS**

To obtain an uracil auxotrophic mutant of *S. acidocaldarius* DSM 639 deletion plasmid pSVA402 was constructed. To this end the upstream and downstream regions of *pyrE* (saci1597) were amplified using primers 390/916 and 917/920, respectively. An overlap extension PCR was performed with both PCR products and the resulting *pyrE* deletion fragment was blunt end cloned into pGEM-T Easy (Promega, Mannheim, Germany) yielding pSV402.

For the construction of the deletion plasmid pSV406 the *pyrEF* (sso0615–sso0616) cassette of *S. solfataricus* together with its own promoter region was amplified using primers 938 and 939 (primer sequences available in the Appendix). This marker cassette was cloned into the *MluI* *NsiI* site of the recirculated pGEM-T Easy vector. Primer 938 contained additional *BamHI* and *AvrII* sites to expand the multiple cloning site of the resulting plasmid.

For construction of the deletion plasmid pSV407 the *lacS* (sso3019) gene of *S. solfataricus*, encoding a β-galactosidase, was amplified with primers 933 and 940 and fused to the maltose inducible promoter of the maltose binding protein (*saci1165*) of *S. acidocaldarius*, which was amplified with primers 937 and 932, via overlap extension PCR. The resulting reporter cassette was cloned into the *NsiI* site of pSV407. A correctly oriented clone was identified by restriction analysis.

The deletion plasmid pSV431 was constructed by amplifying the *pyrEF* (sso0615–sso0616) and *lacS* (sso3019) cassette of *S. solfataricus* from pSV407 using primer 939 and 1072. The resulting marker reporter cassette was cloned into the pGEM-T Easy vector. The pSV431 exhibited two multiple cloning sites up and downstream to the marker reporter cassette, respectively.

For the construction of the *upsE* (saci1949) deletion plasmids via single crossover event the upstream region of *upsE* was amplified with the primers 2010 and 2011 and the downstream region was amplified with the primers 2012 and 2013. With both PCR products an overlap extension PCR was performed and cloned into the *Apal BamHI* sites of pSV406 and pSV407 leading to the plasmids pSV1804 and pSV447. For the construction of the *upsE* deletion plasmid via double crossover the *upsE* gene was amplified with primers 532 and 639. The resulting PCR product was cloned into the *BamHI* and *PstI* site of the MCSII of pSV431. A PCR was performed on pSV1804 with the outer primers 2264 and 2265 to introduce different restriction sites and cloned into the *Neol* and *KpnI* site of MCSII.

For the construction of the ectopic integration plasmid of the *S. solfataricus* *glcV* (sso1165) upstream region was amplified using primers 2256 and 2257. The promoter region together with the signal sequence of the maltose binding protein (*saci1165*) was amplified employing primers 2258 and 986. *glcS* (sso2847) without signal sequence was amplified using the primers 987 and 988. *glcT* (sso2848), *glcU* (sso2849), and *glcV* (sso2850) were amplified in a single PCR with the primers 989 and 2259. The *pyrEF* reporter cassette of *S. solfataricus* was amplified with the primers 2260 and 2261 and for the amplification of the *upsE* downstream region the primers 2262 and 2263 were used. All six PCR products were mixed together to perform an overlap extension PCR. The resulting PCR product was blunt end cloned into pGEM-T Easy following the standard protocol yielding pSV445.
To simplify the purification of saci1274 by affinity chromatography, a One-Strep-tag was fused to this gene in the genome. For the construction of the insertion plasmid 900–1000 bp fragments of the up- and downstream regions of saci1274 were PCR amplified. For the upstream region the forward primer introduced an Apal restriction site at the 5′ end, whereas the reversed primer was designed to incorporate the One-Strep-tag sequence in front of the Stop codon (primers 1818 and 1819, respectively). For the downstream fragment the forward primer 1820 was designed to incorporate the complementary strand of the One-Strep-Tag, while the reverse primer 1821 contained a BamHI restriction site. Both fragments were fused via an overlapping PCR and the amplified PCR fragment was digested with Apal and BamHI and ligated into plasmid pSVA407 yielding plasmid pSVA1224. The correct sequence was confirmed by sequencing. To increase the accessibility of the One-Strep-tag a longer linker sequence was added to the up- and downstream regions of the gene together with the tag. The overlap sequence was confirmed by sequencing. To increase the accessibility of the One-Strep-tag a longer linker sequence was added to the tag. For the downstream region amplification with primer 1821 contained a PstI restriction site at the 5′ end, whereas the reversed primer 1822 contained a BamHI restriction site.

S. ACIDOCALDARIUS

Preparation of competent MW001 cells

Sulfolobus acidocaldarius MW001 were grown in 50 ml Brock media supplemented with 0.1% NZ-Amine, 0.2% sucrose, and 20 μg/ml uracil and adjusted to pH 3 with sulfuric acid. When the culture reached an OD600 nm of 0.5–0.7 (exponential growth phase) an aliquot was transferred to 400 ml fresh medium and harvested at an OD600 nm of 0.2–0.3. The culture was cooled down on ice, then centrifuged for 15 min at 4000 g and washed twice with 250 ml ice cold 20 mM sucrose. The pellet was resuspended with ice cold 20 mM sucrose to a theoretical final OD600 nm of 10 and aliquotted in 50 μl portions. The aliquots were directly used for transformation or frozen at −80°C without using liquid nitrogen for storage.

Transformation of plasmids into S. acidocaldarius

Prior to transformation into S. acidocaldarius, suicide- and shuttle plasmids were methylated to prevent restriction by the SstII restriction enzyme. For that purpose E. coli ER1821 (New England Biolabs) bearing the additional plasmid pM.EsBC4I (New England Biolabs, Frankfurt am Main, Germany) was transformed with plasmid DNAs.

Methylated deletion plasmids were electroporated in electro-competent wild type cells MW001 using a Gene Pulser Xcell (BioRad, München, Germany) with a constant time protocol with input parameters 1.5 kV, 25 μF, 600 Ω in 1 mm cuvettes. Before plating on uracil lacking and NZ-Amine containing plates, cells were regenerated for 30 min at 75°C in two-fold recovery solution (1% sucrose, 20 mM β-alanine, 1.5 mM malate buffer, pH 4.5, 10 mM MgSO4). The plates were sealed in plastic bags to avoid drying-out and incubated for around seven days at 75°C.

 Colonies of S. acidocaldarius

Single colonies appearing on plates were analyzed by colony PCR. To that end, single colonies were picked and lysed in 30 μl 0.2 M NaOH and the solution neutralized with 70 μl 0.2 M Tris pH 7.8. To amplify the genomic region of interest 0.5 μl lysate was used in 30 μl PCR reactions using Phusion High-Fidelity polymerase with Phusion HF buffer and monitored on an agarose gel.

Blue–white screening

Integration of pSVA407 or pSVA431 constructs could be visualized by X-Gal spraying of cells using a 25 mg/ml X-Gal stock solution in DMF diluted 1:5 with water. The 5 mg/ml X-Gal solution was sprayed on plates when single colonies appeared after around one week and the plates were directly put back into the 75°C incubator for 30 min. Transformants on first selection plates and point mutants on second selection plates turned blue while deletion mutants on second selection plates stayed white.

Purification of Saci1274-One-Strep

Ten liter culture of the strain MW001 Saci1274::Saci1274-One-Strep was grown in Brock medium until an OD of 0.8. Cells were harvested by centrifugation (3000 g, 4°C; 20 min). The cell pellet was resuspended in 40 ml buffer A (100 mM NaCl, 100 mM Tris HCl, 1 mM EDTA, pH 8) and lysed by a 20 min sonication with an intensity of 60% and an interval 20 s (Bandelin Sonopuls). Unbroken cells were removed by a low spin centrifugation 3000 g at 4°C for 20 min. The supernatant was centrifuged at 120,000 g at 4°C for 45 min and the membrane pellet suspended in 12 ml of buffer A. 6 ml of the membrane fraction solubilized at 42°C under shaking condition in 30 ml Buffer S (2% n-dodecyl beta-D-maltoside (DDM), 100 mM NaCl, 100 mM Tris HCl, 1 mM EDTA, pH 8) supplemented with PMSF. Undissolved membranes were pelleted by an ultracentrifugation (120,000 g; 4°C for 45 min). The supernatant was added twice to a 0.8 ml STREP-Tag column (Strep-Tactin® Superflow®, IBA, Goettingen, Germany). Before loading the column was equilibrated with 10× column volume of buffer E (0.05% DDM, 100 mM NaCl, 100 mM Tris HCl, 1 mM EDTA, pH 8) and after the loading of the supernatant washed with 15× column volumes of the same buffer. The fusion protein was eluted with the buffer E containing 1 mM desthiobiotin.

Purification of Saci-Strep/His was performed with the difference that the cytoplasm was used for purification. For His affinity chromatography His-SELECT material from Sigma was used.
WESTERN BLOT ANALYSIS

From each purification step 30 μl samples were loaded on a 11% SDS-PAGE and run at 100 V. Transfer to a PVDF membrane and blotting were performed as commonly done. The generated chemiluminescence of the Precision StreptTactin-AP Conjugate antibody (Biorad) or His-AP (Abcam, Cambridge, UK) was measured in a Fujifilm LAS-4000 Luminescent image analyzer (Fujifilm, Duesseldorf, Germany).

PROMOTER ACTIVITY ASSAY

For the promoter activity assay pSVA1673 and pCmalLacS were transformed into MW001. Single colonies containing pCmalLacs were inoculated in Brock Medium with 0.1% NZ-Amine and 0.2% sucrose whereas pSVA1673 containing cells were grown in Brock medium supplemented with 0.2% maltose and 0.2% xylose. Moreover, for these cells the trace element solution only contained Na2B4O7 and MnCl2. For induction, 0.4% maltose or 1 mM CuSO4 were added to the medium, respectively.

Promoter activity was analysed by measuring the cleavage of o-nitrophenyl-β-D-galactopyranosid (ONPG) by LacS of *S. solfataricus* as described in Lassak et al. (2012). All given strains were assayed in biological as well as technical triplicates. The production of ONP was measured at 410 nm over a period of 4 h at 42˚C using an Infinite 200 luminometer (Tecan, Maennedorf, Switzerland). Miller units were calculated as described previously (Lassak et al., 2012). With respect to the temperature optimum of LacS a conversion factor FT = 7 was determined and included in the final equation.

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## APPENDIX

### Table A1 | Primers used in this study.

| Primer | Sequence (5’→3’) | Purpose |
|--------|------------------|---------|
| **PRIMERS FOR pSVA402** | | |
| 390 | GGTACCCCATGCTAAAGGAGCTTTCCTG | ΔpyrE upstream fw |
| 916 | GCCAATATTACCTGCAATTTCCACTTCCCATATCTGATGAGG | ΔpyrE upstream rev ol |
| 917 | GCCGACCATGGGCTGAGTTGACATCC | ΔpyrE downstream fw ol |
| 920 | AGGACCTGAAACAGGCCC | ΔpyrE downstream rev |
| 914 | GCCTCCTGCTATTAGGGTGATGACCAT | ΔpyrE check primer fw |
| 915 | CTAGCTTCTTACCTATATTTACAC | ΔpyrE check primer rev |
| **PRIMERS FOR pSVA406, pSVA407, and pSVA431** | | |
| 938 | CCCTCAGATGTCGCTGTTCACTC | pyEF cassette SSO rev NsiI |
| 939 | CCCTGATGTCGCTGTTCACTC | pyEF cassette SSO fw MluI, BamHI, AvrII |
| 937 | CCCTCAGATGTCGCTGTTCACTC | Saci mbp promoter fw NsiI |
| 932 | GCCTTCTGCTATTAGGGTGATGACCAT | Saci mbp promoter rev ol to Sso lacS |
| 933 | GCCTTCTGCTATTAGGGTGATGACCAT | Sso lacS fw ol to Saci mbp promoter |
| 940 | CCCTCAGATGTCGCTGTTCACTC | lacS cassette SSO reverse NsiI |
| 938 | CCCTCAGATGTCGCTGTTCACTC | ΔupsE upstream SstI |
| 939 | CCCTCAGATGTCGCTGTTCACTC | ΔupsE downstream SstI |
| 940 | CCCTCAGATGTCGCTGTTCACTC | ΔupsE check primer fw |
| 941 | CCCTCAGATGTCGCTGTTCACTC | ΔupsE check primer rev |
| **PRIMERS FOR ΔupsE WITH DIFFERENT METHODS** | | |
| 2010 | GTAGGCGCGCTGTATATAAGGATCACTATATCATCG | ΔupsE upstream Apal |
| 2011 | CTAATATTTTCCAACCTAGGAAGATGATATTGGA | ΔupsE upstream rev ol |
| 2012 | CCTTCTGCTATTAGGGTGATGACCAT | ΔupsE downstream rev ol |
| 2013 | GTGACGACTTAATCAATTCAATTCACTGATGAGG | ΔupsE downstream rev BamHI |
| 532 | GGGCGCGATGTCGCTGTTCACTC | upsE fw BamHI |
| 639 | GGGCGCGATGTCGCTGTTCACTC | upsE rev PstI |
| 2264 | GTAGGCGCGCTGTATATAAGGATCACTATATCATCG | ΔupsE upstream KpnI |
| 2265 | GTAGGCGCGCTGTATATAAGGATCACTATATCATCG | ΔupsE downstream Ncol |
| 2073 | CTAGCTTCTTCCATATATTTACC | ΔupsE check primer fw |
| 2074 | CTAGCTTCTTCCATATATTTACC | ΔupsE check primer rev |
| **PRIMERS FOR ECOTROPIC INTEGRATION OF GLUCOSE TRANSPORTER SYSTEM** | | |
| 2256 | ATGAGCGCGCTGTATATAAGGATCACTATATCATCG | upsE upstream fw + KpnI site |
| 2257 | ATGAGCGCGCTGTATATAAGGATCACTATATCATCG | upsE upstream rev ol to Saci mbp promoter |
| 2258 | ATGAGCGCGCTGTATATAAGGATCACTATATCATCG | Saci mbp promoter rev ol to upsE upstream |
| 986 | CTATATACCTATATATCATATATCATATATCATAC | Saci mbp promoter + ss rev ol to Sso glcS |
| 987 | CCTTCTGCTATTAGGGTGATGACCAT | Sso glcS fw ol to Saci mbp promoter + ss |
| 988 | CCTTCTGCTATTAGGGTGATGACCAT | Sso glcS rev ol to Sso glcT + glcV |
| 989 | CTATATACCTATATATCATATATCATATATCATAC | Sso glcT rev ol to Sso pyrEF cassette |
| 2259 | ATGAGCGCGCGCTGTATATAAGGATCACTATATCATCG | Sso pyrEF cassette framol to Sso glcV |
| 2260 | ATGAGCGCGCGCTGTATATAAGGATCACTATATCATCG | upsE downstream rev ol to Sso pyrEF cassette |
| 2261 | ATGAGCGCGCGCTGTATATAAGGATCACTATATCATCG | upsE downstream rev + KpnI site |
| **PRIMERS FOR IN GENOME TAGGING** | | |
| 1818 | CTCATCGCTGCCCTTTACACACATGATGACCCCTACAC | SacI1274 rev Apal |
| 1819 | TCTATCGCTGCCCTTTACACACATGATGACCCCTACAC | SacI1274 rev with Strep-tag ol to SacI1274 downstream |
| 1820 | GTGATATACCTATATATCATATATCATATATCATAC | SacI1274 downstream rev ol to SacI1274 with Strep-tag |
| 1821 | GTGATATACCTATATATCATATATCATATATCATAC | SacI1274 downstream rev BamHI |
| 1583 | GGGCGCGATGTCGCTGTTCACTC | SacI1210 rev |
| 3106 | GGGCGCGATGTCGCTGTTCACTC | SacI1210 rev with His-tag |
| 3107 | GGGCGCGATGTCGCTGTTCACTC | SacI1210 downstream rev ol to SacI1210 with His-tag |
| 1578 | GGGCGCGATGTCGCTGTTCACTC | SacI1210 downstream rev BamHI |
| **PRIMERS FOR COPPER PROMOTER** | | |
| 3428 | CCCGGGATGTCGCTGTTCACTC | copT rev Sacl |
| 3429 | CCCGGGATGTCGCTGTTCACTC | copT fw copMA promoter Ncol |

*Underline indicates restriction sites.*
Table A2 | Strains and plasmids used in this study.

| Strains | Genotype | Source/reference |
|---------|----------|-----------------|
| **STRAINS** | | |
| DH5α | *Escherichia coli* K-12 cloning strain | Gibco |
| DSM639 | Wild type *Sulfolobus acidocaldarius* | DSMZ |
| MW001 | DSM639 ΔpyrE (Saci1597; Δ91–412 bp) | This work |
| MW109 | MW001 ΔupsE (Saci1494; 1–1410 bp) | This work |
| MW363 | MW001 Saci1210::UpsESaci1494 6×His | This work |
| MW095 | MW001 aglB::aglBOne-Strep | This work |
| MW009 | MW001 ΔupsE::PmbpSaci SSmbpSaci glcSSO glcTSSO glcUSSO glcVSSO | This work |
| **PLASMIDS** | | |
| pGEM-T Easy | | Promega |
| pSVA402 | In-frame deletion of pyrESO cloned into pGEM-T Easy | This work |
| pSVA406 | Gene targeting plasmid, pGEM-T Easy backbone, pyrEFSSO cassette; single crossover method | This work |
| pSVA407 | Gene targeting plasmid, pGEM-T Easy backbone, pyrEFSSO and lacSSO cassette; single crossover method | This work |
| pSVA431 | Gene targeting plasmid, pGEM-T Easy backbone, pyrEFSSO and lacSSO cassette; double crossover method | This work |
| pSVA1804 | In-frame deletion of upsE cloned into pSVA406 with Apal, BamHI | This work |
| pSVA447 | In-frame deletion of upsE cloned into pSVA407 with Apal, BamHI | This work |
| pSVA449 | In-frame deletion of upsE cloned into pSVA431 with Ncol, KpnI and the gen region with BamHI, PstI | This work |
| pSVA445 | Inserting the glucose transporter system of *S. solfataricus* together with the pyrEFSSO cassette into the upsE Sarc site | This work |
| pSVA1224 | Genomically Strep-tag of aglB cloned into pSVA407 with Apal, BamHI | This work |
| pSVA1252 | Insertion of One-Strep linker sequence into pSVA1224 with AccI, PstI | This work |
| pSVA1097 | Genomically Strep-6Xhis-tag of Saci1210 cloned into pSVA406 with Ncol, BamHI | This work |
| pCMaLaS | pRN1 based shuttle vector with lacSSO reporter gene | Berkner et al. (2010) |
| pSVA1673 | copA promoter replacing mbp promoter, cloned into pCMaLaS with SacII, Ncol | This work |

5’ GTATAAATTGTTAATATCATATTATAATTAACTAGTTGCAAGCGGTTGAAGTAGCTTTTCATTGTCGAGTAGTACTCCTCATCCTCATCCTGAGAAAAGAGAAGGTATTGATCGAGGGTTTCAGGTGGAGGTCTTCTGAGATCTCACAATTGAGAAGTACCTAACCCTTTTTATAT TCGAGTATATTATATTTATCGACGCGAGAACACGAGGTATTGATGAATGCTTTC TACACCACAAGATACGACATCTTTTATCGCATATAAGATAGTACCTATTACATTCTTCTTATTG TACTTCTTCAACACACCGAAACGTACGTTCATGGTCATGCTTTATCCTCTTAATCAAGTTGAT TCGTCTGTAAGAAGATTTGATACCAACTCTGACTGTAACATCATGACTCACTCCTACGGG AGCACCATTGTATATTTTATCTTACTACGATCGTACATCACTTTTTTACACTGCAG 3’

FIGURE A1 | Codon optimized One-STrEP-tag sequence.