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Appl. Environ. Microbiol. **2006, 72(1):102. DOI: 10.1128/AEM.72.1.102-111.2006.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2006, p. 102–111 Vol. 72, No. 1
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.1.102–111.2006

Production of Recombinant and Tagged Proteins in the Hyperthermophilic Archaeon *Sulfolobus solfataricus*

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Received 10 July 2005/Accepted 22 September 2005

Many systems are available for the production of recombinant proteins in bacterial and eukaryotic model organisms, which allow us to study proteins in their native hosts and to identify protein-protein interaction partners. In contrast, only a few transformation systems have been developed for archaea, and no system for high-level gene expression existed for hyperthermophilic organisms. Recently, a virus-based shuttle vector with a reporter gene was developed for the crenarchaeote *Sulfolobus solfataricus*, a model organism of hyperthermophilic archaea that grows optimally at 80°C (M. Jonuscheit, E. Martusewitsch, K. M. Stedman, and C. Schleper, Mol. Microbiol. 48:1241–1252, 2003). Here we have refined this system for high-level gene expression in *S. solfataricus* with the help of two different promoters, the heat-inducible promoter of the major chaperonin, thermophilic factor 55, and the arabinose-inducible promoter of the arabinose-binding protein AraS. Functional expression of heterologous and homologous genes was demonstrated, including production of the cytoplasmic sulfur oxygenase reductase from *Acidianus ambivalens*, an Fe-S protein of the ABC class from *S. solfataricus*, and two membrane-associated ATPases potentially involved in the secretion of proteins. Single-step purification of the proteins was obtained via fused His or Strep tags. To our knowledge, these are the first examples of the application of an expression vector system to produce large amounts of recombinant and also tagged proteins in a hyperthermophilic archaeon.

The homologous and heterologous expression of genes is a prerequisite for most biochemical studies of protein function. A vast variety of systems have been developed for protein production in members of the *Bacteria* and *Eukarya*, using numerous combinations of vector and promoter systems. Members of the *Archaea*, the third domain of life, are much less amenable to genetic manipulation. Transformation tools for the production of recombinant proteins exist for only a few species (for a review, see reference 2). A shuttle vector has been described for the expression of bacterial and methanococcal genes in *Methanococcus maripaludis* (12). Heterologous expression of bacterial and eukaryotic genes as well as of homologous genes has been achieved in the genetically most accessible archaea, the mesophilic, salt-dependent *Halobacterium* spp. (14, 17, 33). However, no such system has existed so far for thermophilic or hyperthermophilic archaea. Mesophilic hosts, in particular *Escherichia coli*, have been used to produce thermostable proteins for biochemical characterization and crystallographic studies (e.g., see references 22, 23, 29, and 34). However, a considerable number of proteins of hyperthermophiles fold into their native state only under natural conditions of high temperature or in the presence of their native cofactors. Furthermore, the production of recombinant and tagged proteins in native thermophilic hosts allows the identification of associated factors or even larger protein complexes.

The crenarchaeote *Sulfolobus solfataricus* has developed into an important model organism for molecular and biochemical studies of hyperthermophilic archaea. It grows optimally at 80°C and pH 3 under aerobic and heterotrophic conditions. Since many studies on the transcription, translation, and replication of this extremophile have been performed in vitro (4, 5, 10, 40), it is highly desirable to develop genetic tools for in vivo studies and for high-level production of proteins in this organism. Initial transformation systems and selectable markers have been established for *Sulfolobus solfataricus* in some laboratories (3, 6, 7, 9, 16, 41). We have recently developed a reporter gene system based on the virus SSV1 as well as the selectable marker genes *pyrEF* for the complementation of uracil auxotrophic mutants. The latter genes allow stabilization of the propagation of the vector by growing transformants under selective conditions (16). Moreover, strong, heat-inducible expression of the reporter gene *lacS*, coding for β-galactosidase, was demonstrated when it was placed under the control of the promoter of the major heat shock chaperonin gene *tf55*α (16).

In this study, we have used and improved this vector system for the heterologous and homologous expression of genes in *S. solfataricus*. We have constructed a set of entry vectors and introduced a transcriptional terminator and a second inducible promoter. Our system allows for the high-level production of functional and tagged cytoplasmic and membrane-associated

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TABLE 1. Plasmids used for this study

Plasmid	Description	Purpose of construction	Reference or source
pMJ02	SSV1 in pUC18 with *lacS* under control of *tf55alpha* promoter	Promoter studies in *Sulfolobus* (heat shock induction)	16
pMJ03	*pyrEF* cassette inserted into pMJ02	Selectable marker added for stability	16
pMJ05	*pyrEF* cassette, *tf55alpha* promoter, *lacS* from pBR05 inserted into pMJ02, replacing *tf55alpha* and *lacS* from pMJ02	Destination vector for *Sulfolobus*	This study
pMJ11	Derivative of pMJ03, with NcoI site introduced to separate *tf55alpha* promoter from *lacS;* no peptide spacer	Promoter studies in *Sulfolobus* (heat shock induction)	S. Albers and M. Jonuscheit, unpublished
pBR-05	pBR322 containing *pyrEF* cassette and *lacS* under control of *tf55alpha* promoter, with BssHII site introduced (Fig. 1)	*E. coli* entry vector with heat shock promoter	This study
pASK5	pASK75 containing *sor* gene fused to C-terminal Strep tag	PCR template for amplification of Strep-tagged *sor*	37
pBR-sor	pBR05 containing *sor* gene fused to C-terminal Strep tag	*E. coli* donor vector for *sor*	This study
pMJ05-sor	*pyrEF* cassette, *tf55alpha* promoter, *sor* from pBR05-sor inserted into pMJ02, replacing *tf55alpha* and *lacS* from pMJ02	*sor* expression in *Sulfolobus*	This study
pSVA1	pBR05 with an ApaI restriction site downstream of the stop codon of *lacS*	*E. coli* entry vector with terminator sequence separated	This study
pSVA2	SSO2316-6his inserted into pSVA1 (Fig. 1)	*E. coli* donor vector for SSO2316	This study
pSVA5	pSVA1 containing the *lacS* gene under control of *araS* promoter (Fig. 1)	*E. coli* entry vector with *ara* promoter	This study
pSVA6	*tf55alpha* promoter, SSO2316 from pSVA2 inserted into pMJ05, replacing *tf55alpha* and *lacS* from pMJ05	SSO2316 expression in *Sulfolobus*	This study
pSVA9	*araS* promoter, *lacS* from pSVA5 inserted into pMJ05, replacing *tf55-alpha* promoter and *lacS* from pMJ05	Promoter studies in *Sulfolobus* (arabinose induction)	S. Albers, unpublished
pSVA14	SSO2680-10his inserted into pSVA5	*E. coli* donor vector for SSO2680	This study
pSVA15	*araS* promoter, SSO2680-10his from pSVA14 inserted into pMJ05, replacing *tf55alpha* and *lacS* from pMJ05	SSO2680 expression in *Sulfolobus*	This study
pSVA30	SSO0287-Strep-10his inserted into pSVA5	*E. coli* donor vector for SSO0287	This study
pSVA31	*araS* promoter, SSO0287-Strep-10his from pSVA30 inserted into pMJ05, replacing *tf55alpha* and *lacS* from pMJ05	SSO0287 expression in *Sulfolobus*	This study

proteins in *S. solfataricus*, enabling biochemical characterizations and in vivo studies of protein function.

MATERIALS AND METHODS

Strains and culture conditions. An *S. solfataricus pyrEF* mutant (PH1-16) (25) was grown at 80°C and pH 3 in Brock's medium with or without 10 μg ml^{-1} uracil and with 0.1% tryptone and 0.2% arabinose or as indicated. The optical densities of liquid cultures were monitored at 600 nm (OD_{600}).

Plasmid construction. The plasmids used for this study are listed in Table 1. For the construction of pBR05, the genes *pyrEF* and *lacS* as well as the promoter region of the Tf55α gene were amplified by using the primers *pyr*EF-f-AvrII (CGAAATCACCCTAGGGAATAATGC), *pyr*EF-r-NheI (GTGGTGCTAGC TTCCTCGTGTAG), *ptf*55-f-AvrII (GCATTATTCCCTAGGGTGATTTCG), *ptf*55-r-BssHII (GACTGGCGCGCCCATACCTCA), *lac*S-f-BssHII (TGAGGT ATGGGCGCGCCAGTC), and *lac*S-r-EagI (GCGGTGGCGGCCGGCAATC TAA), with pMJ03 (16) as the DNA template. Because of complementary overlaps of the primers, all PCR products were used as template DNAs in a second PCR using the flanking primers *pyr*EF-f-AvrII and *lac*S-r-EagI, thus generating a fragment of 3.6 kbp. The PCR product was purified (using a QIAGEN Gene-Clean kit), restricted with NheI and EagI, and ligated into pBR322 restricted with NheI and EagI. The *Sulfolobus-E. coli* shuttle vector pMJ05 was constructed by ligating the NheI/EagI fragment of pBR05 to the XbaI/EagI-restricted and dephosphorylated SSV1/pUC18 shuttle vector pMJ02 (16).

The *sor* gene of *Acidianus ambivalens*, including a 10-amino-acid-encoding Strep tag at the C terminus, was PCR amplified by using the primers SorN-Strep-BssHII (CTAGATAACGCGCGCAAAAAATGCCG) and SorC-Strep-EagI (C TTCACCGGCCGA GCTTATTAACC). The construct pASK5 (37), containing the *sor* gene fused to a C-terminal Strep tag, was used as the DNA template. The PCR product was restricted with BssHII and EagI and ligated into pBR05, which yielded vector pBR-sor. This plasmid was cut with NheI and EagI, and the *sor* gene with the promoter was ligated to XbaI/EagI-restricted and dephosphorylated pMJ02, yielding the *Sulfolobus* expression vector pMJ05-sor.

An additional restriction site (ApaI) was introduced into pBR05 such that other genes could be introduced by leaving the terminator from *lacS* in the vector. This was done with a quick mutagenesis kit I (Stratagene). A PCR was performed with the primer ApaIF (GCCATTAAGGCACTAAGGGCCCACT TTCTCAAGTCTC) and its complement ApaIR (containing an ApaI site), with pBR05 as the template, yielding pSVA1. This vector was used as a template to replace the *lacS* gene with SSO2316 (*flaI*). The latter was amplified by PCR from the *S. solfataricus* genomic DNA using the primer pair 2316F (CCCCCGCGC GCCAGTCATGAGCTTTATTGAAGATTAC) and 2316R (CCCCCCGGGC CCTT AATGATGATGATGATGATGAATATTCTTAG), thereby fusing a C-terminal six-His tag to the encoded product. The PCR product was cloned into the BssHII and ApaI sites of pSVA1 to yield pSVA2. For the construction of pSVA5, the promoter region 241 bp upstream of the *araS* gene (SSO3066) was amplified from *S. solfataricus* genomic DNA with the primer pair *ara*SF (CCC CCCCTAGGGCACCATATGTTTAGAGATG) and *ara*SR (CCCCCGAATTC GCCATGGTCTCGGGTACTTTTATGACCTAAC), containing a BlnI and an NcoI restriction site, respectively. In the same manner, the *lacS* gene was amplified with the primer pair *lac*SF (GGGGGCCATGGACTCATTTCCAAATA GCTTTAG) and *lac*SR (CCCCCCGGGCCCTTAGTGCCTTAATGGCTTT AC), containing an NcoI and an ApaI restriction site, respectively. The ATG codon in the NcoI site coincided with the start codon of *lacS*. Both PCR products were digested with NcoI and ligated. The resulting ligation product was digested

with BlnI and ApaI and inserted into pSVA1 to yield pSVA5, which contained the *lacS* gene under the control of the *araS* promoter.

SSO2680 and SSO0287 were amplified from genomic DNA by PCRs using the primer pairs SSO2680f/SSO2680r and SSO0287f/SSO0287r, respectively. This resulted in the introduction of NcoI and ApaI restriction sites in the flanking regions of these genes and the presence of a C-terminal 10-His tag in SSO2680 and a StrepII tag and 8-His tag at the C terminus of SSO0287. Both genes were cloned into pSVA5 using the NcoI/ApaI sites, yielding pSVA14 and pSVA30, which carried SSO2680 and SSO0287, respectively. To transfer the *araS* promoter together with the gene to be expressed into the virus-based vector, the BlnI/EagI inserts from pSVA14 and pSVA30 were ligated into pMJ05, resulting in plasmids pSVA15 and pSVA31, respectively.

Transformation of *S. solfataricus*. Electroporation of *S. solfataricus* PH1-16 and the isolation of single transformants were done as described previously (16, 31). Integration of the viral vector into the genome was confirmed by Southern analysis using standard procedures.

Expression and purification of Sor. Cells containing the pMJ05-sor construct were grown in 300 ml Brock's medium supplemented with 0.1% tryptone and 0.2% D-arabinose. After 3 days of cultivation, cells (1 g) were harvested by centrifugation and resuspended in 25 ml 10 mM Tris-HCl, pH 8.0. The cells were lysed by sonication, and after centrifugation to remove cell debris and the membrane fraction, the supernatant was used for Streptactin affinity chromatography. Twenty milliliters of supernatant was applied to a Streptactin Superflow column (10 ml; IBA, Göttingen, Germany) connected to a fast-performance liquid chromatography apparatus. Purification was performed according to the manufacturer's instructions. The peak fractions were pooled and concentrated first by ultrafiltration (Centrex UF-2 [cutoff, 30 kDa]; Schleicher & Schuell, Dassel, Germany) and subsequently with a Speed-Vac machine.

Expression of FlaI. Cells containing pSVA6 were inoculated into 50 ml Brock's medium supplemented with 0.1% tryptone and 0.2% arabinose. At an OD_{600} of 0.5, the cells were diluted into 400 ml of the same medium and grown to an OD_{600} of 0.8. Cultures were then shifted to 88°C for 24 to 48 h, and the cells were harvested by centrifugation and resuspended in 10 mM Tris-HCl, pH 8, and 100 mM NaCl. Lysis of the cells and the isolation of membranes were done as described below.

Expression and purification of SSO2680. Cells containing pSVA15 were inoculated into 50 ml Brock's medium containing only 0.1% tryptone. After 2 days of growth (OD_{600}, ~0.5), 10 ml of cells was transferred to 400 ml medium containing 0.1% tryptone and 0.2% arabinose to induce the expression of SSO2680. After 2 days of growth (OD_{600}, ~0.8), the cells were harvested and resuspended in buffer A (50 mM NaP_i, pH 8, 100 mM NaCl, and 5 mM imidazole). Lysis of the cells and the isolation of membranes were done as described below. Membranes were resuspended in buffer A and solubilized with 2% *n*-dodecyl-D-β-maltopyranoside (DDM) at a protein concentration of 4 mg/ml for 45 min at room temperature. Nonsolubilized protein was removed by high-speed centrifugation, and the supernatant containing the solubilized membrane protein was applied to a His-Select 1-ml column (Sigma) that was preequilibrated with buffer A and 0.05% DDM. The column was washed with 10 volumes of buffer A and 0.05% DDM and subsequently with 3 volumes of 50 mM NaP_i, pH 8, 100 mM NaCl, 30 mM imidazole, and 0.05% DDM. Bound protein was eluted with 1.5 column volumes of 50 mM NaP_i, pH 8, 100 mM NaCl, 250 mM imidazole, and 0.05% DDM. The protein was dialyzed overnight against 20 mM morpholineethanesulfonic acid, pH 6.5, 100 mM NaCl, and 10% glycerol and subsequently frozen in aliquots at −20°C.

Expression and purification of SSO0287. The growth conditions for cells containing pSVA31 were the same as those described for SSO2680. After 2 days of growth (OD_{600}, ~0.8), the cells were harvested and resuspended in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl. The cytoplasmic fraction was applied to a 1-ml His-Select affinity column, and further purification was performed as described for SSO2680, except that DDM was omitted from the buffers. Strep tag-mediated purification was performed with Streptactin columns (IBA Technology) according to the manufacturer's recommendations. The elution buffer contained 100 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, and 2.5 mM desthiobiotin. Gel filtration was performed on a Smart system (Pharmacia), using a Superdex TM 200 PC 3.2/30 column (Amersham Biosciences) with 20 mM Tris-HCl, pH 7.4, and 100 mM NaCl.

Fractionation of cells. Cells were broken by sonication in a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, England) (10 cycles of 15 s on and 45 s off), and cell debris was removed by low-speed centrifugation. Membranes were collected by ultracentrifugation for 30 min at 100,000 × *g* at 4°C.

Activity assays. β-Galactosidase activity was determined as described previously (16). β-Galactosidase activity staining was performed by incubating native gels in a buffer containing 5 mM ONPG (*o*-nitrophenyl-β-D-galactopyranoside) for 30 min at 75°C. ATPase activity assays were performed with a colorimetric assay (24) as described previously (39).

The Sor activity assay was performed aerobically as previously described (19). Protein aliquots were incubated in 1 ml assay buffer (70 mM Tris-HCl, pH 7.2, 0.1% Tween 20, 2% [wt/vol] sulfur [dispersed by sonication]) at 85°C for different time intervals (0, 4, 8, 12, and 16 min), and the reactions were stopped by cooling on ice. After centrifugation, the products thiosulfate and hydrogen sulfide were examined colorimetrically in the supernatant and quantitated using the respective calibration curves. The reaction velocity was calculated from the linear increase in product concentrations. Since sulfite rapidly reacts to form thiosulfate at 85°C (19), the amounts of thiosulfate and sulfide were quantitated. One unit (U) of enzyme activity was defined as the formation of 1 μmol of thiosulfate (oxygenase activity) or 1 μmol of H_2S (reductase activity) per minute.

Analytical methods. Protein concentrations were determined by the Bradford method (5a), by measuring the OD_{280}, or by using an RC protein determination kit (Bio-Rad). Proteins were separated in 10% sodium dodecyl sulfate (SDS) gels (30a) and visualized with colloidal Coomassie blue (Roti-Blue; Roth, Karlsruhe, Germany). Samples (50 μg) of protein from crude cell extracts of transformants were fractionated in nondenaturing blue native gels (30b). Gels (6.5%; 14 × 14 × 0.15 cm) were prepared and run in an SE400 vertical electrophoresis chamber (Hoefer Scientific Instruments). After activity staining with ONPG, the gels were stained with colloidal Coomassie blue for visualization of the protein bands. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass fingerprints after tryptic digestion of the proteins were done by the Dencher group at the Clemens Schöpf Institute of Organic Chemistry (Darmstadt, Germany).

RESULTS

Entry vectors and introduction of the sugar-inducible *araS* promoter. In the recently described shuttle vector pMJ03 (16), the complete DNA of the virus SSV1 (15 kb) was combined with pUC18 for replication/selection in *Escherichia coli*, the genes *pyrEF* of *S. solfataricus* for complementation of uracil auxotrophic mutants of *Sulfolobus*, and the reporter gene *lacS*, coding for β-galactosidase. The system was used to show the expression of the β-galactosidase gene (*lacS*) under the control of the heat-inducible promoter of the α subunit of the thermosome of *S. solfataricus* (TF55α). This promoter has a strong basal activity and is additionally inducible by shifting cultures from 78 to 88°C. In order to improve the pMJ03-based vector system for the homologous and heterologous expression of genes in *S. solfataricus*, entry vectors were constructed with restriction sites that allowed us to replace the *lacS* gene with other genes of interest by cloning them directly behind the promoter sequence. The putative transcriptional terminator sequences of *lacS* were left intact (Fig. 1). Moreover, the heat-inducible *tf55*α promoter was replaced with an arabinose-inducible promoter in order to allow the induction of high-level expression without imposing stress on the host cells. For this purpose, a 241-bp region upstream of the open reading frame of *araS*, encoding the arabinose-binding protein, a subunit of an ABC sugar transporter in *S. solfataricus* (11), was selected for cloning. In contrast to the original *tf55*α-*lacS* promoter-gene fusion in pMJ03, this construct did not contain the five initial codons of the *tf55*α gene. Instead, the start codon of *araS* coincided with the start codon of the gene to be expressed (Fig. 1). High-level expression of β-galactosidase, which forms a tetramer of 240 kDa in vivo (28), was demonstrated by the separation of cell extracts of transformants in a nondenaturing blue native protein gel and subsequent activity staining with ONPG (Fig. 2). A quantitative estimation of promoter strengths using the LacS reporter activity showed that the arabinose promoter exhibited a considerably lower basal activ-

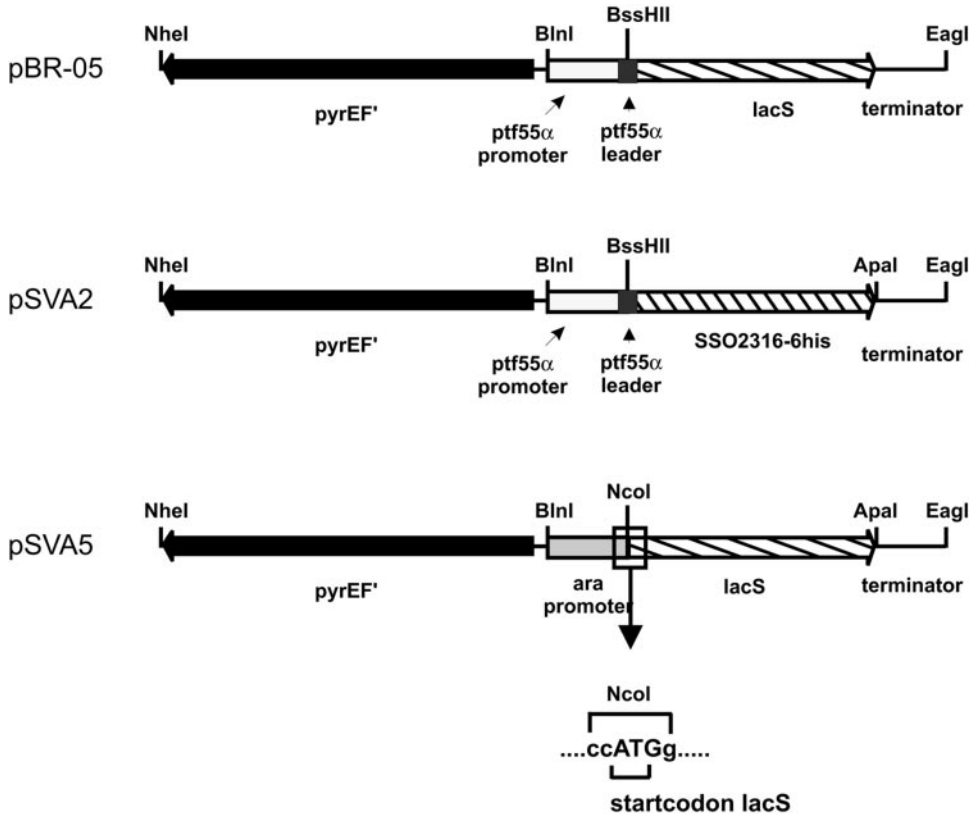

FIG. 1. Schematic representation of *S. solfataricus* entry vectors. The diagrams show the regions of the pBR322 vector with the *pyrEF* gene cassette, the different promoters, and the restriction sites that can be used to clone the gene of interest behind the promoter sequences.

ity than the *tf55*α promoter in the absence of an inducer (Table 2). Upon the addition of arabinose to the medium, the activity levels of β-galactosidase increased about 13-fold. The absolute value of 3.3 U/mg protein was comparable to that obtained with the *tf55*α promoter after heat shock (Table 2, second column). However, transformants with a *tf55*α promoter that contained an NcoI site at the translation start site (comparable to the situation in the *araS* construct) exhibited an even higher activity, at ca. 12 U/per mg of protein, in crude extracts (Table 2, construct pMJ11). A more detailed analysis of promoter variants and the effects of mutations on the initiation of transcription and translation is currently in progress.

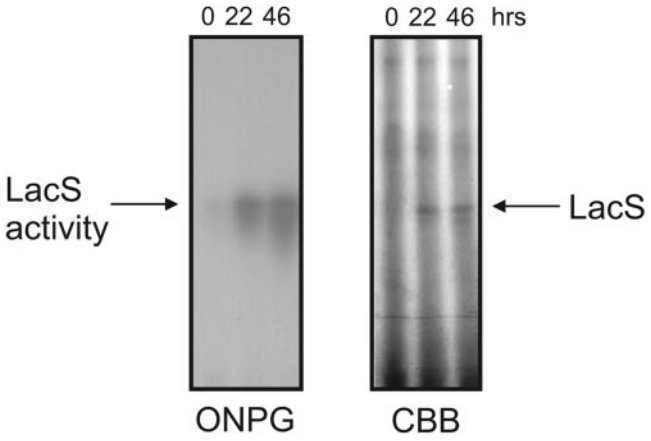

FIG. 2. β-Galactosidase activity monitored in a nondenaturing polyacrylamide gel before and after induction with arabinose. Crude extracts (50 μg of protein) of two independent pSVA9 transformants were separated by blue PAGE 0, 22, and 46 h after the addition of 0.4% arabinose to the growth medium. (Left) ONPG activity staining of β-galactosidase. (Right) Colloidal Coomassie blue-stained gel.

Heterologous expression and purification of the sulfur oxygenase reductase from *Acidianus ambivalens*. The sulfur oxygenase reductase (Sor) from the chemolithoautotrophic crenarchaeote *A. ambivalens* is a cytoplasmic enzyme that catalyzes the initial step in the dissimilatory sulfur oxidation pathway in this organism (19, 20). Sulfite, thiosulfate, and hydrogen sulfide are simultaneously produced from elemental sulfur in an oxygen-dependent reaction. Sor is an icosatetrameric protein with a molecular mass of 840 kDa that is composed of identical subunits of 35.2 kDa (38) and contains a mononuclear nonheme iron center as a cofactor (37). *S. solfataricus* lacks a *sor* homolog (32).

The *sor* gene, including codons for a C-terminally fused Strep tag, was cloned under the control of the *tf55*α promoter. Single transformants of *S. solfataricus* PH1-16 containing the pMJ05-*sor* construct were grown at 78°C and subsequently shifted to 88°C to induce the expression of the *sor* gene. The yield was 0.5 mg of enriched Sor protein per liter of culture after Streptactin affinity chromatography. A band corresponding to a molecular mass of 36 kDa was visible on SDS gels

TABLE 2. Comparison of promoter strengths in the reporter gene system

Construct	Promoter	Beta-galactosidase sp act (U/mg protein)[a]		Fold induction	Inducing agent
		Before induction	After induction		
pMJ03	*tf55alpha*	1.46 (0.13)	5.09 (0.16)	3.50 (0.33)	Heat
pMJ11[b]	*tf55alpha* (NcoI)[c]	1.18 (0.13)	11.83 (0.70)	10.02 (0.67)	Heat
pSVA9	*araS* (NcoI)[c]	0.28 (0.09)	3.31 (0.51)	12.97 (3.84)	Arabinose

[a] Data from 5 (pMJ03), 3 (pMJ11), and 10 (pSVA9) independent experiments (numbers in parentheses indicate standard deviations).
[b] Unpublished construct.
[c] NcoI site introduced at the start codon of *lacS*.

containing the purified protein fraction (Fig. 3A). The protein was confirmed to be identical to the *A. ambivalens* Sor protein plus the additional amino acids from the Strep tag by MALDI-TOF analysis. Other bands were visible besides the Sor band. A prominent band with an apparent molecular mass of 55 kDa was identified by MALDI-TOF analysis as the large subunit of the biotinylated *S. solfataricus* acetyl-coenzyme A (acetyl-CoA)/propionyl-CoA carboxylase (also called AccC or SSO2466 [15]). The specific activities in the Sor preparation, measured as the production of thiosulfate and hydrogen sulfide from elemental sulfur, were 4.5 and 1.2 U mg protein^{-1} (oxygenase and reductase activities, respectively). These values are comparable to those obtained for the enzyme isolated from its native host (Fig. 3B), indicating the successful assembly of the homomultimeric enzyme and the incorporation of the iron cofactor.

Homologous expression of the ATPase FlaI. *flaI* (SSO2316) is part of the potential flagellin operon of *S. solfataricus* and encodes a 59-kDa protein with a nucleotide-binding domain. FlaI is homologous to other ATPases present in archaeal flagellin operons, which may fulfill a role in the assembly of the archaeal flagellum. Gene inactivation experiments with *Halobacterium salinarium* and *Methanococcus voltae* have previously shown that the FlaI protein is essential for flagellum formation (27, 36). In *S. solfataricus*, *flaI* is expressed only in the stationary growth phase (1). The protein was expressed in *E. coli* earlier, purified to homogeneity, and shown to exhibit divalent cation-dependent ATP-hydrolyzing activity (1). In order to produce FlaI in *Sulfolobus*, the gene, including codons for a six-His tag, was cloned under the control of the *tf55*α promoter to yield pSVA6. Single transformants containing the vector were isolated and grown at 78°C with a subsequent shift to 88°C. Strain PH1-16 (*pyrEF lacS* double mutant) was grown as a control. Samples were taken from both cultures at the start of heat incubation and after 1 and 2 days. Cells were harvested, separated into membrane and cytoplasmic fractions, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using antibodies directed against purified FlaI (1). While the heterologously expressed FlaI protein in *E. coli* had been isolated from the soluble cytoplasmic fraction

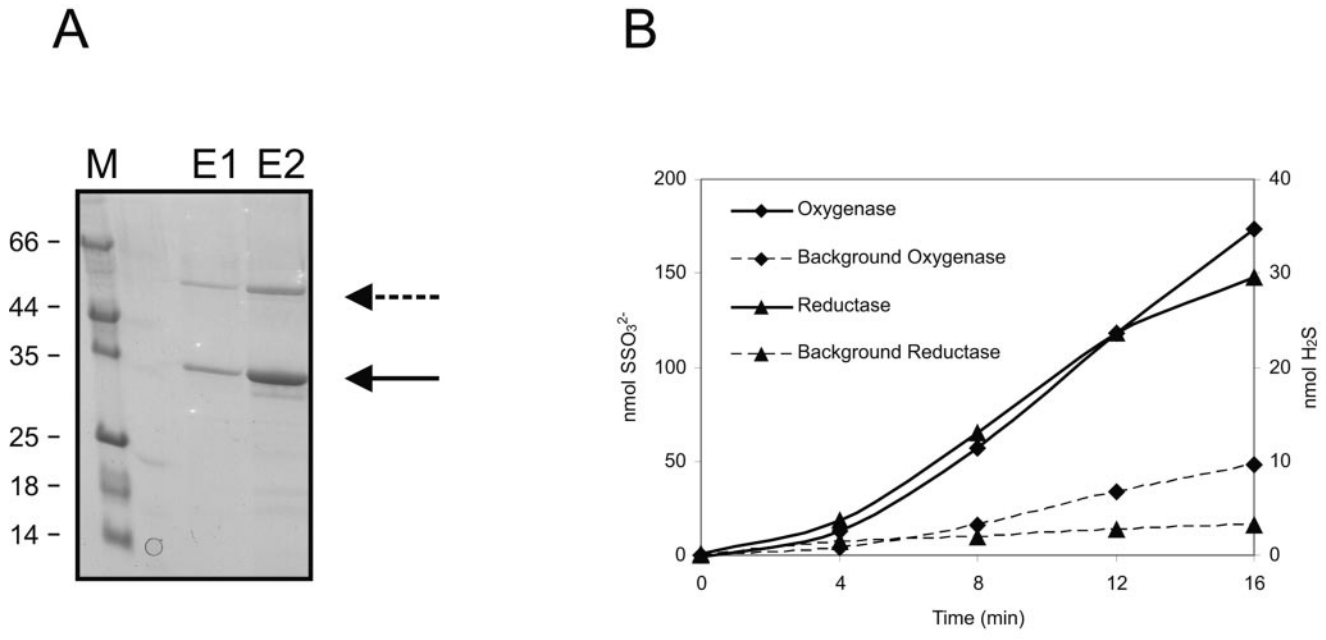

FIG. 3. Purification and activity of *A. ambivalens* Sor isolated from *S. solfataricus* transformed with pMJ05-sor. (A) SDS-PAGE of different fractions (E1 and E2) eluted from the Streptactin column. M, molecular mass marker proteins. Solid arrow, Sor band; dashed arrow, AccC subunit of the acetyl/propionyl-CoA carboxylase (both identified by MALDI-TOF analysis). (B) Sulfur oxygenase and reductase activities of pooled eluate fractions showing time-dependent increases in the amounts of thiosulfate and hydrogen sulfide in the assay mixture (19). Background, nonenzymatic production of thiosulfate and hydrogen sulfide from sulfur disproportionation under the same assay conditions without the enzyme (19).

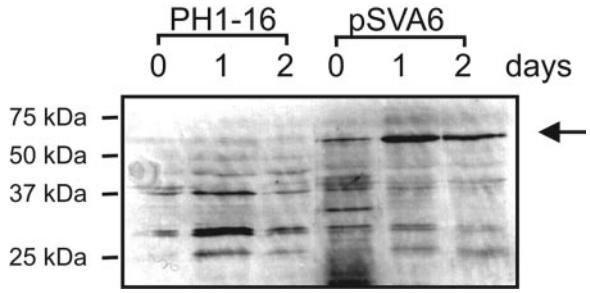

FIG. 4. Western analysis of FlaI expression. *S. solfataricus* cells transformed with pSVA6 and control PH1-16 cells were grown for 2 days at 88°C. Samples were taken at the beginning of the temperature shift from 78 to 88°C and after 1 and 2 days of growth at 88°C. Membranes were isolated from the cells and were analyzed by SDS-PAGE and immunoblotting using FlaI-specific antibodies. The arrow indicates the detected expression product.

(1), the protein produced in *Sulfolobus* could only be detected in the membrane fraction of induced cells (Fig. 4). This observation was confirmed using antibodies directed against the C-terminal His tag of FlaI (data not shown).

Homologous expression and purification of SSO2680. SSO2680 is a hydrophilic nucleotide-binding protein of 59 kDa. It has been shown to be encoded in an operon with five other genes (1) that encode proteins with motifs typically found in subunits of bacterial protein secretion systems. These so-called type II and type IV secretion machineries, as well as type IV pilin assembly systems, are involved in the assembly or secretion of multimeric substrate proteins such as pili. The protein substrate of this putative secretion operon in *Sulfolobus* is unknown. Interestingly, the operon contains a gene for one small protein of 15 kDa, SSO2681, which contains a type IV pilin signal peptide and might therefore be a subunit of a pilus structure (1). SSO2680 shows sequence similarity to bacterial secretion ATPases, such as Virb11 and PilT, which power the secretion process (30, 42). SSO2680 was cloned behind the *araS* promoter with codons for a C-terminal 10-His tag and transferred to the viral vector, yielding pSVA15. After transformation of *Sulfolobus*, single transformants were grown on 0.1% tryptone and subsequently transferred to the same medium supplemented with 0.2% arabinose. After 2 days of growth, cells were harvested, lysed, and separated into membrane and cytoplasmic fractions. These fractions were analyzed by SDS-PAGE and immunoblotting using antibodies directed against the His tag (Fig. 5). Although SSO2680 is predicted to be a soluble protein, it was recovered with the membrane fraction. In *E. coli*, the protein had been recovered with inclusion bodies, and only a small fraction could be isolated from the soluble fraction (1). SSO2680 was purified from detergent-solubilized membranes of *S. solfataricus* by His-Select Ni affinity chromatography, yielding ~1 mg purified enzyme per liter of cells (Fig. 5A). Immunodetection with His tag-specific antibodies confirmed that the purified protein corresponded to the recombinant tagged protein (lower part of Fig. 5A). SSO2680 showed divalent cation-dependent ATPase activity at a high temperature and a preference for Mn^{2+} over Mg^{2+} (Fig. 5B), in agreement with previous studies using the heterologously expressed protein purified from *E. coli* (1). Interestingly, the specific activity of the protein produced and purified from *S. solfataricus* was six times higher (50 nmol of P_i released mg protein^{-1} min^{-1}) than that of the enzyme isolated from *E. coli* (8 nmol of P_i released mg protein^{-1} min^{-1}) (1), which indicated a more native conformation of the homologously expressed protein. In order to analyze the effect of medium composition on the expression level, cells were grown with different amounts of tryptone and arabinose, and the membrane and cytoplasmic fractions of lysed cells were analyzed by SDS-PAGE and immunoblotting. The relative yield of recombinant protein did not change when the arabinose concentra-

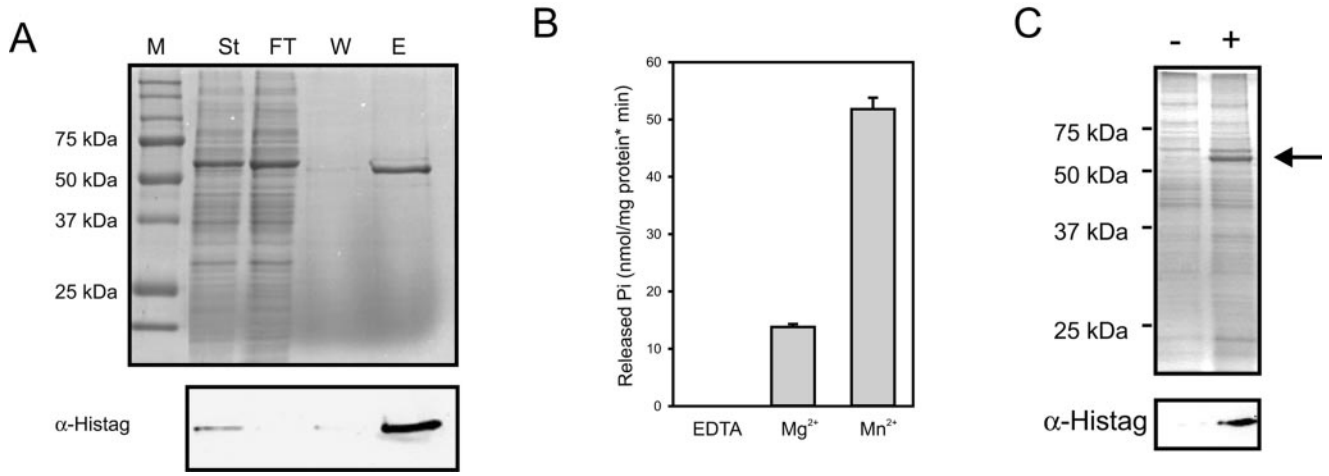

FIG. 5. Overexpression, purification, and activity of SSO2680. (A) Coomassie blue-stained SDS-PAGE gel of His tag-specific affinity chromatography fractions from solubilized membranes derived from *S. solfataricus* cells transformed with pSVA15. In the lower panel, the corresponding Western blot of the same samples, using His tag-specific antibodies, is shown. M, molecular mass marker; St, starting material; FT, flowthrough; W, wash; E, elution fraction. (B) ATPase activity of purified SSO2680 in the presence of EDTA, Mg^{2+}, or Mn^{2+}. (C) Overexpression of SSO2680. Membranes of a single pSVA15 transformant were separated in a Coomassie blue-stained SDS-PAGE gel before (−) and after (+) induction with 0.4% arabinose. The arrow indicates SSO2680. In the lower panel, the corresponding Western blot of the same samples, using His tag-specific antibodies, is shown.

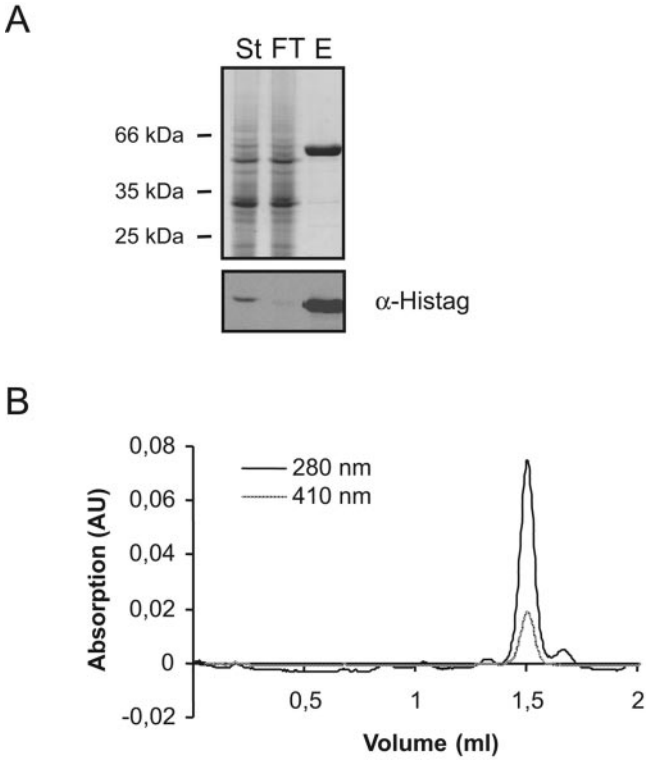

FIG. 6. Purification and gel filtration of SSO0287. (A) Coomassie blue-stained SDS-PAGE gel of Strep tag-specific affinity chromatography fractions of the cytoplasm of SSO0287 transformants. St, starting material; FT, flowthrough; E, elution fraction. (B) Gel filtration of purified SSO0287. Traces were recorded at 280 nm (black line) and 410 nm (dashed line).

tion was varied between 0.2 and 0.4%. Although the amount of tryptone affected the growth rate, it did not affect the expression level (data not shown). We occasionally observed very high expression levels, comparable to those obtained with *E. coli* expression systems, in different transformants of *Sulfolobus*. One such example is shown for SSO2680 in Fig. 5C. The conditions which led to these increased expression levels are currently under investigation.

Expression of SSO0287. SSO0287 (or ABCE1) is a cytoplasmic protein of 68 kDa that contains two nucleotide-binding domains and is predicted to harbor two iron-sulfur clusters. It is homologous to eukaryotic ABC proteins that are essential for cell function and involved in ribosome biogenesis and protein translation (18, 43) but whose specific function is unknown.

The gene was cloned behind the *araS* promoter with codons for a C-terminal tandem tag, including a Strep tag followed by an eight-His tag. Single transformants were grown on 0.1% tryptone and subsequently transferred to medium containing both 0.1% tryptone and 0.2% arabinose. After 2 days, cells were harvested and lysed. The cytosolic fraction was applied to either a Streptactin or His-Select Ni affinity column. Both methods resulted in single-step purification of the tagged protein (Fig. 6). The identity of the protein was verified by means of immunoblotting using antibodies directed against the Strep tag and the His tag and polyclonal antibodies directed against ABCE1 itself. The purified protein exhibited divalent cation-dependent ATPase activity, with a high temperature optimum of 85°C, close to the optimal growth temperature of *S. solfataricus* (S. Dinkelaker and R. Tampé, manuscript submitted). As described above, SSO0287 contains two putative sites for the binding of iron-sulfur clusters. Previous attempts to obtain correct assembly of these clusters by heterologous expression in *E. coli* failed (unpublished results), whereas the homologously produced protein in *S. solfataricus* appeared to contain the iron-sulfur clusters. The protein was subjected to gel filtration chromatography, and elution was monitored at two different wavelengths, 280 and 410 nm (Fig. 6B), to detect the protein and the iron-sulfur clusters, respectively. The isolated protein eluted as a single monodisperse symmetric peak that was devoid of aggregates. The elution profiles recorded at 280 and 410 nm coincided, suggesting that the recombinant SSO0287 protein contained correctly assembled iron-sulfur clusters.

The expression of SSO0287 in *S. solfataricus* was scaled up from 400-ml cultures to fermentation in 45 liters. A fermentor containing medium with 0.1% tryptone and 0.2% arabinose was inoculated with 1.6 liters of SSO0287-expressing *S. solfataricus* culture. After 2 days of growth, the cells were collected at an OD_{600} of ~0.8, and the protein was purified. The yields per liter (0.75 to 1 mg) were comparable to those from small cultures, but when the cells were grown to higher densities (ODs of about 1.5), degradation products of the purified protein were observed.

DISCUSSION

We report here the construction of a virus-based shuttle vector for the heterologous and homologous expression of genes in the hyperthermophilic archaeon *S. solfataricus*. A summary of the expression studies is shown in Table 3. Two promoters were used to drive expression, namely, the heat-inducible promoter of the alpha subunit of the chaperonin TF55 (for Sor, FlaI, and β-galactosidase) and an arabinose-inducible promoter (for SSO2680, SSO0287, and β-galactosidase). Since the TF55α promoter requires a shift of cultures to 88°C, which is close to the maximal growth temperature of *S. solfataricus*, this procedure might impose highly stressful conditions on the cells. For routine expression purposes, we therefore favor the use of the arabinose promoter, which is more easily induced and results in less stressful conditions. Similarly, the pBAD system is often used in *E. coli*, which is based on the *araBAD* operon controlling the arabinose metabolic pathway (13). This is a tightly controlled system that only allows for expression in the presence of arabinose, thereby preventing adverse effects on growth during the stages that are needed to obtain cell mass. In *S. solfataricus*, several sugar binding proteins that are part of ABC transporters are expressed only in the presence of their respective substrates (11). One of these genes is *araS*, which encodes an arabinose-binding protein. The expression of *araS* is induced only when *S. solfataricus* is grown on arabinose (11). We have therefore chosen the promoter region of *araS* to drive the expression of genes in *S. solfataricus*. A thorough characterization of the regulatory elements in this promoter and the construction of minimal in-

ducible promoters for this expression system are under way (M. Jonuscheit, S. V. Albers, et al., unpublished data).

Although the expression system for *Sulfolobus*, as presented here, involves the use of a rather large virus-based vector, we have greatly facilitated the cloning procedures by using small entry vectors and by introducing convenient restriction sites, i.e., an NcoI restriction site at the start codon to allow in-frame expression of newly introduced genes and an ApaI restriction site that leaves the putative terminator region of *lacS*. The latter dismisses the need for cloning of a terminator region for each gene of interest. Indeed, transcripts of defined sizes were observed in Northern analyses of transformants, indicating that the chosen region does indeed contain a transcriptional terminator (not shown).

All proteins produced in this study (except β-galactosidase) were fused either with a His tag, a Strep tag, or both. The data show that *S. solfataricus* tolerates these tags and that they can be used for protein detection and purification. In particular, the use of His-Select material for His tag-specific affinity chromatography resulted in single-step purification to 99% homogeneity, indicating that there is hardly any background of endogenous proteins in *S. solfataricus* that may bind to the affinity matrix. In contrast, the purification of proteins from *Sulfolobus* via a Streptactin column can result in coelution of the biotinylated acetyl-CoA/propionyl-CoA carboxylase. This enzyme is involved in the modified 3-hydroxypropionate cycle used for autotrophic CO_2 fixation in crenarchaeota (15). The function of this enzyme in *S. solfataricus* is not clear since all of our experiments were conducted under heterotrophic conditions. Copurification of the carboxylase was observed only when low, and not high, yields of the recombinant protein were obtained (Fig. 3, Sor, versus Fig. 6, SSO0287).

The proteins produced in this study will be used for biochemical and physiological studies. For example, transformants that express the *sor* gene from *Acidianus ambivalens* will be analyzed for the ability to metabolize sulfur. *S. solfataricus* lacks the *sor* gene but contains other genes that might encode proteins involved in the metabolism of inorganic sulfur compounds, e.g., a *doxDA* operon encoding a thiosulfate:quinone oxidoreductase, an enzyme which acts downstream in the sulfur oxidation pathway in *A. ambivalens* (26). Therefore, it will be interesting to analyze the sulfur-oxidizing capabilities of various *S. solfataricus* transformants carrying the *sor* gene. In the case of SSO2680, we will attempt to identify interacting partners by coimmunoprecipitation. Interestingly, this protein exhibited a much higher activity than the heterologously produced protein from *E. coli*, where it was mainly recovered in inclusion bodies, pointing to a folding defect in the bacterial host (1). The homologously expressed protein was solely localized in the membranes of *S. solfataricus* cells. Since the protein is predicted to be a cytoplasmic ATPase subunit of a membrane-bound protein secretion system, this result might indicate that the protein is associated with native lipids or membrane components that are part of the secretion machinery. Similarly, tagged FlaI can be used for the identification of binding partners. The proteins which play an important role in flagellation in archaea are known from transcription and deletion studies (35), but their subunit interactions during assembly of the flagellum are not known. The *flaI*-expressing recom-

TABLE 3. Summary of expression studies

Protein	Activity	Construct	Promoter	Tag[a]	Detection	Comments
Beta-galactosidase (SSO3019)	Cleaves beta-galactosidase analogs	pMJ05/pSVA5	*tf55*, *araS*		Native stain	Reporter, cytoplasmic
FlaI (SSO2316) (putative ATPase [secretion] with nucleotide binding domain)		pSVA6	*tf55*	Six-His	Immunodetection	In membrane fraction (versus cytoplasmic in *E. coli*)
ATPase (SSO2680) (type II or IV secretion)	ATPase (Mn > Mg)	pSVA15	*araS*	10-His	Immunodetection, ATPase assay	Immunodetection (inclusion bodies in *E. coli*), 5× higher specific activity when produced in *Sulfolobus*
ABCE1 protein (SSO0287) (two nucleotide binding domains and two Fe-S clusters)	ATPase	pSVA31	*araS*	Strep plus eight-His	Immunodetection (anti-Strep, anti-His) and 280/410 nm	Intact Fe-S clusters (410-nm adsorption, not in *E. coli*), upscaling to 45-liter fermenter
Sor (sulfur oxygenase/reductase from *A. ambivalens*)	Sulfur oxidation/reduction	pASK-SOR.05	*tf55*	Strep	Sor activity	Specific activity as high as protein from native host

[a] All tags were positioned at the C terminus.

binant *Sulfolobus* strain can serve as an important tool to address such questions.

Also, in the case of SSO0287, the tagged version can be used for searches for unidentified interacting proteins. The exact function of this protein is unknown, but it contains a unique ABC domain with two iron-sulfur clusters. Strikingly, it appeared that only 50% of these iron-sulfur clusters assembled when the protein was expressed in *E. coli*, whereas in *S. solfataricus* the protein mostly likely followed the endogenous assembly pathway, which ensures the correct incorporation of these iron-sulfur clusters into the protein. The homologous expression of SSO0287 now allows for mutational analysis in *S. solfataricus* in order to assess its in vivo function using interference assays.

In conclusion, we have established a vector system for protein expression in *S. solfataricus*, making use of two different promoters. The versatility of the system was demonstrated by various examples of heterologous or homologous proteins with a cytoplasmic or membrane-associated location, and all recombinant proteins were shown to be functional (except for FlaI). This is the first report of the application of an expression system in a hyperthermophilic archaeon. The expression of tagged proteins in *S. solfataricus* will greatly facilitate the isolation of unknown protein complexes by coprecipitation methods. Moreover, the unique growth conditions of hyperthermophiles and the ability to express proteins at high temperatures enable the expression of natively folded proteins that in mesophilic hosts end up in inclusion bodies. The virus vector allows for the stable production of proteins in large-scale fermentations of *S. solfataricus*, which might be of interest for industrial applications when the system is further improved. While a few studies have recently demonstrated the expression of proteins in hyperthermophilic bacteria, in particular in *Thermus thermophilus* (8, 21), this system will allow the production of proteins from hyperthermophilic archaea.

ACKNOWLEDGMENTS

We thank Gaby Liebing and Renate Fröhlich (Darmstadt University of Technology) for excellent technical assistance.

This project was funded through a BMBF grant, GenoMik, cluster 4.1, given to C.S. S.V.A. was supported by a VENI grant from the Dutch Science Organization (NWO) and a short-term EMBO fellowship. The work of S.D. and R.T. was supported by the Deutsche Forschungsgemeinschaft (DFG).

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