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Improvement of a *Sulfolobus-E. coli* Shuttle Vector for Heterologous Gene Expression in *Sulfolobus acidocaldarius*

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A *Sulfolobus-E. coli* shuttle vector for an efficient expression of the target gene in *S. acidocaldarius* strain was constructed. The plasmid-based vector pSM21 and its derivative pSM21N were generated based on the pUC18 and *Sulfolobus* cryptic plasmid pRN1. They carried the *S. solfataricus* P2 *pyrEF* gene for the selection marker, a multiple cloning site (MCS) with C-terminal histidine tag, and a constitutive promoter of the *S. acidocaldarius gdhA* gene for strong expression of the target gene, as well as the pBR322 origin and ampicillin-resistant gene for *E. coli* propagation. The advantage of pSM21 over other *Sulfolobus* shuttle vectors is that it contains a MCS and a histidine tag for the simple and easy cloning of a target gene as well as one-step purification by histidine affinity chromatography. For successful expression of the foreign genes, two genes from archaeal origins (*PH0193* and *Ta0298*) were cloned into pSM21N and the functional expression was examined by enzyme activity assay. The recombinant PH0193 was successfully expressed under the control of the *gdhA* promoter and purified from the cultures by His-tag affinity chromatography. The yield was approximately 1 mg of protein per liter of cultures. The enzyme activity measurements of PH0913 and Ta0298 revealed that both proteins were expressed as an active form in *S. acidocaldarius*. These results indicate that the pSM21N shuttle vector can be used for the functional expression of foreign archaeal genes that form insoluble aggregates in the *E. coli* system.

Keywords: Archaea, constitutive promoter, enzyme activity, protein expression, shuttle vector, *Sulfolobus acidocaldarius*

Introduction

Sulfolobus species belong to the Crenarchaeota in the archaeal kingdom. Various strains have served as model organisms for studies on metabolism, replication, transcription, translation, and many other cellular aspects [8, 16, 17, 30]. The complete genome sequences of four species, *Sulfolobus solfataricus*, *S. tokodaii*, *S. acidocaldarius*, and *S. islandicus*, have been published [14, 19, 21, 29]. Genomics, transcriptomics, and proteomic studies using these strains have been carried out [7, 22, 25, 30]. However, the comprehensive study of molecular phenomena in any organism requires genetic analysis and manipulation *in vivo*.

A method for introducing homologous or heterologous genes into an organism is essential for genetic complementation in a knockout system. The development of shuttle vectors in *Sulfolobus* is also important for the overexpression of proteins of interest in the native host. One reason is that the expression of the protein in the native host allows for post-translational modification and the determination of the localization of the protein in the native host. Secondly, the expression of the respective protein as a functional form in mesophilic hosts is difficult owing to the unique structural attributes and different codon usages, the absence of cofactors, and potential toxicity of the protein to the host. Although the development of shuttle vector systems in hyperthermophiles such as *Sulfolobus* has retarded compared with other archaea, such as halophiles or methanogens [3],

various *Sulfolobus-E. coli* shuttle vectors have been constructed during the last decade.

The first shuttle vectors, pDM1, pCSV1, and pAG, were designed based on the rDNA intron or plasmid from other archaea; however, they were no longer used because of the instability of these plasmids in the host [4, 31]. The vector pEXSs was developed for protein expression based on the virus SSV1 with a thermostable hygromycin phosphotransferase marker [13]. The first successful shuttle vectors were pMJ03 and its derivatives that rely on *pyrEF* and *lacS* selections based on the virus SSV1 [20]. Other shuttle vectors were developed based on the virus-plasmid hybrid pSSVx and the virus SSV2 [5, 6]. However, the virus vectors have a possibility that they can be integrated into the host chromosome. Most recently, the shuttle vectors pA to pN based on the plasmid pRN1 isolated from *S. islandicus* REN1H1 have been used in *S. solfataricus* and *S. acidocaldarius* [9]. In *S. acidocaldarius*, *pyrEF* was employed as an efficient selectable marker to obtain a shuttle vector with a constitutive or inducible promoter for expression [11, 32]. The antibiotic simvastatin has also been used to select for the shuttle vector in *S. islandicus* [36]. We also tried to use this antibiotic as a selection marker, but it was not successful because of the failure of reproducibility of this antibiotic. In spite of sufficient knowledge on the genetics and molecular biology of these organisms, many genes of *Sulfolobus* species still have not been successfully expressed.

In this study, we tried to improve the pC vector, one of the pRN1-based plasmids, for an efficient expression of the target gene in *S. acidocaldarius* strain. A novel plasmid vector, pSM21, and its derivative (pSM21N) were generated. They included the *pyrEF* gene from *S. solfataricus* P2 ($pyrE_{sso}$) as the selection marker, a multiple cloning site (MCS) with histidine tag, and a strong constitutive promoter of *gdhA* encoding glutamate dehydrogenase (*saci_0155*) from *S. acidocaldarius* for strong expression of the target gene in *S. acidocaldarius*. For a confirmation of the successful expression of the foreign genes, heterologous genes of archaeal origin were cloned into pSM21N and their stable expression was examined by enzyme activity assay.

Materials and Methods

Strains and Growth Conditions

S. acidocaldarius strains DSM639 and MR31 were aerobically grown at 77°C in Brock medium supplemented with 0.1% (w/v) tryptone and 0.005% (w/v) yeast extract, pH 3.0 [27]. For the growth of uracil auxotroph MR31, uracil (10 μg/ml) was added to the medium. After transformation, cells containing the plasmid vector were selected on Brock medium supplemented with 0.1% NZ-Amine (Sigma, St. Louis, MO, USA) and 0.2% (w/v) xylose as the carbon and energy source. For making plates, two times concentration of Brock's basal salt medium was mixed with an equal volume of 1.6% gelrite solution and 10 mM $CaCl_2$ added to solidify the medium. *E. coli* strain DH5α and ER1821 [pM.EsaBC4I] (New England Biolabs, Massachusetts, USA), used as a host for cloning and GGCC sequence methylation of plasmids [23], respectively, were grown at 37°C in Luria-Bertani (LB) broth or LB agar plates, supplemented with 100 μg/ml ampicillin or 30 μg/ml kanamycin.

Construction of the *Sulfolobus-E. coli* Shuttle Vector

The plasmids used for this study are listed in Table 1. The

Table 1. Plasmids used for this study.

Plasmid	Characteristics	Reference or source
pUC18	Cloning vector, Ap^r	Stratagene
pC	pBluescript II with pRN1 compartments containing $pyrEF_{sso}$	Prof. Lipps[a]
pSM21	*E. coli–Sulfolobus* shuttle vector for gene expression, pSM25 with pRN1 region of pC	This study
pSM22	pUC18 derivative carrying promoter, coding sequence, and terminator of $gdhA_{Saci}$	This study
pSM23	pSM22 containing multiple cloning sites and six-histidine tag	This study
pSM24	pUC18 derivative carrying promoter, coding sequence, and terminator of $pyrEF_{Sso}$	This study
pSM25	pSM23 carrying promoter, coding sequence, and terminator of $pyrEF_{Sso}$	This study
pSM21N	pSM21 carrying N-terminus 5 amino acids of $gdhA_{Saci}$ fused with multiple cloning sites	This study
T-blunt	pUC19-based PCR cloning vector, Ap^r, Km^r	SolGent
pT::PH0193	*PH0193* inserted into T-blunt	This study
pSM21N::PH0193	*PH0193* inserted into pSM21N for PH0193 expression	This study
pT::Ta0298	*ta0298* inserted into T-blunt	This study
pSM21N::Ta0298	*ta0298* inserted into pSM21N for Ta0298 expression	This study

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Table 2. Oligonucleotide primers used in this study.

Primer	Nucleotide sequence[a] (5′ → 3′)	Remarks
gdhF	ACAGT**GACGTC**TTCTCCACTGTTTACGTTTAC	Upstream sequence of $gdhA_{saci}$ with *Aat*II
gdhR	TATATA**GACGTC**TGAGCACTGATATCCTT	Downstream sequence of $gdhA_{saci}$ with *Aat*II
Rev_gdhF	AATTA***GGGCCC***GGGGGTACCGTCGACGCGGCCGC-*CCATGG*ATTTTATCCAAGTAA	Upstream sequence of $gdhA_{saci}$ and MCS site with *Apa*I
Rev_gdhR	TAGAG***GGGCCC***GCTAGCCTCGAGCATCATCAT-CATCATCATTGATCAGACTACATAA	Downstream sequence of $gdhA_{saci}$, MCS site, and histag with *Apa*I
pyrF	ATCTC**GACGTC**GAATAATGCTGCCCCAT	*pyrEF* from *S. solfataricus* P2, with *Aat*II
pyrR	ATTAC**ACATGT**TTCCTCGTGTAGATTTT	*pyrEF* from *S. solfataricus* P2, with *BspLU*11I
Rev_prnF	ACTTTA**ACATGT**CCGCCTTTGCTTCTGTTTCTT	pRN1 region with *BspLU*11I
Rev_prnR	AGAAGC**ACATGT**GGTATAGGAGTCTGGATTGG	pRN1 region with *BspLU*11I
promcs_R	GCCGC**CCATGG**CCGCAGAAGAATTCATATT	Addition of N-terminal 16 nucleotides of $gdhA_{saci}$ into pSM21
mcster_F	AATTCTTCTGCGG**CCATGG**GCGGC	Addition of N-terminal 16 nucleotides of $gdhA_{saci}$ into pSM21
PH0193_F	TAA**CCATGG**GCCCAAGGATAAATTTCATC	Forward primer of PH0193 expression
PH0193_R	ATT**CTCGAG**CATACTCTCTTTGAACTTCAA	Reverse primer of PH0193 expression
TA0298_F	GTA**GCGGCCGC**CTTGACGATATCCGA	Forward primer of TA0298 expression
TA0298_R	AGT**CTCGAG**CTTCAACCTTATTATGCC	Reverse primer of TA0298 expression

[a]The restriction enzyme sites are written in bold, MCS sequences are italicized, and complementary sequences for overlap extension PCR are underlined.

shuttle vectors pSM21 and pSM21N controlled by the promoter of *gdhA* encoding glutamate dehydrogenase were constructed using a fusion PCR technique. Using genomic DNA from *S. acidocaldarius* DSM639 as a template, a fragment of 1,952 bp containing the *gdhA* (*saci_0155*) gene and its putative promoter and terminator region was amplified with the primers gdhF and gdhR. The oligonucleotide primers used in the vector construction are shown in Table 2. PCR amplification was carried out on the Swift Max Pro Thermal Cycler (ESCO, Philadelphia, USA) using Prime STAR HS DNA polymerase (Takara, Otsu, Japan). The PCR product was digested with *Aat*II and cloned into pUC18 to generate pSM22. Two reverser primers containing multiple cloning sites (MCS) and six-histidine tag to the end, Rev_gdhF and Rev_gdhR, were designed and used to insert the MCS and histidine tag between the *gdhA* promoter and terminator region. The amplified 4,638 bp PCR product was digested with *Apa*I and self-ligated to generate pSM23. The *gdhA* promoter and terminator region containing MCS was confirmed by colony PCR using gdhF and gdhR primers and *Aat*II digestion. A DNA fragment of 1,712 bp containing the *pyrE* and *pyrF* from *S. solfataricus* P2 along with its putative promoter and terminator was amplified by pyrF and pyrR primers. This *pyrEF* cassette fragment was digested with *Aat*II and *BspLU*11I and cloned into pUC18 to generate pSM24. A DNA fragment of the *gdhA* promoter and terminator region containing MCS digested from pSM23 by *Aat*II was then cloned into pSM24 to generate pSM25. For the propagation of plasmid in *Sulfolobus* sp., the region of pRN1 from pC was amplified with Rev_prnF and Rev_prnR primers and digested with *BspLU*11I and eventually ligated into *BspLU*11I-restricted and dephosphorylated pSM25 to generate the pSM21 shuttle vector. The construction of pSM21 is described in Fig. 1. The nucleotide sequence of the *Sulfolobus-E. coli* shuttle vector was confirmed by sequencing.

For pSM21N, first, the *gdhA* promoter region, containing 425 nucleotides upstream of the *gdhA* coding sequence, was amplified with the primers gdhF and promcs_R, using pSM21 as a template. The promcs_R was designed to incorporate an *Nco*I restriction site and 15 nucleotides of the *gdhA* start region. Secondly, the *gdhA* terminator region, containing 380 nucleotides downstream of the *gdhA* coding sequence, was amplified with the primers mcster_F and gdhR. The mcster_F was designed to incorporate an *Nco*I restriction site and 13 nucleotides of the *gdhA* start region complementary to the promcs_R primer. The two amplified fragments were fused by overlap PCR to obtain the fusion construct containing the promoter, terminator, and first 15 nucleotides of *gdhA* with MCS. Overall, a 778 bp fragment was purified using a PCR purification kit (ELPIS Biotech) and restricted with *Aat*II and then ligated into the *Aat*II-restricted and dephosphorylated pSM25 to generate pSM21N (Fig. 2). The sequence of the fusion region was confirmed by sequencing.

Construction of PH0193 and Ta0298 Expression Plasmids

The gene of amylomaltase (PH0193, E.C. 3.2.1.1) was amplified from genomic DNA of *Pyrococcus horikoshii* using the primers PH0193_F and PH0193_R introducing *Nco*I and *Xho*I restriction sites at the 5′ and 3′ end, respectively, and the fragment was cloned into the T-blunt vector yielding the vector pT::PH0193. This plasmid was digested with the same restriction enzymes, and the insert containing the *PH0193* gene was ligated into pSM21N yielding pSM21N::PH0193. The gene of α-glucosidase (Ta0298, E.C. 3.2.1.20) was also amplified from genomic DNA of *Thermoplasma*

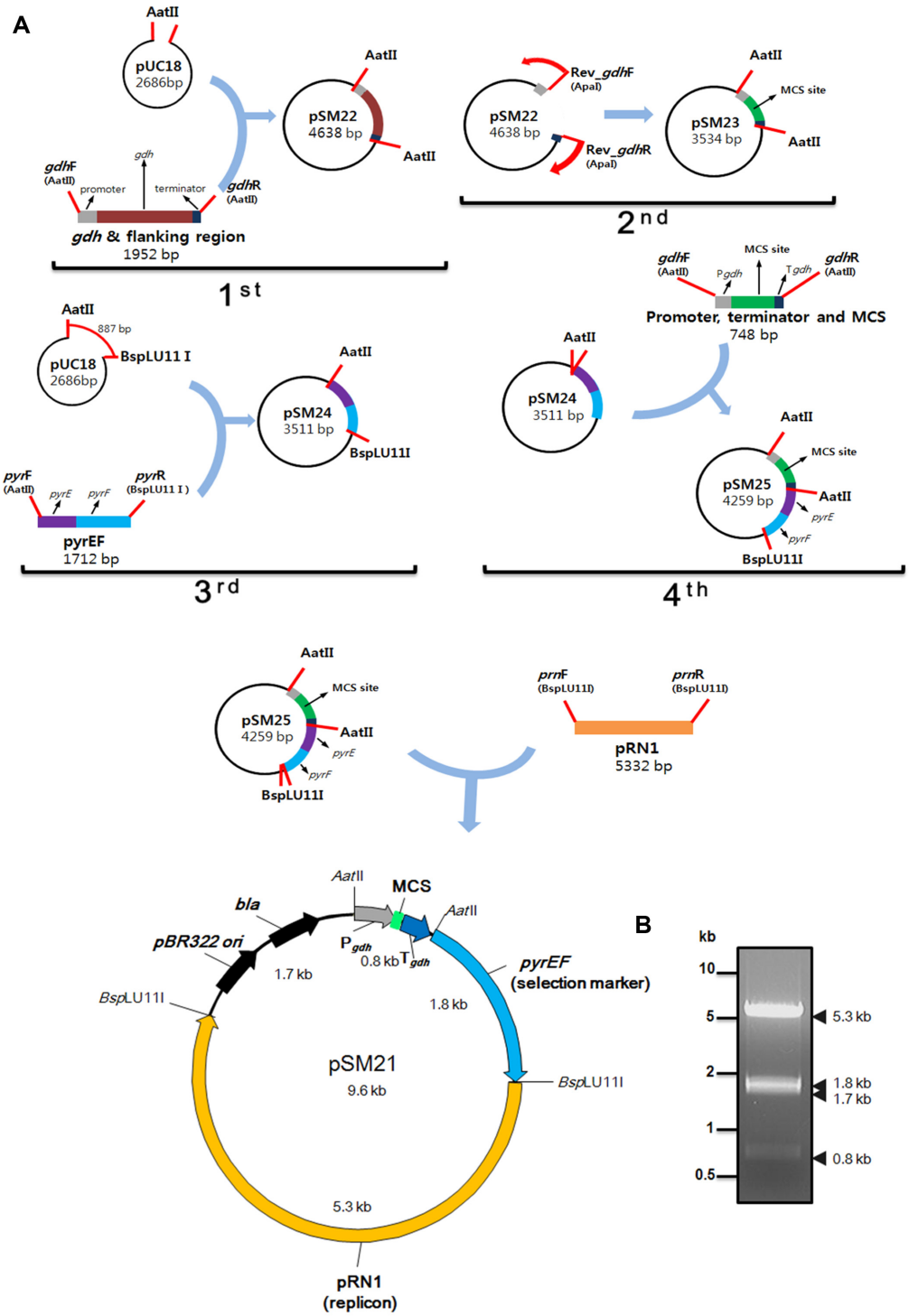

Fig. 1. The strategy for the construction of plasmid pSM21 (**A**) and the confirmation of pSM21 by restriction enzyme digestion (**B**). The expected sizes of the digested pSM21 DNA fragments are shown in the agarose gel.

acidophilum using the primers TA0298_F/TA0298_R introducing *Not*I and *Xho*I restriction sites at the 5′ and 3′ end, respectively, and each fragment was cloned into the T-blunt vector, yielding the vector pT::Ta0298. The recombinant plasmid was digested with the same restriction enzymes, and the inserts containing the *Ta0298* gene was ligated into pSM21N, yielding pSM21N::Ta0298.

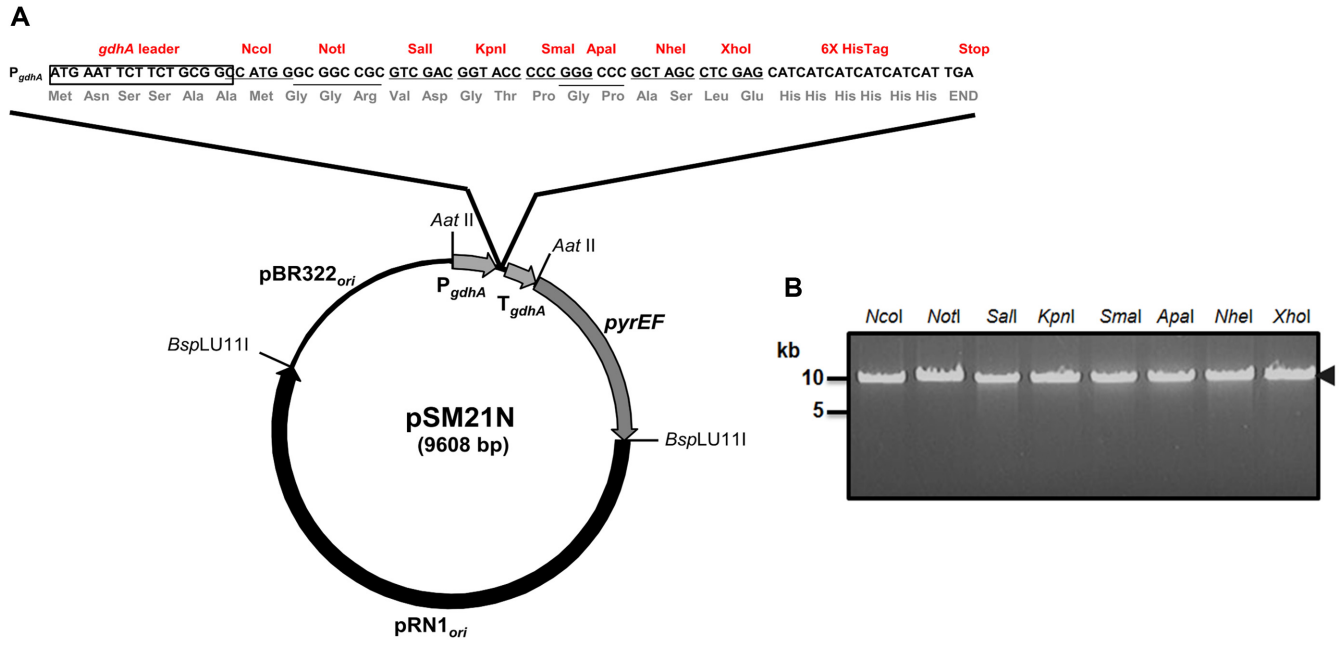

Fig. 2. Map of plasmid pSM21N (**A**), and the unique restriction digestion of pSM21N at the multiple cloning sites (**B**).

The sequence of the cloned genes flanking multiple cloning sites was confirmed by DNA sequencing.

Methylation of Plasmids

For transformation into *S. acidocaldarius*, the *Sulfolobus-E. coli* shuttle vector was methylated at the N^4-position of the inner cytosine residues of GGCC recognition sequences to circumvent restriction by the *Sua*I restriction enzyme [18]. Plasmids were methylated *in vivo* as previously described [23] by transforming the shuttle vector into *E. coli* ER1821 bearing the additional plasmid pM.*Esa*BC4I. Complete methylation was confirmed by the absence of any cleavage after incubation with 5 U *Hae*III for 1– 4 h at 37°C.

Transformation of *S. acidocaldarius*

S. acidocaldarius MR31 was transformed with the plasmids by electroporation, as described previously [15]. The methylated pSM21 or pSM21N DNA (1 μg) for the expression of the target gene was electroporated in electrocompetent MR31 using a Gene Pulser II electroporator (Bio-Rad) with input parameters 1.25 kV, 1,000 Ω, and 25 μF, in 1 mm cuvettes. Following electroporation, an equal volume of 2× recovery solution (1% sucrose, 10 mM $MgSO_4$, 0.18% (w/v) β-alanine, and 0.27% (w/v) DL-malic acid, adjusted to pH 4.5 with 10 N NaOH) was immediately added to the pulsed cells and the mixture was transferred to a 1.5 ml microcentrifuge tube and incubated at 75°C for 30 min. The cells were then spread on solid medium containing 0.1% (w/v) NZ-Amine and 0.2% (w/v) xylose without uracil. The plates were sealed in plastic bags to avoid drying-out and incubated for 8 days at 77°C. Single colonies were picked and identified by colony PCR using the primers pyrEF and pyrER, which are specific to the *pyrE* gene.

Heterologous Expression of Amylomaltase (PH0193) and α-Glucosidase (Ta0298) in *S. acidocaldarius*

Cells containing the pSM21N::PH0193 and pSM21N::Ta0298 constructs were grown in 100 ml of YT medium containing 0.2% (w/v) sucrose at 77°C for 24 h. The cells were harvested by centrifugation (8,000 ×*g* for 30 min at 4°C), and resuspended in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. The cells were then lysed by sonication on ice, and after centrifugation (15,000 ×*g* for 50 min at 4°C), the supernatant was applied to a Ni-NTA affinity column (GE Healthcare, Freiburg, Germany). The column was washed with wash buffer (20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, and 80 mM imidazole), and the bound proteins were eluted using elution buffer (20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, and 200 mM imidazole). All fractions were collected and dialyzed against the same buffer without imidazole and concentrated using Centricon-10 microfilters (Millipore, Darmstadt, Germany). The protein concentration was determined using the Bradford method [12] with bovine serum albumin as a standard. The molecular mass of the purified protein was estimated by 12% SDS-PAGE.

Activity Assays

Amylomaltase activity was determined by measuring the optical change in iodine stain during the conversion of amylose by the enzyme [24]. The reaction mixtures (50 μl) containing 0.1% amylose and 0.4 μg of enzyme solution in 50 mM sodium acetate

buffer (pH 6.0) was incubated at 80°C for 1 and 12 h, respectively. The reaction was stopped by the addition of 50 µl of 1 M HCl and neutralized with 1 M NaOH. Aliquots (100 µl) were then combined with 900 µl of iodine solution (0.02% I_2 and 0.2% KI), and the decrease of the absorbance at 660 nm by amylose degradation was measured immediately.

α-Glucosidase activity was determined using *p*-nitrophenyl α-glucopyranoside (*p*NPαG), as described previously [28]. The reaction mixture (100 µl) containing 0.5 mM *p*NPαG and 0.4 µg of enzyme solution in 25 mM sodium acetate buffer (pH 6.0) was incubated at 80°C for 10 min. The reaction was stopped using 50 µl of 1 M sodium carbonate, and the *p*-nitrophenol (*p*NP) released was measured at 420 nm.

Analytical Methods

The enzyme reaction products were spotted on Whatman K5F silica gel plates (Whatman, Maidstone, UK) activated at 110°C for 30 min. The plates were developed in developing solution composed of *n*-butanol:ethanol:water (5:3:2 (v/v/v)) for maltooligosaccharides. The developed TLC plates were dried completely at room temperature after irrigating once or twice, and visualized by dipping in a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol, followed by heating at 110°C for 10 min.

Stability Measurements

One colony from selective plates was resuspended into 50 µl of YT medium and inoculated into selective medium without uracil. After 2 days of incubation, the cultures were sampled, diluted in sterile YT medium, and plated on plates with and without uracil supplementation. The solid medium was incubated for 4–5 days at 77°C until colonies appeared. The process was repeated after five cycles of transfer to nonselective medium (with uracil). The stability of plasmid in the host cells was examined by counting as well as colony PCR of the colonies on the selective and nonselective media.

Results

pSM21 and pSM21N Characteristics

To construct an easy and convenient gene expression vector working in *S. acidocaldarius*, the *Sulfolobus-E. coli* shuttle vector pSM21 was constructed based on the pUC18 and *Sulfolobus* cryptic plasmid pRN1. The shuttle vector carries the *pyrEF* promoter and structural genes of *S. solfataricus* for uracil selection and a MCS with unique restriction sites for target gene expression under the *gdhA* gene promoter and terminator. It also contains the β-lactamase coding sequence and *ori* site from the pBR322 vector for cloning and propagation of this plasmid in *E. coli*. The regions including the *gdhA* promoter and cloned gene(s) as well as the *pyrEF* cassette were amplified by PCR and ligated into pUC18, and then finally combined with pRN1 to complete pSM21 (Fig. 1A). Previously, the transcriptional activity and regulation of the copy number of replication genes of pRN1 have been examined and revealed that this plasmid is a good backbone for successful propagation in *Sulfolobus* hosts [9]. The advantage of pSM21 is that it contains MCSs and a six-histidine tag for simple and easy cloning of target genes as well as one-step purification by histidine affinity chromatography. The verification of pSM21 was carried out using restriction enzyme digestion with *Aat*II and *BspLU11*I and sequencing (Fig. 1B). Unfortunately, this vector has not succeeded in our hands to express the foreign gene.

Expression of foreign genes in a cell at high levels requires the ability to control the regulatory mechanisms governing expression. This requires suitable regulatory sequences that can function with the desired foreign genes. It includes mutating regulator regions to increase transcription as well as mutation within the coding sequence to enhance protein stability. In order to enhance the expression of a desired protein in a cell, a leader sequence to the 5′ terminus of a desired gene was placed. In our construction, the leader sequence is placed before the natural ATG translation codon for the desired gene. The modified vector pSM21N included the N-terminus sequences encoding the first six amino acids of gene *gdhA* controlled by the *gdhA* promoter just before the *Nco*I site (Fig. 2A). This *gdhA* leader sequence helped to increase the expression of the cloned gene. The unique restriction site for cloning of the foreign gene was confirmed by the restriction digestion of the enzymes at the MCS (Fig. 2B).

The presence of restriction/modification activity can interfere with the successful establishment of a shuttle construct in the recipient strain. It is known that the low transformation efficiency in *S. acidocaldarius* is caused by the restriction enzyme *Sua*I [10, 26]. *In vivo* methylation of plasmid by *E. coli* strain ER1821 containing a low-copy number of a kanamycin-resistant plasmid, pM.*Esa*BC4I, protects the transformed DNA, and thus increases the transformation efficiency in *S. acidocaldarius* [18, 23]. The methylation of plasmid DNA was confirmed by restriction digestion by *Hae*III. As shown in Fig 3, *in vivo* methylated pSM21N was protected completely against *Hae*III digestion, whereas unmethylated plasmid was digested by this restriction enzyme.

To test if the selection for uracil prototrophy ensures that every cell contains a shuttle vector, cells were plated on selective and on nonselective media supplemented with uracil. After five transfers (more than 40 generations) in

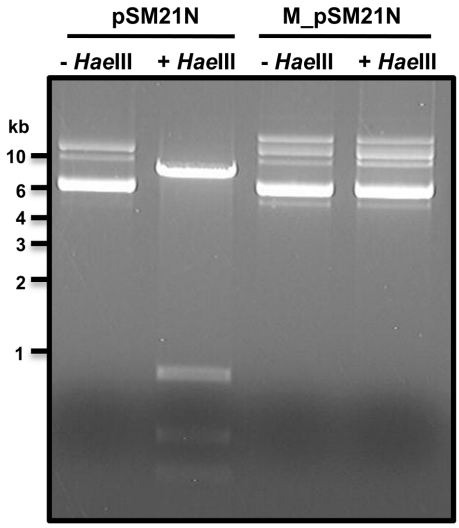

Fig. 3. Analysis of the restriction digestion of *in vivo* methylated pSM21N.

In vivo methylated pSM21N (about 100 ng) and unmethylated pSM21N (about 100 ng) were treated with 5 units of *Hae*III at 37°C for 4 h.

nonselective medium, the cultures were diluted and plated on both media. Colonies appeared on both plates after 4–5 days. To prove that most of the cells contained a shuttle vector, cells were examined by colony PCR with *pyrE* specific primers. More than 90% of the cells examined contained a shuttle vector. The result shows that the vector pSM21N has a high retention under selective conditions and is only slowly lost under nonselective conditions.

Heterologous Expression and Purification of PH0193

The amylomaltase (PH0193) from the obligately heterotrophic euryarchaote *P. horikoshii* is a cytoplasmic enzyme that catalyzes a reversible transglycosidase reaction involved in maltodextrin metabolism. The protein was expressed in *E. coli* earlier, but, turned out to be insoluble in spite of various trials to make the protein soluble. The *PH0193* gene, including codons for a C-terminally fused Histag, was cloned under the control of the *gdhA* promoter. A single transformant of *S. acidocaldarius* containing the pSM21N::PH0193 construct was grown in YT medium containing 0.2% sucrose for 24 h at 77°C. The yield was ~1 mg of protein per liter of cultures after Histag affinity chromatography. A band corresponding to a molecular mass of 75 kDa was visible on SDS-PAGE gels (Fig. 4A). The amylomaltase activity of the purified protein was examined by amylose-iodine binding assay. The amylose content was decreased by half when 0.4 μg of purified protein was incubated with 0.1% amylose for 1 h at 80°C. After 12 h, more than 80% of amylose had disappeared (Fig. 4B). The disproportionation activity was the major characteristic of typical amylomaltase. The disproportionation activity of the purified protein was also investigated with maltopentaose as a substrate at 80°C. TLC analysis indicated

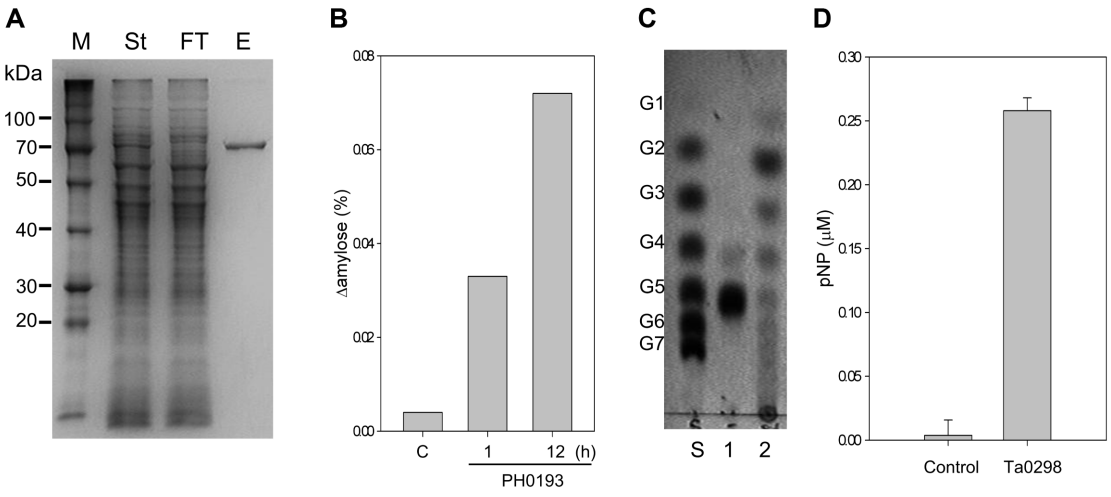

Fig. 4. Expression and enzyme activity measurements of recombinant PH0193 and Ta0298.

(**A**) SDS-PAGE gel of Histag affinity chromatography from solubilized proteins derived from *S. acidocaldarius* cells transformed with pSM21N::PH0913. M, molecular mass marker; St, starting material; FT, flowthrough fraction; E, elution fraction. (**B** and **C**) Amylomaltase activity of PH0913. The enzyme activity was measured using amylose iodine assay and maltopentaose disproportionation activity. C, control; S, maltooligosaccharides standard (G1–G7); 1, without enzyme; 2, with enzyme. (**D**) α-Glucosidase activity of Ta0298. The enzyme activity was determined using *p*NPαG as a substrate. The *p*NP released from the hydrolysis of the substrate by the enzyme was measured at 420 nm.

that various lengths of maltooligosaccharides were produced (Fig. 4C). The amylomaltase activity of purified protein confirmed that the purified protein corresponded to the recombinant tagged protein, being PH0193.

Heterologous Expression of Ta0298

The gene (*Ta0298*) encoding an α-glucosidase from hyperthermophilic archaeon *T. acidophilum* was previously expressed in *E. coli* [28]. *Ta0298* was cloned under the control of the *gdhA* promoter with codons for a C-terminal fused Histag and transferred to the plasmid vector pSM21N, yielding pSM21N::Ta0298. After methylation by *E. coli* strain ER1821, the methylated plasmid was transformed into *S. acidocaldarius*. Single transformants were grown in YT medium containing 0.2% sucrose. After 24 h, the cells were harvested and lysed, and the enzyme activity of the soluble protein fractions was examined using *p*NPαG as a substrate. The hydrolysis of *p*NPαG into *p*NP was detected by the appearance of yellow color in the reaction mixture, and the released *p*NP content was calculated by the absorbance at 420 nm. The expression level of Ta0298 was much lower than that of PH0913. It is supposed that the codon usages of the target gene may affect the expression level of each gene. The soluble protein fractions of the *S. acidocaldarius* cells without *Ta0298* gene showed little activity towards *p*NPαG, whereas the transformants with *Ta0298* gene showed high activity towards *p*NPαG (Fig. 4D). The enzyme activity of Ta0298 was 25 times higher than that of the control, indicating that the expression was successful. Therefore, the expression vector pSM21N we constructed can be used for the efficient expression of archaeal genes whose protein products are rendered insoluble when expressed in *E. coli*.

Discussion

Sulfolobales are the only family for the crenarchaeota that are amenable for genetic manipulation so far. The genetic systems for heterologous gene expression and integration in *S. solfataricus*, and *S. acidocaldarius* have been developed and proved to be successful [1, 33, 35]. *E. coli-Sulfolobus* shuttle vectors based on pRN1 have been constructed by random insertion of the *E. coli* replicon (R6Kγ origin of replication and *cat* gene) and addition of the *pyrEF* genes of *S. solfataricus* as selectable markers [9]. The recipient strains with uracil auxotroph were tested for whether various shuttle vectors pA−pN were stable and did not integrate into the host genome. Vector instability in the transformed cells has been observed in *S. solfataricus* PH1-16 and the *S. islandicus* mutants, but most of the constructs were stable in *S. acidocaldarius* MR31 [9].

In this study, we also tried to construct an efficient *E. coli-Sulfolobus* shuttle vector for heterologous gene expression in *S. acidocaldarius*. The *gdhA* promoter and *sac7d* promoter were considered and examined based on the β-galactosidase reporter assay [11]. In our hands, the *gdhA* promoter and *sac7d* promoter increased the β-galactosidase activity by 30-fold and 26-fold, respectively, compared with the promoterless vector (data not shown). Furthermore, the *gdhA* promoter activity was detected in all growth phases, but *sac7d* promoter activity was decreased in the late exponential phase. Therefore, we chose the *gdhA* promoter for shuttle vector construction. To determine whether the expression vector we constructed can work efficiently in *S. acidocaldarius*, the genes from two different archaeal origins were cloned into pSM21N and the stable expression was examined by enzyme activity assay. The PH0193 from *P. horikoshii* and the Ta0298 from *T. acidophilum* were tested. The enzyme activity measurements (Fig. 4) of PH0193 and Ta0298 revealed that both proteins were expressed as active forms in *S. acidocaldarius*. These results suggest that the pSM21N vector can be used for stable expression of foreign archaeal genes that form as insoluble aggregates in the *E. coli* system.

Although the *gdhA* promoter was successful for the expression of foreign genes, there is still a need to improve the shuttle vector for high-level gene expression. A selection of a suitable promoter is one of the important points. An inducible promoter with low basal activity would allow for controlled expression in *S. acidocaldarius*. The pJ-based vector with inducible *tf55α* promoter upon heat shock together with the *lacS* gene of *S. solfataricus*, pJlacS, successfully expressed the β-glycosidase in *S. acidocaldarius*. The maltose-induced pCmalLacS was also constructed, and LacS expression under control of the *malE* promoter was detected in the maltose or dextrin medium [11]. Recently, a pCmalLacS derivative, which contains a copy of *malR* under its own promoter and the mutated *malE* promoter, has been constructed to improve the expression of the foreign gene [34]. The expression of the PP_i-dependent PFK from *Thermoproteus tenax* cloned into pSVA1450 resulted in a significantly increased yield of recombinant protein compared with pCmalLacS. However, the *malE* promoter is leaky, even in the absence of maltose.

The development of a suitable shuttle vector for archaea such as hyperthermophiles is still difficult and complicated. If these hard trials succeed in the near future, we can explore the features that make archaea so fascinating as well as challenging.

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