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A Novel Integrative Expression Vector for *Sulfolobus* Species

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With the purpose of facilitating the process of stable strain generation, a shuttle vector for integration of genes *via* a double recombination event into two ectopic sites on the *Sulfolobus acidocaldarius* chromosome was constructed. The novel chromosomal integration and expression vector pINEX contains a *pyrE* gene from *S. solfataricus* P2 ($pyrE_{sso}$) as an auxotrophic selection marker, a multiple cloning site with histidine tag, the internal sequences of *malE* and *malG* for homologous recombination, and the entire region of pGEM-T vector, except for the multiple cloning region, for propagation in *E. coli*. For stable expression of the target gene, an α-glucosidase-producing strain of *S. acidocaldarius* was generated employing this vector. The *malA* gene (*saci_1160*) encoding an α-glucosidase from *S. acidocaldarius* fused with the glutamate dehydrogenase ($gdhA_{saci}$) promoter and leader sequence was ligated to pINEX to generate pINEX_malA. Using the "pop-in" and "pop-out" method, the *malA* gene was inserted into the genome of MR31 and correct insertion was verified by colony PCR and sequencing. This strain was grown in YT medium without uracil and purified by His-tag affinity chromatography. The α-glucosidase activity was confirmed by the hydrolysis of *p*NPαG. The pINEX vector should be applicable in delineating gene functions in this organism.

Keywords: Chromosome integration, cloning vector, α-glucosidase, *Sulfolobus acidocaldarius*

Introduction

Research on archaea has long been limited by the lack of genetic tools to study gene functions *in vivo*. The major reason is that the antibiotics used in microbial genetics as selectable markers are readily degraded at the conditions at which various archaea live [14]. Genetic systems were described for hyperthermophilic archaea in the early 2000s and have constantly improved since then. In *Thermococcus kodakariaensis*, an effective gene deletion system was established by Sato *et al*. [20, 21] for which also a complementary expression vector using prototrophic (arginine/citrulline, tryptophan, agmatine, or uracil) or antibiotic (simvastatin or mevinolin) selection markers has been optimized [12, 19]. Recently, the trials for construction of a stable shuttle vector and selection of targeted mutants from *Pyrococcus furiosus* have been carried out [8, 9, 26]. Hence, nowadays, genetic toolboxes are available for a variety of hyperthermophilic euryarchaeota.

In crenarchaeota, only Sulfolobales genetic systems have been developed. The first shuttle vectors and a gene deletion method were based on the β-galactosidase, LacS, as a selection marker in *Sulfolobus solfataricus* [2, 3, 29] and this method was employed in a number of studies [10, 15, 22, 23, 25, 33]. A system for gene deletion mutants including a shuttle vector has also been reported based on the *pyrEF* selection in *S. islandicus* [7, 16] and successfully used in different studies [11, 30]. The antibiotic simvastatin has also been used to select for the shuttle vector and a knockout system in this strain [31, 32]. Other *Sulfolobus* shuttle vectors that rely on *pyrEF* selection were constructed based on the virus SSV1 and the plasmid pRN1 [3, 13]. In *S. acidocaldarius*, *pyrEF* was employed as a selectable marker to obtain the insertional deletion mutants and persistence of a shuttle vector with a constitutive or inducible promoter for expression [4, 27]. Recently, we also developed the shuttle vector using the *pyrE* gene for the expression of foreign genes in *S. acidocaldarius*. However, the segregrational

and structural instability of the plasmid sometimes made it difficult to obtain stable expression of foreign genes [17].

Therefore, we established an alternative method to express homologous or heterologous target genes, relying on homologous recombination with a chromosome using the pop-in and pop-out method. A novel plasmid vector, pINEX, was generated and included the *pyrE* gene from *S. solfataricus* P2 ($pyrE_{sso}$) for selection, a multiple cloning site with histidine tag, and the internal sequences of *malE* and *malG* for homologous recombination in *S. acidocaldarius*, as well as regions of the pGEM-T vector, for the replication in *E. coli*. For the testing of the successful expression of foreign genes, the *malA* gene (*saci_1160*) encoding an α-glucosidase from *S. acidocaldarius* was selected and examined for the stable expression in this strain.

Materials and Methods

Strains and Growth Conditions

S. acidocaldarius uracil auxotroph MR31 was provided by Dennis W. Grogan (University of Cincinnati, USA) and aerobically grown at 77°C in Brock medium supplemented with 0.1% (w/v) tryptone, and 0.005% (w/v) yeast extract containing uracil (50 μg/ml), adjusted to pH 3.0 [18]. After cell transformation, the cells containing pINEX vector were selected on Brock medium supplemented with 0.1% NZ-Amine (Sigma, St. Louis, MO, USA), and 0.2% (w/v) xylose as a carbon and energy source. *E. coli* strain DH5α was used for the propagation of plasmids and was incubated in Luria-Bertani (LB) medium (1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl), containing ampicillin (100 μg/ml), at 37°C.

pINEX Vector Construction

For construction of the ectopic integration plasmid, the internal regions of the *malE* (*saci_1165*) and *malG* (*saci_1163*) genes were amplified using primers SA1165_F/SA1165_R and SA1163_F/SA1163_R, respectively. The oligonucleotide primers used are shown in Table 1. For the *malE* region, the forward primer introduced an *Apa*I restriction site at the 5′-end, whereas the reversed primer was designed to incorporate the multiple restriction enzyme sequence. For the *malG* region, the forward primer was designed to incorporate the complementary strand of the multiple restriction enzyme sequence with a six-histidine tag sequence of SA1165_R primer, and the reverse primer contained a *Sac*I restriction site. Two PCR fragments were fused *via* an overlapping PCR, and the resulting amplified PCR fragment was digested with *Apa*I and *Sac*I, and ligated into the pGEM-T vector, yielding plasmid pSM27. To add a selection marker by *pyrE* gene insertion, a DNA region from 280 nucleotides upstream to 71 nucleotides downstream of the *pyrE* gene from *S. solfataricus* P2 ($pyrE_{sso}$) was amplified using the primers KH71 and KH72 [6]. The 770 bp PCR product was digested with *Bam*HI and cloned into pSM27 to generate pINEX (Fig. 1). The transcription direction of the *pyrE* gene was confirmed by PCR using primers SA1165_F and pyrEF as well as DNA sequencing.

pINEX-malA Construction

For the construction of pINEX_malA, first, an overexpression cassette controlled by the promoter of *gdhA* encoding glutamate dehydrogenase (*saci_0155*) was constructed using a fusion PCR technique. Using the genomic DNA of *S. acidocaldarius* as a template, the *gdhA* promoter and N-terminus region containing 403 nucleotides upstream of the *gdhA* coding sequence was amplified with the primers Gdhsac_F and Gdhfus_R. The forward

Table 1. The oligonucleotide primers used in this study.

Primer	Nucleotide sequence[a] (5′ → 3′)	Remarks
SA1165_F	TATC**GGGCCC**TACACGTTCAAGTCA	*malE* internal region with *Apa*I
SA1165_R	**CTGCAGTGCGGCCGCCCGCGGCCATGG** **GCATGCGACGTCGGATCC**CATAAGTTAAGTTGT	*malE* internal region with MCS at C-terminus
SA1163_F	**CCGCGGGCGGCCGCACTGCAGCATATG** CACCACCACCACCACCACTGACTGTGGCTACTGTAA	*malG* internal region with MCS and 6 histag at N-terminus
SA1163_R	AAAT**GAGCTC**TTACTTACCTCCTGA	*malG* internal region with *Sac*I
KH71	**GGATCC**AATGAAAACTACTTTCCCTGATAGATAA	*pyrE* from *S. solfataricus* P2, with *Bam*H1
KH72	**GGATCC**CTACTTTTCAACATTCTTCACCAAA	*pyrE* from *S. solfataricus* P2, with *Bam*H1
Gdhsac_F	AGT**CCGCGG**TTCTCCACTGTTTACGTT	Promoter of *gdhA* with *Sac*II
Gdhfus_R	TGCTTTAATCTCCACCGCAGAAGAATTCATATTTTA	Leader of *gdhA* following N-terminus of *malA*
MalAfus_F	TATGAATTCTTCTGCGGTGGAGATTAAAGCAGAG	*malA* with *gdhA* leader
MalA_R	GTT**CTGCAG**ACCCTTATTCGGTCTCCTC	*malA* with *Pst*I
PyrEF	TTTCGTTTTAACATCAGGTAAGG	Internal region of *pyrE* from *S. solfataricus* P2
PyrER	TGTGAAGCCCCTTCTTGTCT	Internal region of *pyrE* from *S. solfataricus* P2

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

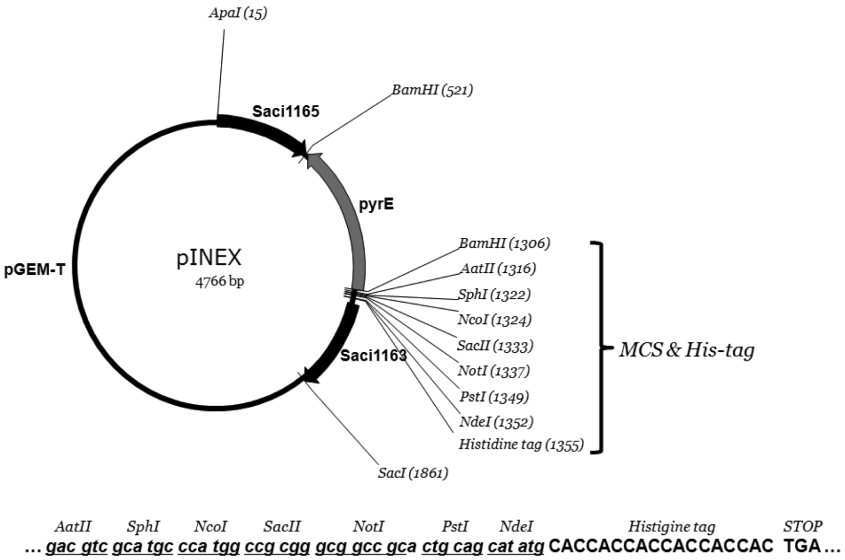

Fig. 1. Map of plasmid pINEX.
The unique restriction sites from the multiple cloning sites are indicated outside of the map.

primer was designed to incorporate a *Sac*II restriction site, and the reverse primer was designed to incorporate a *gdhA* leader sequence (15 nucleotides of the *gdhA* start region) and 15 nucleotides of the *malA* (*saci_1160*) start region. PCR was carried out using 2.5 U of PrimeSTAR HS DNA polymerase (Takara, Japan) using the following conditions: 94°C for 3 min; 30 cycles of 98°C for 10 sec, 55°C for 5 sec, and 72°C for 50 sec; 72°C for 5 min. The *malA* gene along with *gdhA* leader sequence containing 1,906 bp was amplified using the primers MalAfus_F and MalA_R. The forward primer contained 31 nt 5′ overhanging ends (underlined) complementary to the 3′ end of the primer Gdhfus_R, whereas the reverse primer was designed to incorporate a *Pst*I restriction site. Both fragments were fused by overlap PCR to obtain the fusion gene P_{gdhA}-*malA*. Overall, a 2,001 bp fragment was purified using a PCR purification kit (ELPIS Biotech, Korea) and cloned into pINEX to generate pINEX_malA. The construction and sequence of pINEX_malA is shown in Fig. 2. The sequence of the overexpression cassette was confirmed by DNA sequencing.

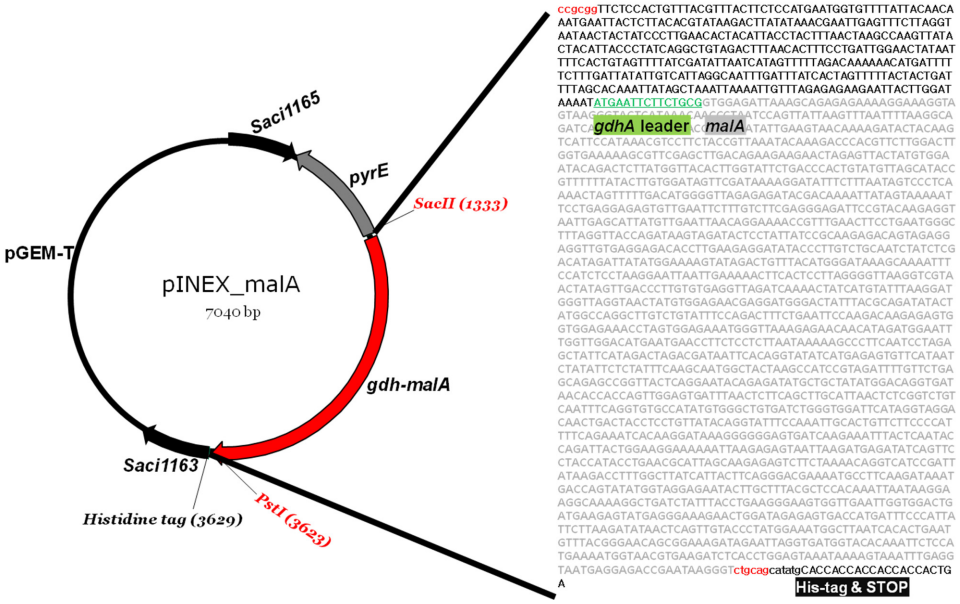

Fig. 2. Map of plasmid pINEX_malA and the nucleotide sequence of the overexpression cassette of the fusion gene P_{gdhA}-*malA*.

Transformation of *S. acidocaldarius*

S. acidocaldarius MR31 was transformed with plasmids by electroporation, as described previously [6]. The non-methylated or methylated pINEX_malA DNA (1 μg) for the chromosomal integration was electroporated in electrocompetent MR31 using a Gene Pulser II electroporator (Bio-Rad), with input parameters 1.25 kV, 1000 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) N-Z-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 gene *pyrE*.

Chromosomal Integration of the *malA* Gene

After the transformation of *S. acidocaldarius* MR31 by a pINEX_malA plasmid, two steps were needed to carry out the replacement of the *malEFG* gene by overexpression cassette including the target gene: a pop-in (vector insertion) by one crossover event, and a pop-out recombination (Fig. 3A). For this purpose, the colonies that contained the *pyrE* gene by colony PCR were grown in uracil-free YT medium containing 0.2% xylose to eliminate the false positives as well as induce the second pop-out recombination at this step. PCR amplification using primers Pgdh_F and SA1163_R was performed to check whether the pop-out recombination resulted in a replacement of the overexpression cassette or wild-type (MR31) genotype recurrence.

Expression and Purification of MalA from *S. acidocaldarius*

S. acidocaldarius integration mutant SMmalA carrying the MalA overexpression cassette into its chromosome was grown in 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 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 160 mM imidazole). All fractions were collected and dialyzed against the same buffer without imidazole and concentrated using Centricon-10 microfilters (Millipore, Darmstadt, Germany). Protein concentration was determined using the Bradford method [5] with bovine serum albumin as a standard. The molecular mass and purity of the purified MalA were estimated by 12% SDS-PAGE.

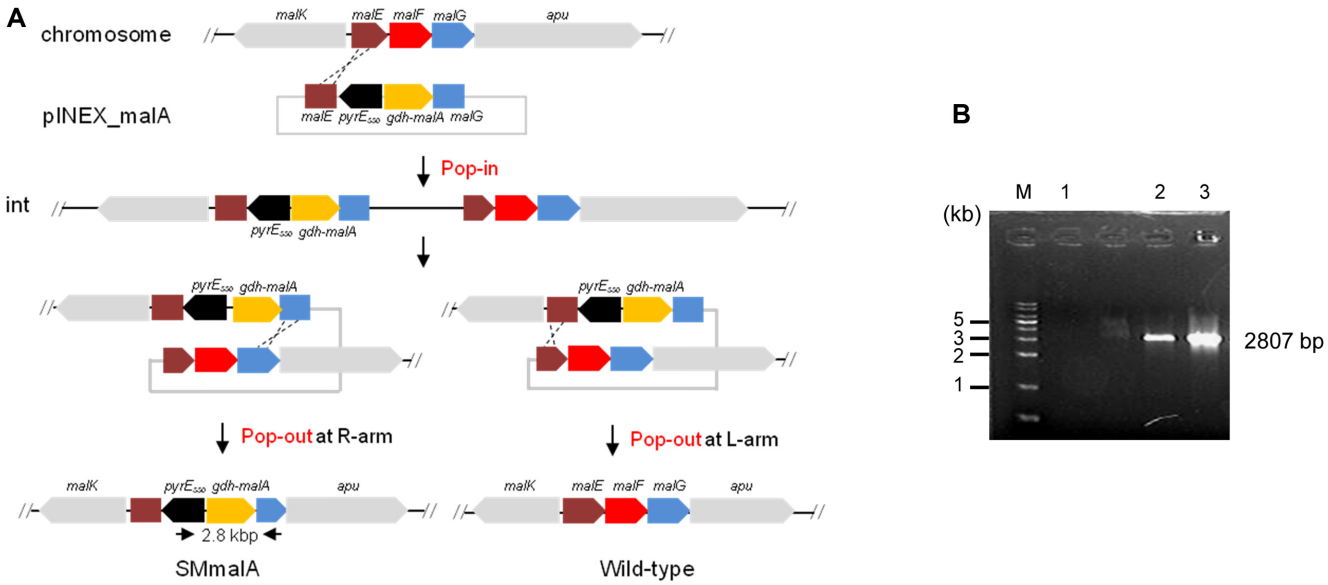

Fig. 3. Strategy for the integration of *malA* into the *malE* and *malG* loci in the chromosome of *S. acidocaldarius*.

(**A**) After transformation, the plasmid was integrated into the genome by a first crossover event in the homologous-region fragment. The second step was the pop-out recombination (or excision) event. There are two possibilities: a recombination between the other homologous fragments, resulting in the replacement of the overexpression cassette, or a recombination between the same homologous fragments of the first recombination, which gives the wild-type genotype. (**B**) To verify the different genotype configurations, PCR amplification was performed with the primers matching Gdhsac_F and SA1163_R regions: primers for identification of *pyrE* and target gene insertion. Lane M, 1 kb size marker; lane 1, PCR result of MR31 (wt) strain; lane 2, PCR result of integrant transformants (int); lane 3, PCR result of colony obtained from the second single crossover of int transformant (SMmalA).

Assay for α-Glucosidase Activity

α-Glucosidase activity was measured by enzymatic determination of D-glucose liberated from maltose, using a glucose oxidase assay kit (Sigma). The reaction mixtures (50 µl) containing 5 mM maltose in 20 mM sodium acetate buffer (pH 5.0) were incubated with 0.1 µg of enzyme at 95°C for 5 min. One hundred microliters of glucose oxidase assay reagent was added to the mixture and incubated at 37°C for 30 min. The reaction was stopped by the addition of 100 µl of 12 N H_2SO_4, and 200 µl aliquots were measured at 540 nm. The assay for *p*-nitrophenyl α-glucopyranoside (*p*NPαG) hydrolysis was performed in 100 µl of 20 mM sodium acetate buffer (pH 5.0) containing 5 mM *p*NPαG and 0.1 µg of enzyme. The mixture was incubated at 95°C for 5 min and the reaction was stopped using 100 µl of 1 M sodium carbonate. Absorbance of the *p*-nitrophenol (*p*NP) released from *p*NPαG was determined at 420 nm.

Results

Characteristics of pINEX

To facilitate the process of gene expression and integration into specific regions of the *S. acidocaldarius* chromosome, we constructed plasmid pINEX (Fig. 1). It carries the *pyrE* promoter and structural gene of *S. solfataricus* as a selection marker and a multiple cloning site (MCS) with unique restriction sites for target gene expression flanked by *malE* and *malG* loci, thus enabling excision by host RecA family recombinase. pINEX also contains the β-lactamase coding sequence and *ori* site from the pGEM-T vector for the cloning and propagation of this plasmid in *E. coli*. The region including expression sequences, cloned gene(s), and the *pyrE* cassette can be amplified by PCR and ligated into the regions of homology to cause integration into a specific chromosomal site in an MR31 strain.

Construction of a Strain Expressing MalA from the Chromosome

pINEX_malA, a plasmid that harbored the *malA* gene under the constitutive *S. acidocaldarius gdhA* promoter, was constructed and used to transform the host strain MR31, a uracil auxotroph derived from *S. acidocaldarius* DSM639. For efficient expression under the *gdhA* promoter and easy purification by affinity chromatography, the *malA* gene was fused to five amino acids of glutamate dehydrogenase at the N-terminus and six histidines at the C-terminus (Fig. 2). A transformant exhibiting uracil prototrophy was selected in medium lacking uracil, and the integration of the plasmid into the *S. acidocaldarius* chromosome was confirmed by colony PCR using *pyrE* internal primers pyrEF and pyrER, and this led to an amplification of a band corresponded to 400 bp. After PCR verification, selected clones were incubated in uracil-free medium for 2 days at 77°C to derive the second pop-out recombination. The second crossover event was verified by PCR amplification of a 2,807 bp fragment by using primers Gdhsac_F and SA1163_R (Fig. 3B).

Characterization of a Strain Expressing MalA

The transformant SMmalA harboring the *malA* gene was grown in YT medium containing 0.2% sucrose for 24 h. Expression of MalA was examined in the cell lysates. The α-glucosidase activity was examined using maltose and *p*NPαG as substrates. The cell-free extracts from the SMmalA strain showed enzyme activity, whereas the cell-free extracts from MR31 (which is the control strain) did not show the enzyme activity. The hydrolysis of *p*NPαG into *p*NP was detected by the appearance of a yellow color in the reaction mixture, indicating that the expression was successful (Fig. 4A). The recombinant MalA was tagged with a 6×His-tag. This enabled us to test purification by His affinity chromatography using this strain. His affinity chromatography of MalA resulted in highly pure MalA in one step (Fig. 4B). The purified MalA estimated by SDS-PAGE was approximately 72 kDa, which is in good agreement with the size obtained from the plasmid expression vector pKHmalA [6]. The characteristics of the purified

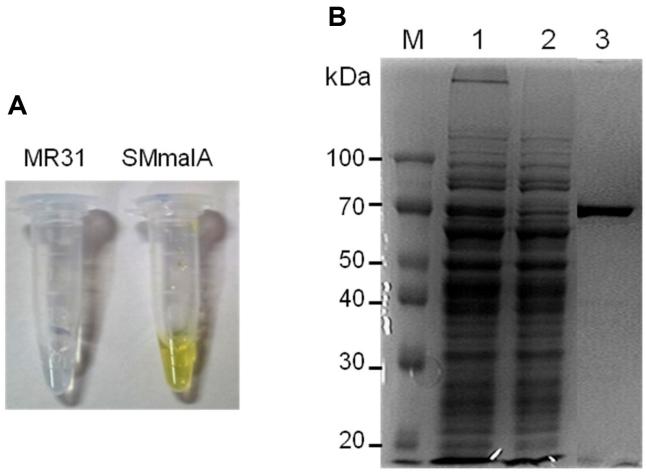

Fig. 4. Enzyme activity measurement (**A**) and SDS-PAGE analysis (**B**) of recombinant MalA.

The α-glucosidase activity was measured using *p*NPαG. The yellow color development by *p*NP produced from the hydrolysis of *p*NPαG was observed in the *S. acidocaldarius* SMmalA strain. MalA was purified by His affinity chromatography. Lane M, protein size standards; lane 1, cell-free extract of SMmalA harboring *malA*; lane 2, flow-through fraction; lane 3, elution fraction with 200 mM imidazole.

MalA was exactly matched with recombinant MalA expressed from a plasmid-based *S. acidocaldarius* strain [6]. Therefore, the integrative expression vector pINEX we constructed can be used for the efficient expression of archaeal genes whose protein products are rendered insoluble when expressed in *E. coli*.

Discussion

Hyperthermophilic archaea have been regarded as a useful organism in the research for cellular aspects of the archaeal kingdom and biotechnology owing to their heat adaptation. In order to understand the physiology, metabolism, and many other cellular aspects, the availability of genetic tools for these organisms is essential. Sulfolobales are the only family for the crenarchaeota that are amenable for genetic manipulation so far. In the last few years, various genetic systems in the Sulfolobales, including *S. solfataricus*, *S. islandicus*, and *S. acidocaldarius*, have been developed and proved to be successful [1, 24, 28, 29]. Although *E. coli-Sulfolobus* shuttle vectors have been used for promoter studies and adapted for the homologous and heterologous expression of tagged proteins in *Sulfolobus* spp., the segregational and structural instability of the plasmid sometimes made it difficult to obtain a stable expression of foreign genes.

As a part of the development of expression technology, we constructed the pINEX vector based on *pyrE* selection for chromosomal gene integration and expression. This plasmid can be utilized as a typical expression vector having a MCS at which the expression cassette including the target gene, along with a strong constitutive or regulated promoter, can be simply inserted into it by specific restriction enzymes. In addition, pINEX was designed to facilitate the process of chromosomal gene integration. Genes cloned in this vector with a *pyrE* auxotrophic cassette can be integrated into the chromosome by homologous recombination at the *malE* and *malG* loci and selected by uracil prototrophy. The integration of the target gene at the *malE* and *malG* loci causes the disruption of a *malEFG* operon in the chromosome, thus preventing the strain from growing in maltose or starch medium [6]. As a result, the integration of the target gene into the chromosome of *S. acidocaldarius* by transformation of the pINEX vector can also be identified by growth defects in the specific growth conditions. By employing various useful promoters derived from Sulfolobales, the strain can control the regulated or constitutive gene expression. As this plasmid was designed to add a six-histidine tag at the C-terminus of the target gene, the expressed protein can be simply purified by affinity chromatography. To determine whether the expression vector we constructed can work efficiently in *S. acidocaldarius*, the *malA* (*saci_1160*) gene encoding for α-glucosidase was tested. Enzyme activity measurements (Fig. 4A) as well as SDS-PAGE analysis (Fig. 4B) of the *S. acidocaldarius* SMmalA strain carrying the *malA* gene revealed that the plasmid pINEX_malA was successfully integrated into the chromosome of strain MR31, and the integrated *malA* gene was stably expressed in this strain. These results imply that in addition to being a plasmid expression vector, the pINEX vector can be used for the stable expression of foreign archaeal genes that are not well expressed in the *E. coli* system.

In conclusion, we constructed a plasmid vector that can genomically express target genes with tag. The expression level of the target genes would be varied depending on the selected promoters, and the His-tag fused at the C-terminus would help the proteins to be easily separated from other proteins in the genomic background. Therefore, vector pINEX might be useful as a tool for the construction of genetically stable and robust *S. acidocaldarius* strains that express foreign genes.

Acknowledgments

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