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The *apt*/6-Methylpurine Counterselection System and Its Applications in Genetic Studies of the Hyperthermophilic Archaeon *Sulfolobus islandicus*

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ABSTRACT

***Sulfolobus islandicus* serves as a model for studying archaeal biology as well as linking novel biology to evolutionary ecology using functional population genomics. In the present study, we developed a new counterselectable genetic marker in *S. islandicus* to expand the genetic toolbox for this species. We show that resistance to the purine analog 6-methylpurine (6-MP) in *S. islandicus* M.16.4 is due to the inactivation of a putative adenine phosphoribosyltransferase encoded by *M164_0158* (*apt*). The application of the *apt* gene as a novel counterselectable marker was first illustrated by constructing an unmarked α-amylase deletion mutant. Furthermore, the 6-MP counterselection feature was employed in a forward (loss-of-function) mutation assay to reveal the profile of spontaneous mutations in *S. islandicus* M.16.4 at the *apt* locus. Moreover, the general conservation of *apt* genes in the crenarchaea suggests that the same strategy can be broadly applied to other crenarchaeal model organisms. These results demonstrate that the *apt* locus represents a new tool for genetic manipulation and sequence analysis of the hyperthermophilic crenarchaeon *S. islandicus*.**

IMPORTANCE

Currently, the *pyrEF*/5-fluoroorotic acid (5-FOA) counterselection system remains the sole counterselection marker in crenarchaeal genetics. Since most *Sulfolobus* mutants constructed by the research community were derived from genetic hosts lacking the *pyrEF* genes, the *pyrEF*/5-FOA system is no longer available for use in forward mutation assays. Demonstration of the *apt*/6-MP counterselection system for the *Sulfolobus* model renders it possible to again study the mutation profiles in mutants that have already been constructed by the use of strains with a *pyrEF*-deficient background. Furthermore, additional counterselectable markers will allow us to conduct more sophisticated genetic studies, i.e., investigate mechanisms of chromosomal DNA transfer and quantify recombination frequencies among *S. islandicus* strains.

Diverse *Sulfolobus islandicus* strains belonging to the hyperthermophilic crenarchaea thrive in geographically isolated populations in hot springs around the world (1). These organisms provide an excellent system for studying microbial evolutionary ecology (2) and may be used as a genetic model system for studying novel molecular mechanisms in the TACK (*Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota*, and *Korarchaeota*) lineage of the archaeal domain, which has been hypothesized by some to be the most recent common ancestor of the eukaryotes on the tree of life (3). To date, the genomes of 20 diverse *S. islandicus* strains have been sequenced (2, 4–6). Versatile genetic tools have been developed for a few representative strains of *S. islandicus* (6–8), including two efficient plasmid shuttle vectors (9, 10), a set of new selectable markers (8, 11, 12), and conventional and novel methods of genetic manipulation (13), as well as clustered regularly interspaced short palindromic repeat-Cas-mediated genome editing protocols (14). Nevertheless, the *pyrEF*/5-fluoroorotic acid (5-FOA) counterselection system, which was first employed for forward mutation assays in *Sulfolobus acidocaldarius* (15), remains the sole counterselection marker in crenarchaeal genetics. Since most *Sulfolobus* mutants constructed by the research community were derived from the genetic hosts lacking the *pyrE* and *pyrF* genes (13, 16), a new genetic marker suitable for counterselection and forward mutation assays is of great importance for the genetic study of genome integrity and DNA damage repair in the *Crenarchaeota*. Furthermore, the availability of additional counterselectable markers would enable more sophisticated genetic studies, such as chromosomal DNA transfer and recombination among *S. islandicus* strains (17) and analyses of mutational frequency at various locations in the chromosome (18).

In addition to *pyrEF*/5-FOA, other counterselectable markers have been reported for bacteria and eukaryotes, especially genes that code for phosphoribosyltransferases (PRTases) and that are involved in the pyrimidine and purine salvage pathways (19–22). The counterselection is based on the fact that the active PRTases convert pyrimidine or purine analogs into toxic metabolites that kill the host cells, whereas mutant cells survive cell killing due to

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
S. islandicus M.16.4	Wild type	4
S. islandicus REY15A	Wild type	5
S. solfataricus P1	Wild type	DSMZ
S. solfataricus P2	Wild type	DSMZ
S. acidocaldarius	Wild type	DSMZ
S. islandicus RJW004	*S. islandicus* M.16.4 with *argD*, *pyrEF*, and *lacS* deletions	12
S. islandicus RJW009	*S. islandicus* RJW004 with an in-frame *apt* deletion	This study
S. islandicus RJW010	*S. islandicus* RJW009 with an in-frame *amyA* deletion	This study
S. islandicus pMID-apt-T	RJW004 double-crossover transformants generated with pMID-apt via downstream insertion	This study
S. islandicus pMID-amyA-T	RJW009 double-crossover transformants generated with pMID-amyA via upstream insertion	This study
S. islandicus pC-apt-T	Δ*apt* Δ*argD* Δ*pyrEF* Δ*lacS*::*Sso-argD-Sso-apt*, *apt* complementation strain	This study
Plasmids		
pRJW8	pUC19 carrying the *lacS*, *pyrEF*, and *argD* expression cassettes from *S. solfataricus* P2	12
pRJW9	pUC19 carrying the *lacS*, *apt*, and *argD* expression cassettes from *S. solfataricus* P2	This study
pMID-apt	pRJW8 carrying the Up-arm and Dn-arm of *apt* and a partial *apt* gene (Tg-arm), *apt*-knockout plasmid	This study
pMID-amyA	pRJW9 carrying the Up-arm and Dn-arm of *apt* and a partial *amyA* gene (Tg-arm), *amyA*-knockout plasmid	This study
pC-SsoargD	pUC19 carrying the Up-arm and Dn-arm of *lacS* and the *Sso-argD* marker cassette	12
pC-Ssoapt	NheI/NcoI-cut pC-SsoargD into which the *apt* expression cassette from *S. solfataricus* P2 was inserted, *apt* complementation plasmid	This study

the lack of enzyme activity (23–26). A few archaeal PRTases have been characterized, but most of them are from methanogens or haloarchaea, which are members of the *Euryarchaeota* (27–31). These studies show that archaeal *hpt* (coding for hypoxanthine phosphoribosyltransferase) mutants exhibit resistance to purine analogs, such as 8-aza-2,6-diaminopurine (8-ADP), 8-azahypoxanthine (8-AHP), and 6-methylpurine (6-MP) (32, 33). This observation has facilitated the development of unmarked gene deletions based on *hpt* genes in different euryarchaea, including *Methanosarcina acetivorans*, *Methanococcus maripaludis*, and *Methanosarcina mazei* (34–36). More recently, the *hpt* gene (also named the *xgprt* gene) was used as a marker for developing genetic tools for use in the anaerobic hyperthermophiles *Thermococcus kodakarensis* and *Pyrococcus furiosus* (37, 38). In contrast to several observations of purine salvage pathways in euryarchaea, the purine salvage pathway in crenarchaea is poorly understood, although annotations of some key enzymes, such as purine PRTases, in the genomes of most crenarchaea have been made. Recently, the adenine and hypoxanthine-guanine-xanthine phosphoribosyltransferase of *Sulfolobus solfataricus* P2, encoded by *Sso2342* and *Sso2424*, respectively, have been biochemically characterized (39); however, their use in genetic manipulations has not been demonstrated.

Here we tested the susceptibility of several *Sulfolobus* species, including *S. islandicus* M.16.4, a genetic model isolated from an acidic terrestrial hot springs in Kamchatka, Russia (4), to a set of purine analogs. 6-Methylpurine-resistant (6-MP^r) mutants of this archaeon were obtained, and characterization of their genetic determinant of resistance revealed that it resulted from the loss function of an adenine phosphoribosyltransferase gene (the *apt*, or *M164_0158*, gene). Then, a new counterselectable method of genetic manipulation based on the *apt* gene was developed and employed to delete an α-amylase-encoding gene (*amyA*, *M164_1052*) in *S. islandicus*. Furthermore, the *apt* gene was used in a forward mutation assay to investigate the spectrum of spontaneous mutations at the *apt* locus in *S. islandicus*. Together, the findings of this study demonstrate that *apt*/6-MP functions as an efficient counterselection marker system in *S. islandicus*.

MATERIALS AND METHODS

Strains, media, and growth conditions. All *Sulfolobus* strains (Table 1) were grown aerobically in standard DT medium at 75 to 78°C and pH 3.5 without shaking, as described previously (12). Plate medium was solidified with 1.6% (wt/vol) Phytagel or Gelrite agent (Sigma-Aldrich, USA). For the cultivation of a triple mutant derived from *S. islandicus* M.16.4, *S. islandicus* RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*) (12), 20 μg/ml uracil and 20 μg/ml agmatine were added to the DT medium (designated DTUA medium). For the growth of *S. islandicus* strains with mutations in the *apt* gene, the liquid medium was supplemented with 0.5 mM GMP disodium salt hydrate (Sigma-Aldrich, USA) or 0.5 mM AMP disodium salt (Sigma-Aldrich, USA). The purine analogs 6-MP, 6-thioguanine, 8-azaguanine, 2,6-diaminopurine, 2-aminopurine, 2-amino-6-methylmercaptopurine, and 6-methylaminopurine (Sigma-Aldrich, USA) were added from sterile stocks at concentrations ranging from 1 μM to 3 mM. In particular, 80 μM 6-MP was used to isolate spontaneous 6-MP^r colonies from wild-type *Sulfolobus* strains and 150 to 300 μM 6-MP was used for counterselection procedures when the Δ*apt* and Δ*amyA* deletion mutants were constructed.

Screening and sequencing of spontaneous *Sulfolobus apt* mutants. Mid-log-phase *Sulfolobus* cells were spun down for 10 min at 10,000 rpm and then resuspended in DT medium with a normalized optical density at 600 nm (OD_{600}) of 0.5. An aliquot of 400 μl of cells was plated undiluted via overlay on selective medium containing 80 μM 6-MP. Single 6-MP^r colonies were picked and resuspended in 400 μl DT medium. Two microliters of cell culture was used as the DNA template for PCR amplification according to a procedure described previously (12). The *apt* gene, together with its putative promoter and terminator regions, from different *Sulfolobus* strains was PCR amplified using the primers *Sispt-3*-seq-F/R, whose sequences are shown in Table 2. The resulting PCR products were treated with ExoSAP-IT for the PCR product cleanup kit (Affymetrix, USA) and then sent to the University of Illinois at Urbana-Champaign Core DNA Sequencing Facility for sequencing. In particular, to extensively study the spontaneous mutation spectrum of the *apt* gene in *S.*

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)[a]
Sispt-1-seq-F	GCTGTAGAGAAGGCTAAGAGAGA
Sispt-1-seq-R	CCAGTTGTAGTGTCAATAAGCG
Sispt-2-seq-F	GATTACCGAGCATTCATATTTAA
Sispt-2-seq-R	AGAGAGAGATGGCATGAAAGTTA
Sispt-3-seq-F	TACCCGGATCATATAACCCAG
Sispt-3-seq-R	AAGGTTTTTGTGGTTGGTGAT
Sacapt-seq-F	CCTATTATTCCTATTTTTGCTTT
Sacapt-seq-R	TTGAAAAGTTGAGCCAAGAAG
Ssoapt-seq-F	GTATAGTCCAGATCCGCCAA
Ssoapt-seq-R	GAAATTAGCACAAGATGCAGAA
Stoapt-seq-F	TTAAATTGCCTAAGATACCTGTT
Stoapt-seq-R	AAAGACATTTGGTGGAGCTAT
apt-Up-F	TCGCGTCGACGACCTCCTCTGCTGTAACTGG
apt-Up-R	ACTAGCTAGCGGGCAAAATTAACTCCCTAA
apt-Dn-F	ACTAGCTAGCATAATCTAATTAATATAGCCTATACCTTA
apt-Dn-R	ACGCGGATCCTGGTCTTATAATTGTGGAGAGG
apt-Tg-F	ACATGCATGCTGAAAGTAGTCACATGGGATGA
apt-Tg-R	GAAACGGCCGTTAGATTATTTTTCTTCTTTTCATTTC
apt-flankP-F	GTAACCTTTGAATTAACGCATA
apt-flankP-R	ATAGAACTGACAAGGAGTTTCA
apt-intP-F	GATAGCAAGAGGAGGTTTAGTTC
apt-intP-R	TGAGATTGGTGGGTCATTTAT
lacS-flankP-F	TACGGGAAGTAACACGGAGC
lacS-flankP-R	TACGGGAAGTAACACGGAGC
Sso-apt-F	CATGCCATGGTCATTTTGCTCAATCATAAGATG
Sso-apt-R	TGATGCTAGCTTTGTTGTTGGTGATGAAGTG
Sso-argD/apt-F	ACTAACGCGTTACTTTCTTACTGCT
Sso-argD/apt-R	GAAACGGCCGTTTGTTGTTGGTGATGAAGTG
argD-F	CATGCCATGGATTCTCCAATATATGGGGTTT
lacS-R	AAAACGCGTCCTAGTGTTGCAAGGCAGAT
amyA-Up-F	CGCGGATCC TTCAGTAGTGTTTGGAGGATATG
amyA-Up-R	CGGGGTACCTTTAGGGAGATTAACCATTGAT
amyA-Dn-For	CGGGGTACCGTAATGAAGAGAGGTCACATTTAG
amyA-Dn-Rev	AAGCGTCGACACCTAATCGCATTTTTAGTCC
amyA-Tg-F	GAAACGGCCGATGATAAAAGCCTCATGTTTAT
amyA-Tg-R	ACATGCATGCAGTGTGTAGCCATACCCAAG
amyA-flankP-F	AAATCTATATCCGTATTCATCACC
amyA-flankP-R	TTATCGGGACAATCCTAGTGT

[a] Added restriction sites are underlined.

islandicus M.16.4, 215 6-MP^r isolates in total from 24 independent cell cultures were examined, whereas 10 6-MP^r isolates of each of the other *Sulfolobus* strains were screened.

Construction of gene deletion and complementation vectors. Plasmid pRJW8 harboring a hybrid marker cassette, *argD-pyrEF-lacS* (12), was used to construct the *apt* deletion plasmid via the recently established marker insertion and unmarked target gene deletion (MID) method (12, 40). The regions upstream (the upstream arm [Up-arm], 773 bp) and downstream (the downstream arm [Dn-arm], 703 bp) of the *apt* gene were amplified from *S. islandicus* M.16.4 genomic DNA using the primer sets *apt*-Up-F/R and *apt*-Dn-F/R, respectively (Table 2). SalI/NheI and NheI/BamHI sites were introduced into the products obtained by PCR with primer sets *apt*-Up-F/R and *apt*-Dn-F/R, respectively. The reverse primer *apt*-Up-R started at position +6 relative to the *apt* TTG start codon, and the forward primer *apt*-Dn-F started 9 bp 5′ to the *apt* TAA stop codon. A triple ligation with Up-arm, Dn-arm, and the cloning vector pRJW8 resulted in plasmid pKapt-UD, which contained the in-frame Δ*apt* allele. Afterwards, a partial targeted *apt* gene (Tg-arm; 617 bp) was amplified using the primer pair *apt*-Tg-F/R. *apt*-Tg-F started at position +16 relative to the *apt* start codon and the added SphI site, whereas *apt*-Tg-R started at the end of the *apt* stop codon and contained an EagI site. SphI/EagI-digested Tg-arm was cloned into pKapt-UD at the corresponding sites, yielding the *apt* deletion plasmid pMID-apt.

To construct the *apt* complementation plasmid pC-Ssoapt, the wild-type copy of *apt* from *S. solfataricus* P2 (*Sso-apt*), constituting the open reading frame of *Sso2342* as well as its native promoter and terminator region (824 bp in total), was PCR amplified with primer pair *Sso-apt*-F/R (Table 2), with NcoI and NheI sites being introduced at the 5′ and 3′ ends, respectively. Then, the resulting PCR fragment was digested with NcoI/NheI and inserted at the corresponding sites of a nonreplicative plasmid, pC-SsoargD, harboring the *S. solfataricus argD* (*Sso-argD*) expression cassette flanked by the upstream and downstream regions of *lacS* in *S. islandicus* M.16.4 (12).

An α-amylase-knockout plasmid, pMID-amyA, was also constructed by the MID method (40), with slight modifications. The *argD-apt* cassette was amplified from pC-Ssoapt with the primer sets *Sso-argD/apt*-F and *Sso-argD/apt*-R (Table 2) and replaced the *argD-pyrEF* cassette in pRJW8, generating pRJW9. Similarly, three homologous arms, namely, the *amyA* Up-arm, *amyA* Dn-arm, and *amyA* Tg-arm, were sequentially inserted into pRJW9, thereby generating the *amyA* deletion plasmid pMID-amyA.

Construction of an *S. islandicus* mutant with unmarked in-frame *apt* and *amyA* deletions. *S. islandicus* RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*) (12) was used as the host strain to construct the Δ*apt* mutant (RJW009). The linearized knockout plasmid pMID-apt was introduced into RJW004 by an electroporation-mediated transformation procedure that was described earlier (41) and modified as described in reference 12. The transformants with the hybrid marker cassette *argD-pyrEF-lacS* integrated into the RJW004 chromosome were screened on selective plates without agmatine. The sequence of one candidate strain (pMID-apt-T) was further confirmed by PCR using the primers *apt*-flankP-F/R, which were specific for sequences located outside the *apt* flanking region (Table 2). To obtain unmarked *apt* deletion strains, colonies in a 6-methylpurine-resistant culture were first enriched in liquid medium containing uracil (20 μg/ml), agmatine (1 mg/ml), GMP (0.5 mM), and 6-MP (150 to 300 μM) and then plated on solid medium. Single colonies were screened by colony PCR using two distinct primer sets, *apt*-flankP-F/R and *apt*-intP-F/R, which annealed outside the flanking regions and internal sequences of *apt*, respectively (Table 2). The sequences of the resulting PCR fragments amplified by primer set *apt*-flankP-F/R were further confirmed by sequencing of the *apt* locus.

The Δ*amyA* mutant (RJW010) was constructed by using RJW009 (RJW004 Δ*apt*) as a parent strain. Again, the transformant (pMID-amyA-T) with a hybrid maker cassette *argD-apt-lacS* integrated into the RJW009 chromosome was selected without agmatine, and successful integration was confirmed by PCR using the proper primer sets shown in Table 2. Cell cultures positive for pMID-amyA-T were then directly subjected to counterselection in DT medium containing uracil (20 μg/ml), agmatine (1 mg/ml), GMP (0.5 mM), and 6-MP (150 to 300 μM). The in-frame deletion of *amyA* was confirmed by PCR and sequencing analysis at the *amyA* locus with appropriate primers (Table 2).

Complementation of the Δ*apt* deletion mutant. Complementation of the Δ*apt* strain was achieved upon electroporation with BamHI-linearized pC-Ssoapt. The transformants with the *Sso-argD* and *Sso-apt* cassette inserted at the Δ*lacS* locus via double-crossover homologous recombination were screened by selection for agmatine prototrophs. Colonies prototrophic for agmatine were examined with the primer sets *lacS*-flankP-F/R and *apt*-flankP-F/R (Table 2), whose sequences are specific for regions located outside the *lacS* and *apt* flanking regions, respectively, and one positive candidate was colony purified three times for further study.

Phylogenetic analysis of *apt* homologs. Sequences to be analyzed were retrieved from the NCBI microbial protein database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The sequences of *S. islandicus* M164_0158 (Apt) and M164_0233 (Gpt) were aligned with the selected sequences using the MUSCLE program (v3.7), which was configured to achieve the highest accuracy. Phylogenetic analysis was performed on the Phylogeny.fr platform (www.phylogeny.fr) (42). After alignment, ambiguous regions (i.e., regions containing gaps and/or poorly aligned regions) were removed by use of the Gblocks program (v0.91b), which was performed using the following parameters: the minimum length of a block

TABLE 3 MICs of various purine analogs for wild-type strain *S. islandicus* M.16.4[a]

Purine analog	MIC (mM)
6-Methylpurine	0.04
6-Thioguanine	0.5
8-Azaguanine	0.1
2,6-Diaminopurine	3.0
2-Aminopurine	>3.0
2-Amino-6-methylmercaptopurine	3.0
6-Methylaminopurine	>3.0

[a] *Sulfolobus* cell cultures were inoculated into 45 ml DT liquid medium containing various concentrations of purine analogs. The initial OD_{600} of the cells was normalized to 0.008 by calculation. Cells were incubated at 75 to 78°C without shaking, and the growth was monitored by measuring the cell density after 10 days.

after gap cleaning was 10, no gap positions were allowed in the final alignment, all segments with contiguous nonconserved positions of greater than 8 were rejected, and the minimum proportion of sequences for a flank position was 85%. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). The default substitution model was selected by assuming an estimated proportion of invariant sites of 0.021 and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 1.044). The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed with a bootstrap analysis using 1,000 replicates. The tree was represented by use of the TreeDyn program (v198.3).

α-Amylase plating assay. The α-amylase activities of RJW010 Δ*amyA* and its parent strain, RJW009, were detected by a method described previously (43, 44). Briefly, cell cultures were washed twice and resuspended in Brock's medium (45), and then 8 μl cells (with a normalized OD_{600} of 0.5) was spotted on Brock's medium plates supplemented with 0.2% starch (Sigma-Aldrich, USA) as a carbon source and 5 mM L-glutamic acid (Sigma-Aldrich, USA) as an alternative carbon and energy source (43). After 11 days of incubation at 78°C, the plates were flooded with 5 ml Gram's iodine solution (Sigma-Aldrich, USA) to visualize the halos generated by starch hydrolysis.

RESULTS

***S. islandicus* M.16.4 is highly sensitive to the purine analog 6-MP.** First, we examined the growth of wild-type strain *S. islandicus* M.16.4 in the presence of a few purine analogs (Table 3). We found that 6-methylpurine (6-MP), 8-azaguanine, and 6-thioguanine apparently inhibited cell growth in liquid medium, with 6-MP showing the lowest MIC (Table 3). The influence of this drug on cell growth was studied in more detail. As shown in Fig. 1A, the culture exhibited slightly retarded growth in the presence of 1 μM 6-MP but could reach approximately the same optical density as cultures growing in the absence of 6-MP. Furthermore, concentrations of 6-MP above 5 μM significantly affected the growth of *S. islandicus* M.16.4, and the cell growth in liquid medium was completely inhibited by 40 μM 6-MP.

To examine the effect of 6-MP on the growth of *S. islandicus* M.16.4 on plates, an exponentially growing culture was used to prepare a series of dilutions of cell suspensions, which were then spotted on DT medium plates containing different concentrations of 6-MP and incubated for 9 days. As shown in Fig. 1B, 6-MP at a concentration of 40 μM completely inhibited cell growth (Fig. 1B), consistent with the results obtained with liquid medium. We tested 6 different *Sulfolobus* species for their 6-MP tolerance in

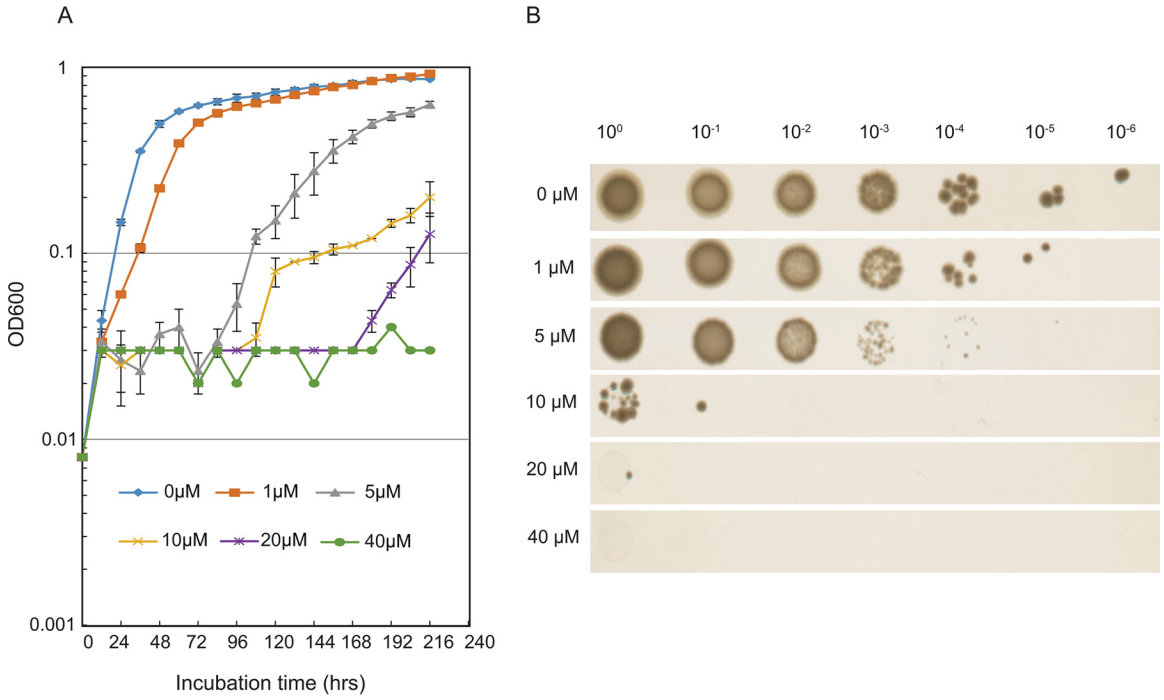

FIG 1 Growth-inhibiting effect of various concentrations of 6-methylpurine on wild-type *S. islandicus* M.16.4. (A) Growth curves of *S. islandicus* M.16.4 in DT liquid medium containing 0, 1, 5, 10, 20, and 40 μM 6-methylpurine. The initial OD_{600} of the different cell cultures was normalized to 0.008, cells were grown without shaking, and cell growth was monitored every 12 h. Error bars represent standard deviations from three independent experiments. (B) Spotting assay with *S. islandicus* M.16.4 on a plate with medium containing various concentrations of 6-methylpurine. Eight-microliter cell dilutions were dropped onto the appropriate plates, and the growth was checked after 9 days of incubation at 78°C.

liquid medium and found that the MICs fell into the range of 20 to 100 μM. Interestingly, *S. acidocaldarius* showed a much higher level of resistance to 6-MP, with the MIC for *S. acidocaldarius* being more than 400 μM (see Table S1 in the supplemental material).

6-MP^r results from the loss of function of M164_0158. To reveal the genetic determinant of the 6-MP resistance in *S. islandicus* M.16.4, 10 6-MP^r colonies were picked up from a DT medium plate containing 80 μM 6-MP, and then the putative promoter and coding regions of each annotated PRTase gene, i.e., *M164_1910* (*Sispt-1*), *M164_0233* (*Sispt-2*), and *M164_0158* (*Sispt-3*), were screened by PCR. Sequencing of the PCR products revealed that 6-MP^r mutations were exclusively located in the *Sispt-3* gene, either on its promoter or in the coding region. A phylogenetic analysis showed that M164_0158 is a homolog of SSO2342 (see Fig. S1 in the supplemental material), a biochemically well-characterized adenine PRTase (APRTase) in *Sulfolobus solfataricus* P2 (39), indicating that *M164_0158* (named *apt* here) codes for a putative APRTase in *S. islandicus* M.16.4.

Deletion of *M164_0158* (*apt*) confers resistance to 6-MP, but AMP or GMP is required to maintain normal cell growth. To verify that the *apt* gene could be the sole target of 6-MP, we sought to create an *apt* deletion mutant following the recently established MID methodology (12). Transformants of strain RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*) that had acquired the marker cassette via double homologous recombination were selected by growth on a plate with medium lacking agmatine (Fig. 2A), and the transformation/recombination efficiency was estimated to be 10 to 50 CFU/μg DNA. The sequence of a representative transformant, designated strain pMID-apt-T, was then confirmed by PCR analysis (Fig. 2B). An ~2.2-kb fragment was obtained with wild-type DNA (RJW004), whereas two fragments with expected sizes of ~9.5 kb and ~1.6 kb were amplified with DNA from the transformant. These corresponded to the recombination allele and the *apt* deletion allele, respectively, as illustrated in Fig. 2B. To obtain the *apt* deletion mutants, 6-MP^r cells were propagated by cultivating the pMID-apt-T recombinant in 6-MP-containing liquid medium, and then the cells were plated to isolate single 6-MP^r colonies (see Fig. S2 in the supplemental material). The 6-MP^r colonies were generated by either spontaneous *apt* mutation or excision of the marker cassette of pMID-apt-T recombinants, which were distinguishable by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. Figure 2B shows the results of PCR analysis of a representative *apt* deletion mutant with two different primer sets, *apt*-flankP-F/R and *apt*-intP-F/R. PCRs with both primer sets revealed that the 6-MP^r strain carried the designed allele with the *apt* deletion (Fig. 2B). This was further confirmed by sequencing of the 1.6-kb PCR products amplified from the 6-MP^r strain with primers *apt*-flankP-F/R.

The growth profiles of the Δ*apt* strain, named RJW009 (Fig. 2C), and its parent strain, RJW004 (Fig. 2D), were documented by cultivating them in different types of media. It was found that the Δ*apt* strain was resistant to a high concentration of 6-MP (0.15 mM, 3.75-fold the MIC); however, AMP or GMP was required to maintain its normal cell growth (Fig. 2C). In contrast, the growth of wild-type strain RJW004 was completely inhibited by 6-MP within 12 days of incubation, even in the presence of AMP or GMP (Fig. 2D).

Finally, the *apt* deficiency was complemented by the insertion of the *S. solfataricus apt* gene (*Sso2342*) at the *lacS* locus of *S. islandicus* via double crossover, as illustrated in Fig. 3A. The sequences of the recombinants (strain pC-apt-T; Fig. 3B), in which *Sso-apt* and *Sso-argD* were integrated at the *lacS* locus, were confirmed by PCR analysis using primers with sequences specific for regions located outside the *lacS* and *apt* flanking regions, respectively (Fig. 3A and B). Growth analysis of one representative complemented Δ*apt* strain (pC-apt-T) in liquid medium showed that its sensitivity to 6-MP was restored, and no GMP or AMP was required to maintain normal growth (Fig. 3C). Together, these results indicate that the *apt* gene is solely responsible for 6-MP sensitivity in this organism.

Unmarked chromosomal gene deletion using the *apt* gene as a counterselection marker in *S. islandicus*. Next we studied the application of the *apt*/6-MP-based counterselection system to conduct unmarked chromosomal gene deletions in *S. islandicus*. A new integrative vector, pRJW9, containing a marker cassette consisting of the *lacS*, *argD*, and *apt* genes was made and employed to construct knockout plasmids. We chose to delete *M164_1052*, a putative α-amylase gene (*amyA*) coding for a protein whose amino acid sequence shared 92% and 61% identity with the amino acid sequences of the well-characterized α-amylases of *S. solfataricus* (SSO1172) and *S. acidocaldarius* (Saci_1162), respectively (43, 46, 47). The knockout plasmid was introduced into *S. islandicus* Δ*apt* (i.e., strain RJW009 [Δ*argD* Δ*pyrEF* Δ*lacS* Δ*apt*]) by electroporation, and transformants were selected by growth on a plate with medium lacking agmatine (Fig. 4A). PCR amplifications of two representative transformants (pMID-amyA-T-1 and pMID-amyA-T-2) confirmed that the knockout plasmid was integrated into the host chromosome via homologous recombination, as illustrated in Fig. 4A and C. We found that the 6-MP^r colonies with unmarked *amyA* deletions (recombinants; Fig. 4B, white) could be effectively selected during a counterselection step with 0.15 mM 6-MP, 1 mM agmatine, and 0.5 mM GMP, whereas the frequency of spontaneous 6-MP^r colonies (spontaneous mutants; Fig. 4B, blue) occurred at a ~500-fold lower frequency. The *amyA* deletions from three randomly selected white colonies were confirmed by PCR with primers whose sequences bind to regions outside the *amyA* flanking regions (Fig. 4C, lanes 8 to 10).

To reveal the phenotype of the deletion mutants, the amylase activities of one of the Δ*amyA* strains (RJW010) and its parent strain (RJW009 Δ*apt*) were assayed on starch-containing plates, and this revealed a large halo surrounding the colony of the wild-type strain (Fig. 5B), which was absent from the colony of the Δ*amyA* mutant (Fig. 5B). Taken together, the successful construction of a strain with an unmarked *amyA* deletion indicated that *apt*/6-MP represents a counterselection system useful for genetic manipulation in *S. islandicus*.

Application of the *apt* gene in a forward mutation assay. Subsequently, we tested the usefulness of the *apt* gene as a genetic marker to evaluate the spectrum of spontaneous mutation in *S. islandicus* M.16.4. This was done by selecting 215 independent 6-MP^r colonies from 24 independent experiments and analyzing their *apt* mutant alleles. The *apt* coding sequence and its flanking regions, including 122 bp upstream of the start codon and 62 bp downstream of the stop codon, were amplified by PCR using primers *Sispt-3*-F/R (Table 2). The sequences of the resulting PCR fragments were determined by DNA sequencing and compared with the sequence of the wild-type gene. Mutations of different types were observed, including base substitutions (BPSs; 46.5%); small indels (<3 bp) causing frameshift mutations (38.6%); and

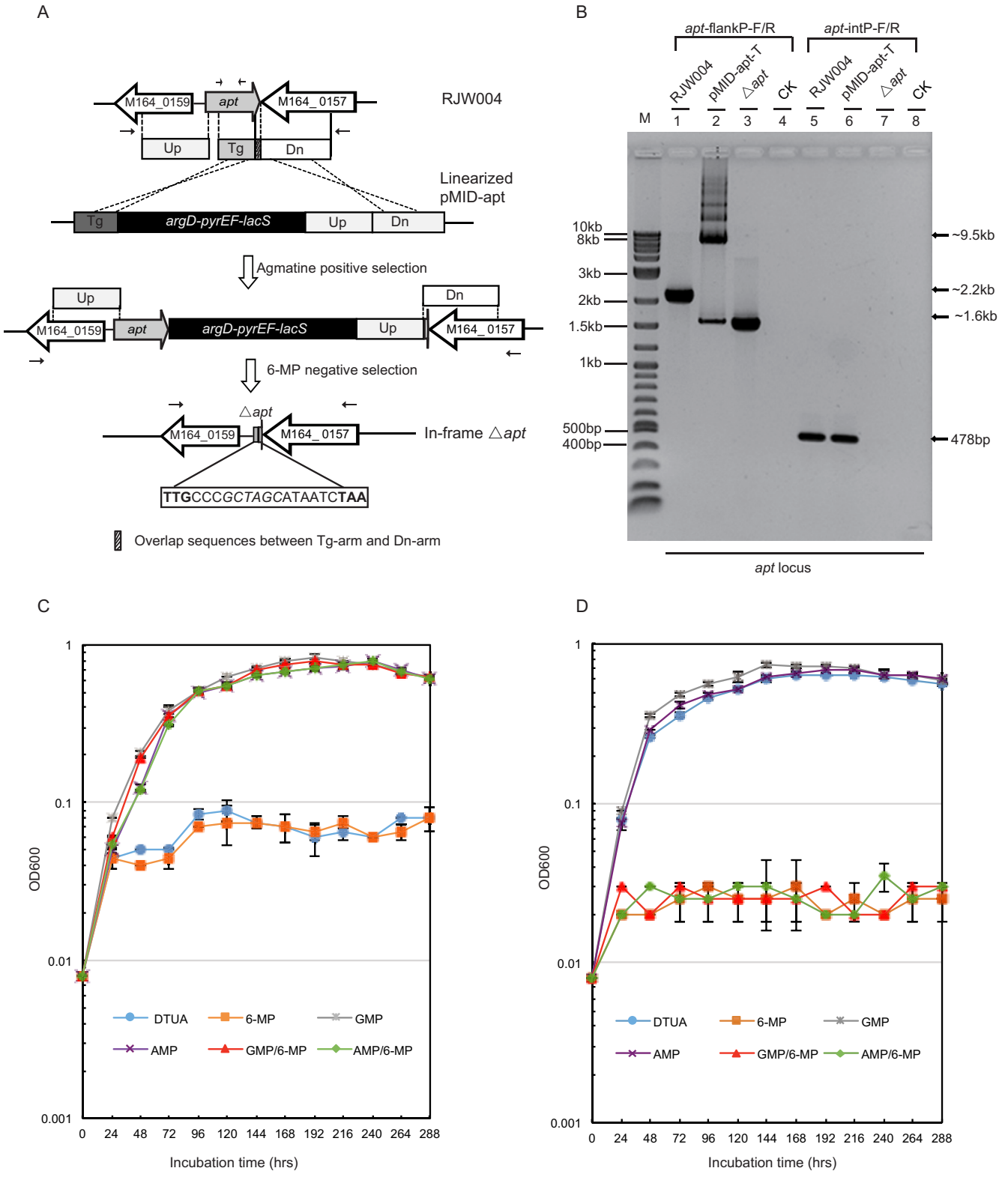

FIG 2 Construction and phenotype characterization of the Δ*apt* mutant. (A) Schematic of the *apt* deletion construct in the background of RJW004 (Δ*argD* Δ*pyrEF* Δ*lacS*). Arrows, positions of PCR primer sets. (B) PCR analysis of the *apt* locus in host strain RJW004, transformant pMID-apt-T, and the Δ*apt* mutant using two different primer sets (*apt*-flankP-F/R and *apt*-intP-F/R). The expected sizes of the PCR products are indicated. Lane M, 2-log DNA ladder (New England Biolabs [NEB]); lanes CK (lanes 4 and 8), no DNA template was added when PCR amplifications were performed. (C) Growth profiles of the Δ*apt* mutant (RJW009) in DTUA liquid medium with or without 6-MP, GMP, AMP, GMP and 6-MP, or AMP and 6-MP. These growth experiments were conducted with each cell culture at an initial OD_{600} of 0.008 and 78°C without shaking. Cell growth was monitored very 24 h for 12 days. Error bars represent standard deviations from three independent experiments. (D) Growth profiles of host strain RJW004 in DTUA liquid medium with or without 6-MP, GMP, AMP, GMP and 6-MP, or AMP and 6-MP.

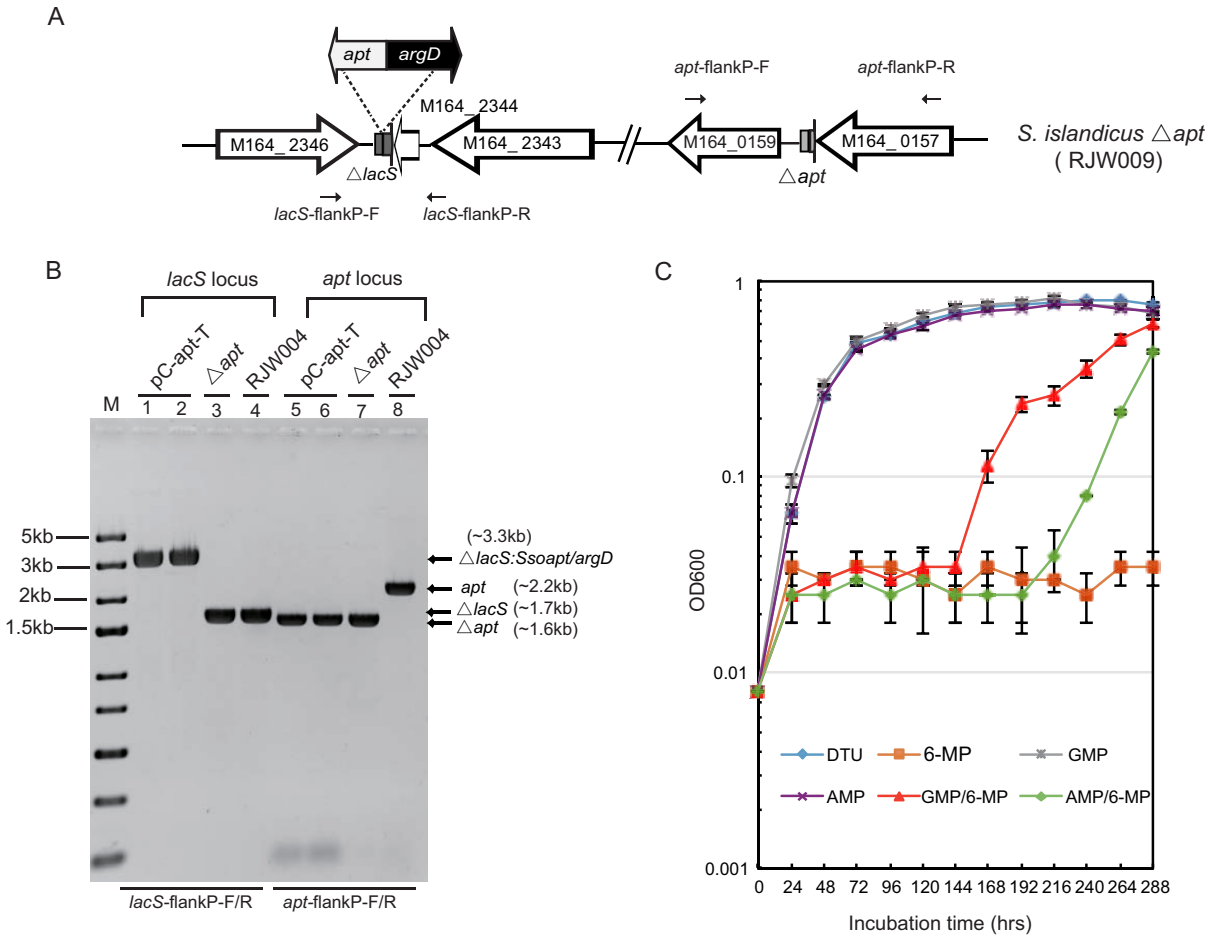

FIG 3 Complementation of the *S. islandicus* Δ*apt* mutant. (A) Integration of the *apt* expression cassette into the *lacS* gene deletion locus of *S. islandicus* Δ*apt* via double crossover. The *apt* deletion strains were transformed with linearized pC-apt containing two homologous flanking regions of *lacS*, selectable markers for the agmatine prototroph (*Sso-argD*), and the *apt* expression cassette from *S. solfataricus* P2 (*Sso-apt*). The transformants were selected for agmatine prototrophs. (B) PCR analysis of the *S. islandicus* Δ*apt* complemented strain. Primers with sequences specific for regions outside the 5′ and 3′ flanking regions of the *lacS* (*lacS*-flankP-F/R) or *apt* (*apt*-flankP-F/R) gene were designed. Lane M, DNA markers; lanes 1 to 4, the results of PCR with two individual pC-apt-T transformants, a Δ*apt* mutant, and the host strain (RJW004), respectively, and primers *lacS*-flankP-F/R; lanes 5 to 8, the results of PCR with two individual pC-apt-T transformants, a Δ*apt* mutant, and the host strain (RJW004), respectively, and primers *apt*-flankP-F/R. Arrows, the amplified fragments expected. (C) Growth profiles of representative complemented strain pC-apt-T in DT liquid medium with uracil (DTU) with or without 6-MP, GMP, AMP, GMP and 6-MP, or AMP and 6-MP.

large indels, including large deletions (1.4%) and large insertion mutations of tandem duplications and an insertion sequence (IS) element (ISC*1205*) (see Fig. S3 in the supplemental material)-based mutation (13.5%) (Table 4). The molecular characteristics of all these mutations, except for a large deletion occurring in the region from positions −40 to +455 of the gene, are illustrated graphically in Fig. 6. This indicated that *apt* constitutes another useful reporter gene suitable for use in forward mutation assays.

DISCUSSION

In this study, we provide genetic evidence that *M164_0158* encodes an adenine phosphoribosyltransferase (APRTase) in the hyperthermophilic crenarchaeon *S. islandicus* M.16.4 and a mutation in the *apt* gene confers 6-MP resistance by preventing lethal incorporation of this toxic purine analog. For this reason, the *apt*/6-MP system represents another counterselection marker, in addition to the *pyrEF*/5-FOA system. We show that this marker has two important applications in *S. islandicus*: (i) the *apt*/6-MP system serves as an efficient counterselection marker for constructing markerless gene deletions, and (ii) it is a useful reporter gene for studying the profile of spontaneous mutations.

Recently, *Sso2342* (*apt*) and *Sso2424* (*gpt*) of *S. solfataricus* P2 have been characterized biochemically, and they have been found to encode adenine and hypoxanthine-guanine-xanthine phosphoribosyltransferases, respectively (39). Here we show that *S. islandicus apt* is the genetic target of 6-MP selection. Since the amino acid sequence of the *apt*-encoded protein (M164_0158) shares 90% identity with the *S. solfataricus* SSO2342 amino acid sequence, this suggests that *M164_0158* also encodes an APRTase. Furthermore, phylogenetic analyses of proteins that show sequence similarity to the characterized APRTase (SSO2342, Apt) and guanosine PRTase (GPRTase; SSO2424, Gpt) of *S. solfataricus* P2 (39) show that they fall into different clades (see Fig. S1B in the supplemental material). This indicates that the Apt and Gpt clades of proteins are well conserved in crenarchaea and perhaps the result of duplication at this locus before divergence within this

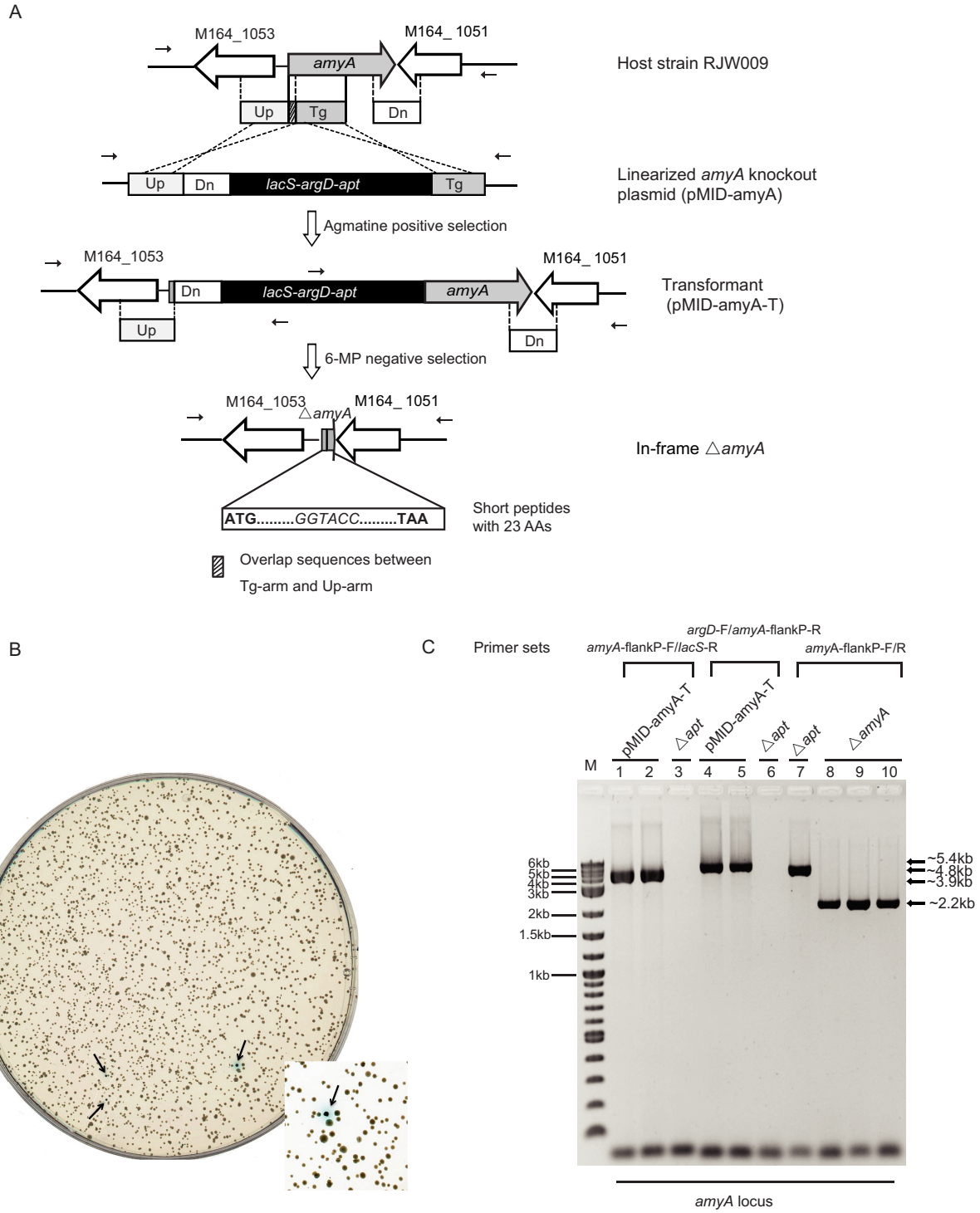

FIG 4 Construction of mutants (RJW010) with an unmarked *amyA* gene deletion based on the *apt*/6-MP counterselection system. (A) Schematic overview of the recombination strategy used to generate an unmarked mutant with an in-frame *amyA* gene deletion via 6-MP counterselection. *S. islandicus* RJW009 (Δ*argD* Δ*pyrEF* Δ*lacS* Δ*apt*) was used as the host strain for genetic manipulation. Cells that had undergone a double-crossover homologous recombination were selected by agmatine prototrophy. Cells (strain pMID-amyA-T) that suffered from a second single crossover at two repeated homologous Up-arms generated mutants with an in-frame unmarked *amyA* gene deletion in the presence of 150 to 300 μM 6-MP. AAs, amino acids. (B) X-Gal staining of 6-MP^r colonies on 6-MP-containing plates. (C) PCR verification of the *amyA* locus in the host strain RJW009 Δ*apt*, pMID-amyA-T transformants, and the Δ*amyA* mutant (RJW010). The pMID-amyA-T sequence was verified with two primer sets, *amyA*-flankP-F/*lacS*-R and *argD*-F/*amyA*-flankP-R, and the sequences of the Δ*amyA* mutants were analyzed with primers whose sequences were specific for regions located outside the 5′ and 3′ flanking regions. The expected sizes of the PCR products are indicated. Lane M, 2-log DNA ladder (NEB).

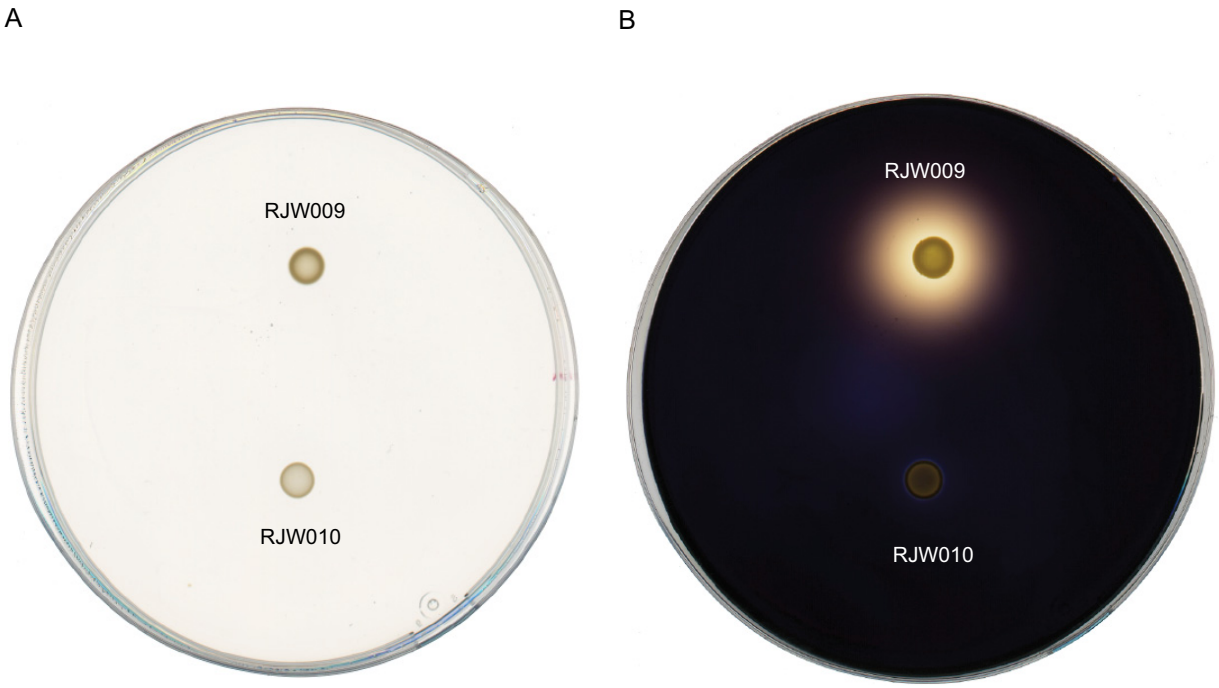

FIG 5 Starch hydrolysis test with the Δ*amyA* mutant (RJW010) and its parent strain, RJW009. Cells were grown on solid medium containing 0.2% starch and 5 mM glutamic acid for 11 days at 78°C. (A) No treatment; (B) treatment with Gram's iodine solution.

clade. We also found that a few other *Sulfolobus* species, including *S. acidocaldarius*, *Sulfolobus tokodaii*, and *S. islandicus* REY15A, that carry an *apt* gene (i.e., *Saci_0998*, *St0484*, and *SiRe_0139*, respectively) and that are sensitive to 6-MP (see Table S1 in the supplemental material) and exhibit 6-MP counterselection. Together, this finding suggests that the APRTase is conserved in the genus *Sulfolobus* and probably in all crenarchaea whose genomes code for an *apt* homolog and they could be tested for application of the *apt*/6-MP counterselection system in genetic studies.

TABLE 4 Summary of spontaneous mutations at the *apt* locus in wild-type strain *S. islandicus* M.16.4

Mutation	No. of 6-MP^r *S. islandicus* M.16.4 isolates (% of total)
BPSs	100 (46.5)
Transitions	71 (33.0)
G:C→A:T	60 (27.9)
A:T→G:C	11 (5.1)
Transversions	29 (13.5)
G:C→T:A	13 (6.0)
G:C→C:G	2 (0.9)
A:T→C:G	3 (1.4)
A:T→T:A	11 (5.1)
Frameshifts	83 (38.6)
−1 bp	58 (27.0)
+1 bp	24 (9.6)
−2 bp	1 (0.5)
Insertions	29 (13.5)
Tandem duplications	10 (4.7)
IS-mediated insertions	19 (8.8)
Deletions	3 (1.4)
3 bp	2 (0.9)
495 bp	1 (0.5)
Total	215 (100)

Characterization of the growth of the Δ*apt* strain showed that cell growth was significantly impaired in liquid medium with or without 6-MP during a period of 12 days of incubation (Fig. 3C); however, cell growth recovered after a prolonged cultivation (~ 3 weeks; not shown in Fig. 3C). Interestingly, the addition of AMP or GMP was required to maintain robust growth (Fig. 3C). The reason that GMP can also support cell growth was probably due to the interconversion between GMP and AMP in purine metabolism, as is reported to occur in most microorganisms (48). The molecular mechanism for the requirement for AMP or GMP for the growth of *apt*-deficient strains of *S. islandicus* remains unknown, but a similar phenomenon was also described in the hyperthermophilic euryarchaeon *P. furiosus*, in which the growth of the Δ*hpt* mutant was maintained well in the presence of 6 mM GMP (38). In contrast, in another hyperthermophilic euryarchaeon, *T. kodakarensis*, no retarded growth was observed in the medium by testing the *hpt* deletion strain (37) or 6-MP^r strains with spontaneous mutations or indels in the *hpt* gene (T. J. Santangelo, personal communications). It was also noteworthy that the *apt*-deficient strain also showed relatively slower growth on Gelrite plates, which could be explained by the observation that single 6-MP^r colonies appeared after approximately 14 days of incubation on the overlay plates to which AMP or GMP was not added.

The purine PRTase-encoding genes have been broadly applied in forward mutation assays for bacteria and eukaryotes, which were based on the inability of mutants to enzymatically convert a

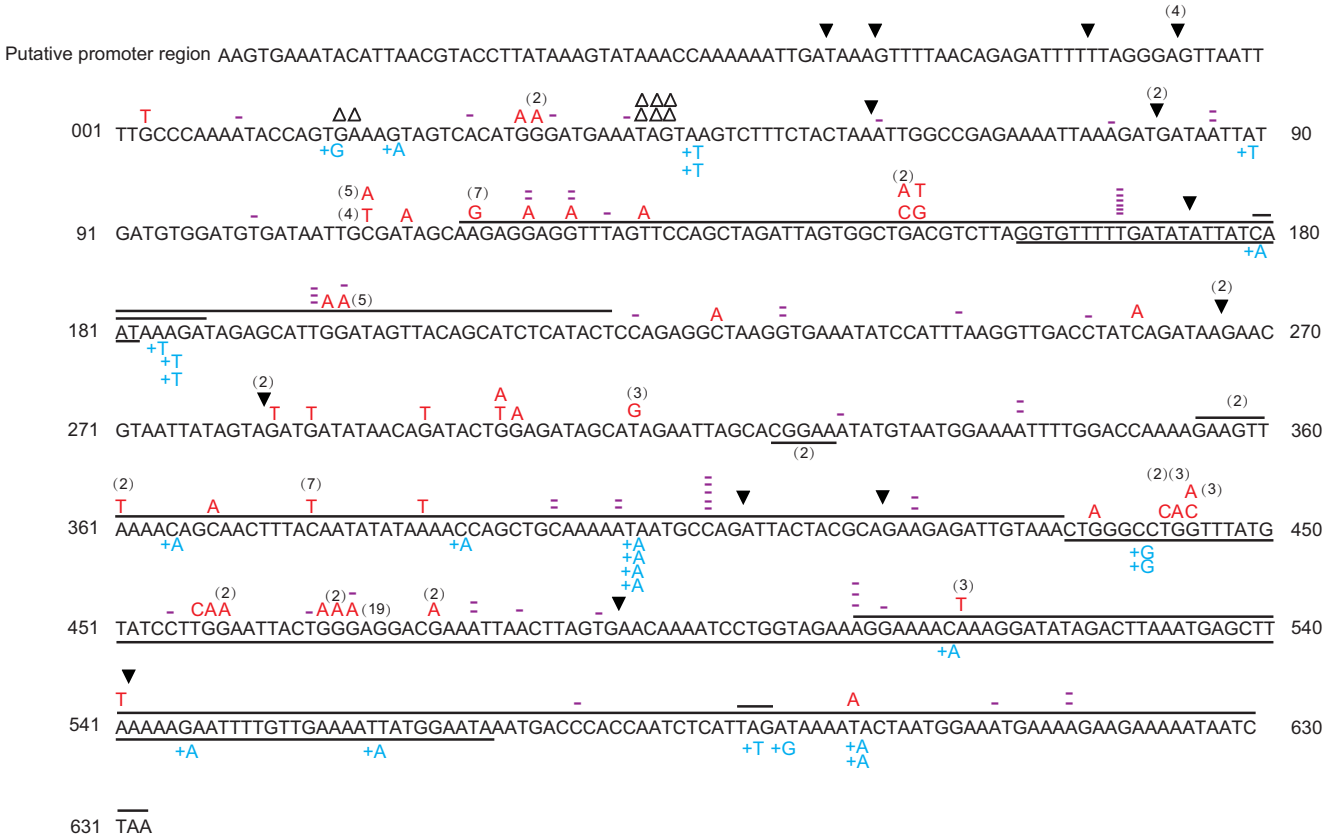

FIG 6 The spontaneous mutational spectra in *S. islandicus* M.16.4 at the *apt* locus. The coding region of the *apt* gene is labeled starting from base 1 (T) and going to base 633 (A). The intergenic region between *apt* (*M164_0158*) and *M164_0159* is listed as base −83 (A) to base −1 (T). BPSs are denoted with a base in red above the sequence. Single-base-pair insertions or deletions are represented by a base with a plus sign in blue or a minus sign in purple, respectively. △△ and △△△, deletions of 2 and 3 bp, respectively; underlining or overlining, tandem duplications of the corresponding sequences; ▼, insertion sequence element (IS*1205*)-mediated insertions. A large deletion of the fragment of the *apt* gene from the A at position −40 to the C at position +455 is not shown. The numbers in parentheses indicate the number of independent isolates with the same mutation event.

drug to a toxic metabolite (23–25). Recently, this counterselection system has been tested in *T. kodakarensis*, in which the hypoxanthine-guanine phosphoribosyltransferase-encoding gene *hpt* was used to compare the patterns of spontaneous mutations that occurred in wild-type and DNA polymerase B mutant strains (49). Here we have used the *apt* gene for the forward mutation assay in *S. islandicus* M.16.4. Another reporter gene that has been used in the forward mutation assay is the *pyrEF*/5-FOA system, with which mutation profiles have been obtained for *S. acidocaldarius*, *S. solfataricus*, and *S. islandicus* (50, 51). The profile of frameshifts, tandem duplications, and larger deletion and BPSs obtained in this work was generated from molecular analysis of the *apt* locus from over 200 6-MP^r strains. We found that the mutation profile is comparable to that of *T. kodakarensis*, also assayed by 6-MP counterselection with an *hpt* gene (49). Interestingly, a 5-FOA-based forward mutation assay in *S. acidocaldarius* revealed that frameshift mutations are dominant in this organism (50). Furthermore, this and a few other studies have revealed that the transitions outnumbered transversions by more than 2-fold, and a very strong strand bias for G or C-to-A or T transitions was observed (49, 50, 52, 53). These should be further studied by generating mutants deficient in certain DNA repair mechanisms and investigating their effect on the profile of spontaneous mutations. Since most mutants are generated using *pyrEF* as a selection marker (e.g., see references 7 and 13), the *pyrEF*/5-FOA system for use in forward mutation assays is no longer available. The demonstration of an *apt*/6-MP counterselection system for the *Sulfolobus* model renders it possible again to study the mutation profile in mutants that have already been constructed in the background of *pyrEF* deficiency. *Vice versa*, when *apt*/6-MP is used for genetic manipulations, the mutation profile in the generated mutants can then be investigated using the *pyrEF*/5-FOA system. In conclusion, the *apt* marker developed here will further facilitate genetic studies in *Sulfolobus* organisms, which serve as the most broadly applicable genetic model in the *Crenarchaeota*.

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